AN INVESTIGATION OF TELOMERASE-BASED DIAGNOSTIC AND THERAPEUTIC STRATEGIES IN CANINE CANCER

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Abstract

In 1881, the German biologist August Weissman first proposed the theory that the ageing of tissues is related to the finite ability of somatic cells to replicate. Confirmation of this theory did not arrive until the 1960s with the work of Leonard Hayflick, who showed that somatic cells possess a finite ability to divide, before entering a state known as 'replicative senescence'. This finite replicative lifespan was first proposed to be related to the 'end replication problem' shortly after the discovery of DNA and the DNA replication machinery by Watson and Crick in the 1970s. The end replication problem describes the inability of the DNA replication machinery to fully replicate the 3' end of linear chromosomes. As a result, shortening of the terminal end of each chromosome occurs with every mitotic division. The structure that forms the end of linear chromosomes had been termed the 'telomere' by Muller in 1941, and proof that shortening of the telomere with cumulative mitotic divisions is responsible for the finite ability of somatic cells to divide was finally provided by scientists at the Geron corporation in 1998.

The DNA structures that make up the telomere are highly conserved across a wide range of vertebrate and invertebrate species, and in all vertebrates a key feature is a variable number of repeats of the hexanucleotide sequence TTAGGG. In humans the TTAGGG repeat sequence comprises approximately 5-15 kb in length, as compared with 20 - 150kb in mice and 11 – 20 kb in dogs. Together with telomere-associated proteins, the telomeric DNA has been shown to form a number of three dimensional structures which are thought to be important for telomere function. Telomere shortening with accumulated mitotic divisions ultimately results in the onset of replicative senescence, activated through the p53 pathway in cells from a number of different lineages. This has been termed the M1, or mortality 1, checkpoint. If the M1 checkpoint is bypassed, further cell divisions and telomere shortening occur, up to second checkpoint, known as the M2 checkpoint. Bypassing the M2 checkpoint in the absence of telomere-length maintenance mechanisms leads to end-to-end chromosome fusion through the non-homologous end joining (NHEJ) pathway, resulting in crisis and cell death. For this reason, telomere-shortening has been proposed as a major mechanism underlying the ageing of somatic cells. Additional functions have been ascribed to telomeres, including putative roles in gene regulation and nuclear organisation.

A small number of cells can bypass the M2 checkpoint and achieve an immortal phenotype spontaneously. In the vast majority of these immortal cells, this occurs through the re-activation of telomerase, an RNA-dependent reverse transcriptase first characterised by Carol Greider. This enzyme, composed of a reverse transcriptase catalytic subunit (TERT), an RNA template (TR) and a number of associated proteins, is capable of adding nucleotides to the end of telomeres, and thus circumvents telomere shortening. *In vitro*, enzyme function can be reconstituted with the TERT and TR components alone, and studies with fibroblasts have shown that expression of the TERT

subunit is the principal determinant of telomerase activity. Telomerase activity *in vivo* is absent from the vast majority of somatic tissues in dogs and humans, but is present in some stem cell and germ cell populations. It is also present in the majority of cancers in humans *in vivo* and in cancer cell lines *in vitro*, suggesting that it is a potentially valuable target for novel anti-cancer strategies. Recent research has shown that dogs represent a potentially valuable model in which to investigate novel anti-cancer strategies, and are genetically more similar to humans than existing mouse models. In addition, telomerase activity in humans is more closely paralleled by telomerase activity in dogs than mice, adding to the value of this model. For these reasons, this thesis aims to investigate the telomerase as a diagnostic and therapeutic tool in canine cancer.

Several large studies have shown that 85-90% of all human tumours possess telomerase activity. Brain tumours represent an important subset of these because of the debilitating symptoms associated with their effects and because survival rates for the most malignant subtype, glioblastoma multiforme, remain unchanged despite advances in anticancer treatment strategies over the past thirty years. Their canine counterparts show strikingly similar features both at the histological and genomic levels, and for this reason offer an attractive model for the development of novel therapies. Given the ubiquity of telomerase activity in other tumour types, telomerase activity in human brain tumours has been extensively examined as a potential prognostic indicator. However, there appears to be a wide variation in the reported incidence of telomerase activity in brain tumours which is likely to be the result of problems associated with the methods used to date. Studies have suggested that methods detecting telomerase activity *in situ* may resolve some of these problems. For this reason, we set out to investigate the immunohistochemical detection of telomerase activity using a number of antibodies directed at TERT. One antibody, NCL-hTERT, was found to stain telomerase-positive D17 and CMT7 cells and telomerase-positive, formalin-fixed canine testis, at a concentration of 1/200. This antibody was then used to examine the correlation between TERT staining and malignancy in a panel of 93 canine brain tumours and to correlate telomerase activity with proliferation as assayed using the Ki-67 monoclonal antibody in a smaller subgroup. TERT staining was found to correlate significantly with malignancy in brain tumours overall as well as in the meningioma and oligodendroglioma subgroups. When TERT staining was correlated with proliferation, TERT staining was also found to correlate significantly with the MIB-1 labelling index in all tumours, and again in the meningioma and oligodendroglioma subgroups. These results suggest that the NCL-hTERT antibody represents a promising method with which to identify telomerase expression in formalin-fixed, paraffin-embedded material, and that the prevalence of telomerase activity in canine brain tumours is similar to their human counterparts.

Given the importance of telomerase activation as a means for attaining an immortalised phenotype, considerable research has focused on its inhibition. Several approaches have been investigated: using strategies interfering with the function of either TR or TERT, strategies targeting telomerase-

associated proteins, and strategies interfering with the interaction between telomerase and the telomere. A large number of naturally-occurring and synthesised molecules are now in existence that fall into one of these categories. We set out to investigate two of these in canine telomerase-positive cell lines: the dominant negative mutant of human TERT, DN-hTERT, and the G-quadruplex inhibitor telomestatin, isolated from *Streptomyces anulatus*. We tested the DN-hTERT dominant negative mutant by establishing cell lines stable transfected with this construct carried by the selection vector pCINeo, and found that over 50 population doublings, the DN-hTERT mutant failed to inhibit telomerase activity in telomerase-positive D17 and CMT7 cells. Over this period, growth rates and telomere lengths of transfected cells were similar to those of negative control mock-transfected cells. Surprisingly, the same construct reactivated telomerase activity in telomerase-negative K9SF fibroblasts. Moreover, K9SF cells transfected with DN-hTERT appeared to undergo morphological changes consistent with a cancerous phenotype and exhibited accelerated growth as compared to negative controls. Investigation of telomestatin treatment of telomerase-positive cell lines yielded more encouraging results. Treatment of D17 and CMT7 cells with telomestatin at varying concentrations showed that a concentration of 2 μM caused a decrease in telomerase activity by approximately 80% as compared with negative controls. Over a longer treatment period, telomestatin was also shown to cause slower growth and telomere shortening in these cell lines, together with an increase in the proportion of cells undergoing apoptosis. Treatment of K9SF cells, however, did not cause any change in growth rate or apoptosis. These findings suggest that telomestatin treatment specifically abrogates telomerase activity in canine telomerase-positive cell lines and warrants further investigation of this promising telomerase inhibitor in canine cells.

Several anticancer therapies have been developed using either tissue-specific or cancer-specific gene promoters to drive the expression of transgenes. The only truly cancer-specific promoters currently known are the promoters of the TR and TERT genes involved in telomerase activity. Since telomerase activity is dependent principally on regulation of the TERT gene, research as focused on the activity of this gene in normal and cancer tissues. Upregulation of the TERT gene depends primarily on transcriptional activity of its promoter, although post-transcriptional modification of the TERT transcript and methylation of the TERT promoter may also play a role. The human TERT promoter is GC-rich, has no TATA box and contains multiple binding sites for transcription factors including Sp1, c-Myc and p53. Using this promoter, several groups have developed a number of promoter-driven therapies to induce apoptosis or cell death by other means both *in vitro* and *in vivo* in nude mouse models, with considerable success. Some groups have also utilised the TR promoter in a similar fashion with good results.

In order to develop future TERT and TR promoter-driven therapies in dogs, we set out to sequence and characterise the canine TERT and TR promoters. We successfully sequenced approximately 5.4 Kb of the canine TERT promoter and created deletion constructs to identify the core promoter

responsible for activity in telomerase-positive cells following luciferase assays. The core promoter was identified as a region 314 bp upstream of the ATG start codon, and conveyed approximately 50% of the activity of a positive control vector in CMT7 and D17 cells. Importantly, inclusion of the initial part of the TERT gene with the core promoter reduced activity by approximately 75%, suggesting that important negative regulatory factors exist within the canine TERT gene. Transfection of other telomerase-positive cells with the core promoter construct showed promoter activity varied from between approximately 8% to 50% of the activity of the positive control. In addition, transfection of telomerase-negative fibroblasts with all deletion constructs showed that the activity of the TERT promoter was restricted to telomerase-positive cells. A number of potentially important putative transcription factor binding sites were identified within the core promoter, as well as within the TERT gene itself, although there were also significant differences between the canine promoter and its human counterpart. In addition to sequencing and characterising the TERT promoter, initial sequencing and analysis of the canine TR promoter was performed. In this experiment, genome walking was used to generate approximately 700 bp of the canine TR promoter sequence amplified from genomic DNA. The most remarkable finding in the TR promoter sequence was the presence of multiple $(TAAAA)_n$ repeats. Although the significance of these repeats is currently unknown, there is some evidence from the human literature to suggest that they are important for transcriptional regulation of some genes. Together, these experiments, by elucidating the canine TERT and TR promoters, provided an important tool that can be used in the design of future telomerase-specific promoter-driven therapies.

This thesis set out to investigate telomerase as tool for the diagnosis and treatment of canine cancer. The NCL-hTERT antibody investigation showed that the immunohistochemical recognition of TERT in paraffin-embedded tissue may provide a useful tool for the detection of telomerase in a wide range of canine cancers. It may also be useful as a means for examining telomerase activity in non-tumour tissues. However, further work still needs to be performed to characterise the true extent of telomerase activity in canine cancers, and to evaluate whether telomerase activity correlates with clinical outcome in dogs. The second part of this project was to investigate TERT as a target for therapy in canine cancers. We have shown that it is possible to inhibit telomerase activity in canine cancer cells, and that this leads to slowed growth, telomere shortening and cell death over a period of approximately 20 days in the cell lines examined. Finally, we have sequenced the canine TERT and TR promoters, and identified that core activity of the canine TERT promoter resides within a 314 bp region immediately upstream of the ATG start codon. Thus, we have identified two potential ways of utilising telomerase as a target in canine cancer therapy – firstly through direct inhibition of the enzyme, and secondly by making it possible to design TERT promoter-directed gene expression for the targeted killing of telomerase-positive cancer cells.

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DECLARATION

I, Sam Nicholas Long, do hereby declare that the work carried out in this thesis is original, was carried out by either myself or with due acknowledgement, and has not been presented for the award of a degree at any other university.

Sam N. Long
October 2005

PUBLICATIONS

Articles

Gene (2005); 358:111-20

THE CANINE TELOMERASE CATALYTIC SUBUNIT (dogTERT): CHARACTERISATION OF THE GENE PROMOTER AND IDENTIFICATION OF PROXIMAL CORE SEQUENCES NECESSARY FOR SPECIFIC TRANSCRIPTIONAL ACTIVITY IN CANINE TELOMERASE POSITIVE CELL LINES.

Long S, Argyle DJ, Gault EA, Campbell S, Nasir L.

Submitted (Neoplasia, 2005):

CORRELATION BETWEEN TELOMERASE REVERSE TRANSCRIPTASE (TERT) EXPRESSION AND MIB-1 EXPRESSION IN CANINE BRAIN TUMOURS – AN IMPORTANT ANIMAL MODEL FOR DEVELOPING TELOMERASE BASED THERAPEUTICS.

Long S, Nicholson I, Argyle DJ, Nixon C, Botteron C, Olby N, Platt S, Smith K, Rutteman G, Nasir L.

Conference Presentations

2005:

American College of Veterinary Internal Medicine Annual Conference, Baltimore, USA

SEQUENCING AND CHARACTERIZATION OF THE CANINE TELOMERASE REVERSE TRANSCRIPTASE (TERT) PROMOTER

Long, S.N., Gault, E., Nasir, L.

2004:

British Small Animal Veterinary Association Annual Conference, Birmingham, UK

ASSOCIATION BETWEEN TELOMERASE ACTIVITY AND MALIGNANCY IN CANINE BRAIN TUMOURS

Long S, Rutteman G, Platt S, Olby N, Nasir L

Poster Presentations:

2003:

Cancer Research UK / Beatson Laboratories Cell Cycle Conference, Glasgow, UK

TARGETING TELOMERASE IN CANINE TUMOURS

Long SN, Gault L, Argyle DJ, Nasir L.

CHAPTER I:

GENERAL INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 Abstract

In 1881, the first theories concerning the ageing of somatic cells were proposed by a German biologist, August Weissman. However, definitive proof that somatic cells possess a finite proliferative lifespan was not published until the 1960s, by Leonard Hayflick and Paul Moorhead. In demonstrating that somatic cells are unable to replicate indefinitely, they introduced the concept of an internal mechanism, or replicometer, capable of counting the number of cell divisions that a cell has undergone. This replicometer was then shown to be a structure comprising the terminal end of linear chromosomes, the telomere, in 1998. This structure is composed of a variable number of highly conserved repeat sequences, shown in vertebrates to be TTAGGG. The functions of the telomere are thought, at least in part, to be due to its ability to assume a number of tertiary structures, the most important of which is the T-loop. With every cell division, the terminal portion of the telomere is lost due to the inability of the DNA replication machinery to fully replicate the end of linear chromosomes, a phenomenon known as the 'end replication problem'. Ultimately, cumulative telomere shortening following successive rounds of mitotic division has been shown to trigger the onset of replicative senescence or crisis in somatic cells of a number of different lineages, mediated by the p53 and p16/Rb pathways. Thus, the discovery of telomere shortening introduced a major new hypothesis explaining the ageing of somatic tissues.

A number of organisms such as protozoa are able to replicate indefinitely, and a means by which cells of these organisms bypass the ageing process was uncovered in 1985 by Carol Greider and Elizabeth Blackburn through the discovery of the enzyme telomerase. This enzyme, composed of a reverse transcriptase catalytic subunit (TERT), an RNA template (TR) and a number of associated proteins, is capable of adding nucleotides to the end of telomeres, and thus circumventing telomere shortening. *In vitro*, enzyme function can be reconstituted with the TERT and TR components alone, and studies with fibroblasts have shown that expression of the TERT subunit is the principal determinant of telomerase activity. Telomerase activity *in vivo* is absent from the vast majority of somatic tissues in dogs and humans, but is present in some stem cell and germ cell populations. It is also present in the majority of cancers in humans *in vivo* and in cancer cell lines *in vitro*, suggesting that it is a potentially valuable target for novel anti-cancer strategies. Recent research has shown that dogs represent a potentially valuable model in which to investigate novel anti-cancer strategies, and are genetically more similar to humans than existing mouse models. In addition, telomerase activity in humans is more closely paralleled by telomerase activity in dogs than mice, adding to the value of this model. For these reasons, this thesis aims to investigate the value of telomerase as a diagnostic and therapeutic tool in canine cancer.

1.2 Historic Perspective

In 1881, German biologist August Weismann proposed that 'death takes place because a worn-out tissue cannot forever renew itself, and because a capacity for increase by means of cell division is not everlasting, but finite' (Shay & Wright 2000). However, this concept was challenged, and the opposite dogma – namely that normal human cells are (at least *in vitro*) able to replicate indefinitely – held sway up until the 1960s. Then, Leonard Hayflick and Paul Moorhead performed a series of experiments demonstrating the finite replicative capacity of normal human fibroblasts (Hayflick & Moorhead 1961; Hayflick 1965). In 1961, Hayflick and Moorhead showed that populations of cultured human fibroblasts doubled a finite number of times, after which cells stopped dividing and entered what Hayflick termed the 'phase III' phenomenon. He called the primary culture period phase I, the subsequent period of expansive growth phase II, and the period when cell replication diminished and ultimately stopped, phase III (Hayflick & Moorhead 1961). The experiments which showed this were elegant in their simplicity. Normal human male fibroblasts at the fortieth population doubling were mixed in equal numbers with female fibroblasts at the tenth population doubling. Cells from each population were also maintained unmixed to serve as controls. At the point at which the male control population stopped dividing, Hayflick showed that the only remaining dividing fibroblasts in the mixed culture were female. This showed that the older cells retained a 'memory' of their age, even when surrounded by young cells, and that other factors, such as culture conditions, could not be the reason for the cessation of cell division. From these experiments arose the concept that normal cells have a finite capacity to replicate. Eventually, this work led Sir Macfarlane Burnett to coin the phrase 'the Hayflick Limit' for the first time in 1974 as a means of describing a cell's natural limit to replication (Burnett 1974).

1.2.1 The Consequences of Senescence

Hayflick, as well as noting that normal somatic cells have a finite proliferative lifespan, also made the observation that the end of that proliferative lifespan results in a quiescent, yet viable state which is now known as replicative senescence (Wynford-Thomas 1999). In this state, the cell-cycle is arrested in the G0 phase of division and a number of changes in gene expression occur that differentiate senescent cells from younger, actively growing cells (Shelton *et al.* 1999). These changes vary depending on the cell type studied, but include permanent growth arrest, repression of c-fos, repression of cyclin A and B and β galactosidase activity (summarised in (Kipling 2001)). Similar behaviour has been observed in a wide variety of normal cell types, both mesenchymal and epithelial (Wynford-Thomas 1999). In the approach to replicative senescence, cells show a

gradual decline in the proportion of dividing cells within the population, and the timing of this decline varies between cell types and between sister clones (Thomas *et al.* 1997).

One of the most important implications of the finite lifespan of cells within tissues concerns the ageing process. It is perhaps useful when considering ageing in general to look at the interrelated processes that may be involved within any given tissue. Firstly, ageing may occur due to changes in the extracellular environment (such as the extracellular matrix, basement membranes etc). Secondly, ageing may occur due to changes within cells that are incapable of further division (eg mature neurons). Finally, ageing may occur due to changes within populations of dividing cells (Kipling 2001). Replicative senescence is likely to contribute to the ageing of any tissue which contains a significant number of dividing cells. For example, in those tissues for which cell division is an important part of day-to-day function such as the gastrointestinal tract, immune system and hair follicles, a reduction in the number of actively dividing cells is likely to have an impact on normal activity. Cell division is also important in other tissues for which proliferation is not an active feature, but which retain the ability to divide in response to damage or cell loss. This includes, for example, the ability of skin to undergo a localised burst of cell proliferation as part of wound healing following trauma. In addition, cell division is required within tissues to compensate for physiological cell loss through wear and tear. In all of these situations, the process of cell division in older cell populations has the potential to produce senescent cells. Accumulation of senescent cells could then result in an altered tissue microenvironment, and produce an aged phenotype in affected tissues. For example, delayed wound healing is a hallmark of old age in people, consistent with this theory (Kipling 2001).

1.2.2 Telomeres: the 'replicometer'

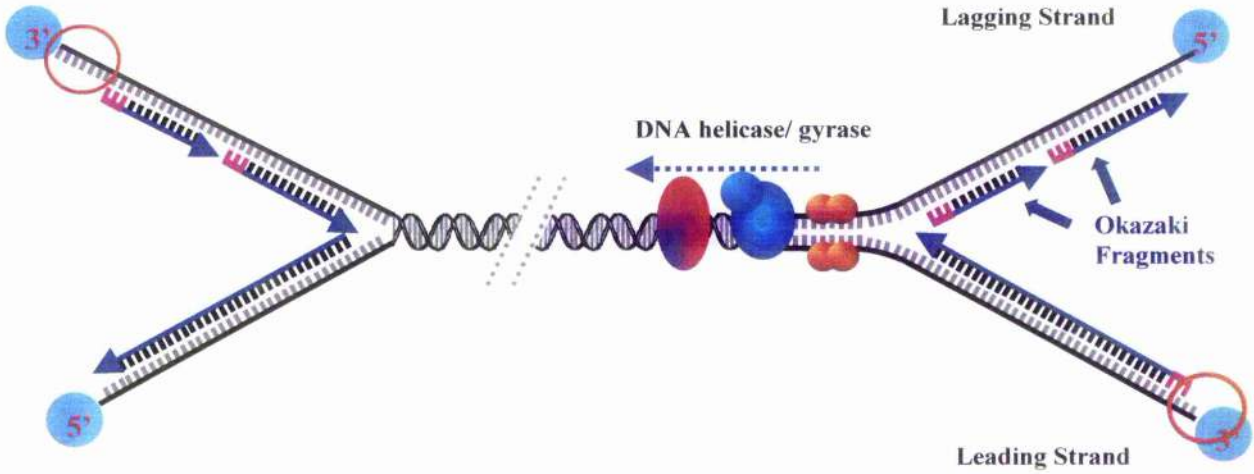
In order to explain a limit to cellular proliferation, it is necessary to introduce another concept – that of a clock, or counting mechanism within each cell that could somehow track the number of divisions performed by the cell. This was first implied by two of Hayflick's findings – first, that normal cultured fetal cells undergo a finite number of population doublings, and second, that cryogenically preserved cells can 'remember' how many times they have divided, even after being frozen. From this, the term 'replicometer' arose (Hayflick 1998), which in 1975 was shown to be located within the nucleus (Wright & Hayflick 1975).

Meanwhile, the discovery of DNA structure and replication in the early 1970s had revealed an inherent problem. Because of the nature of lagging strand synthesis, it was realised that cells are unable to fully copy the ends of linear DNA. This is termed the 'end replication problem'. During the S phase of the cell cycle, DNA polymerase is unable to replicate the 3' end of a linear DNA molecule. Lagging strand DNA synthesis proceeds as a series of discrete events with the

formation of Okazaki fragments, each of which requires an RNA primer. As there is no DNA beyond the 3' end of the chromosome to which an RNA primer can anneal, DNA polymerase cannot fill in the gap between the final Okazaki fragment and the end of the chromosome. This is shown in Figure 1.1. Therefore, with every round of cell division, the terminal portions of the chromosome shorten slightly, with daughter chromatids inheriting slightly shorter chromosomes than their parent chromosomes.

Figure 1.1: DNA replication and the end replication problem

During DNA replication, the lagging strand is synthesised using Okazaki fragments. However, at the 3' end of the lagging strand (circled) there is a gap that can not completely be reproduced because of the absence of a suitable primer.



Eukaryotic chromosome undergoing
DNA replication

The first postulated mechanism that could act as a replicometer was proposed by Olovnikov in 1973, not long after Watson and Crick had unravelled DNA replication (Olovnikov 1973). He proposed that the progressive erosion of the ends of chromosomes with each cell division as a result of incomplete replication of one end of each DNA strand could act as a biological clock. Prior to this, the term *telomere* itself had first been used by Muller in 1938 in order to describe the terminal elements of linear chromosomes (Muller 1962). The theory that, in humans at least, the functions of the replicometer are provided by telomeres, was finally confirmed by scientists at the Geron institute in 1998, with the finding that mechanisms that extend telomeres in fibroblasts bypass replicative senescence (Bodnar *et al.* 1998). Since then, a large body of research has advanced our knowledge of the structure and function of telomeres. A review of the current understanding of telomere structure and function will be presented here as a background prior to discussing telomerase.

1.3 Telomeres: structure and function

1.3.1 Telomere Structure

Telomeres comprise the terminal portion of linear chromosomes and consist of a complex of DNA together with a number of telomere-associated proteins (TAP), the function of many of which are as yet unknown.

1.3.1.1 Telomeric DNA

The DNA structure at the ends of chromosomes is remarkably conserved across all eukaryotes. The telomeric DNA sequences have been generally subdivided into 3 distinct areas: telomere associated sequences (TAS), double stranded telomeric repeats, and the terminal structure at the very end of the chromosomal DNA.

1.3.1.1.1 Telomere Associated Sequences

In all organisms in which sequencing of subtelomeric areas has been performed, the region of DNA proximal to the chromosome terminus contains a variety of repeated elements known as Telomere Associated Sequences (TAS) (Biessmann & Mason 1992; Louis 1995). The actual amounts of TAS vary between chromosomes, even within the same species, and may make up a considerable portion of the total genome (Wellinger & Sen 1997). For example, allelic variation in the short arm of human chromosome 16 can lead to variation in TAS length of between 170 and 450 kb (Wilkie *et al.* 1991). There are two different types of elements found in these subtelomeric areas. Relatively complex, middle-repetitive elements, varying from a few hundred to a few

thousand bp have been found in several species, including plants, fungi, invertebrates and vertebrates (including humans) (Bedbrook *et al.* 1980; Richards *et al.* 1992; Louis *et al.* 1994; Rubin 1978; Lopez *et al.* 1996; de Lange *et al.* 1990; Rouyer *et al.* 1990), although the location and copy number of individual repeats are variable. Interspersed in between and within these complex repeats are shorter, tandemly repeated satellite-like sequences. These repeats of 20-1000 bp seem to recombine and rearrange with high frequency, and may be the reason for the high degree of polymorphism in subtelomeric chromosomal regions (Louis 1995; Simmler *et al.* 1985; de Bruin *et al.* 1994). In addition to the complex repeats and short satellite-like repeats, in some yeasts, copies of genes have been found interspersed within some (but not all) of these subtelomeric regions (Louis 1995; Michels *et al.* 1992). However, in general, the subtelomeric areas contain very low numbers of genes and transcriptional activity (Wellinger & Sen 1997).

The reasons for the existence of these large regions of (generally) non-encoding DNA are unknown. A number of theories concerning their possible function have been proposed. Since, in yeast and Drosophila, genes located close to an actual chromosome end are subject to transcriptional repression (Levis *et al.* 1985; Gottschling *et al.* 1990; Nimmo *et al.* 1994), it has been argued that TAS provide a buffer zone between the actual terminal telomeric structure and more internal chromosomal domains (Louis 1995; Wright & Shay 1992). Secondly, they could also provide a region in which genes can undergo adaptive changes via amplification and recombination (de Bruin *et al.* 1994). Thirdly, since terminal telomeric repeats are identical for all telomeres in any given species, it could be that the highly variable arrays of the different TAS specify telomere to telomere interactions. This would explain the finding that telomeres interact with each other non-randomly during mitosis and meiosis in several species (Gilson *et al.* 1993). Finally, TAS have been shown to provide alternative mechanisms for telomere maintenance via recombination or transposition in cases where the main telomere replication mechanism via telomerase is absent (Lundblad & Blackburn 1993).

1.3.1.1.2 Double-stranded Telomeric Repeats

Whilst the repeat sequence of the TAS of different species bear little resemblance to each other, the sequence found at the very end of the chromosome is astonishingly well conserved (see Table 1.1). The first terminal sequences to be identified were those of some ciliates such as *Tetrahymena* (Blackburn & Gall 1978). For most species, the terminal repeats consist of short (6-8 bp) tandemly repeated sequences. In all vertebrates so far characterised, the telomere repeats are comprised of the 6 bp sequence TTAGGG. The actual number of repeats per telomere varies greatly between organisms. In some ciliates, such as *Oxytricha* and *Euplotes*, there may be as few as 2-3 repeats, 20-30 bp in all (Klobutcher *et al.* 1981). In others, such as mice, there may be more than 10,000 copies, occupying more than 60-100 kb (Kipling & Cooke 1990; Starling *et al.* 1990).

As well as varying between organisms, the amount of telomeric repeats also varies between the chromosomes of the same cell, and may also change with growth and ageing (Slijepcevic & Hande 1999). The actual length of telomeric repeats are the result of a complicated interplay of competing activities which either lengthen or shorten the telomere (Biessmann & Mason 1992; Zakian 1995). For instance, whilst the telomeres in human cells contain from 500 to >2000 repeats (3 – 12 kb of telomeric repeat sequence), they gradually diminish in cycling cells of most somatic lineages (Moyzis *et al.* 1988; Harley *et al.* 1990). This variability in telomere length from a given species results in a very heterogenous collection of restriction fragments following digestion with endonucleases specific to the telomere sequence, and consequently a 'smeary' appearance of telomeric bands on southern blots.

Due to the base composition of the telomeric repeat sequence, one strand of the telomere is usually rich in clusters of G residues. This strand, which in most species lacks any C residues, is known as the G strand, with its complementary partner known as the C strand. In those species which possess a G strand, the strand always forms the extreme 3' end of the chromosome (Wellinger & Sen 1997).

Whilst the majority of telomeric repeats occur at the end of chromosomes, it is important to note that telomeric repeats also occur at subtelomeric and non-telomeric chromosomal loci (Delany *et al.* 2000). The reason for these repeats existing at locations removed from the chromosome ends is unknown, but they may be remnants of recombination or transposition events involving telomeric repeats (Cherry & Blackburn 1985; Rossi *et al.* 1993). Alternatively, they may indicate telomere fusion points of ancestral chromosomes now joined together (Ijdo *et al.* 1991).

Table 1.1: Telomeric repeat sequences of selected organisms

Organism	Repeat Sequence
Protozoa	
Tetrahymena spp.	TTGGGG
Euplotes spp.	TTTTGGGG
Trypanosoma spp.	TTAGGG
Fungi	
Histoplasma spp.	TTAGGG
Kluyveromyces spp.	ACGGATTTGATTAGGTATGTGGTGT
Candida albicans	ACGGATGTCTAACTTCTTGGTGT
Saccharomyces cerevisiae	$(TG)_{1\text{-}6}TGG_{2\text{-}3}$
Invertebrates	
Ascaris spp.	TTAGGC
Parascaris spp.	TTGCA
Vertebrates	
Homo sapiens	TTAGGG
Mus musculus	TTAGGG
Many other vertebrates	TTAGGG

1.3.1.1.3 The terminal part of the chromosome

Although several of the functions of telomeres are likely to relate to the functions conferred by the structures at the very end, relatively little is known about these physical structures. End-labelling studies using linear rDNA molecules suggest that an overhang of the G strand over the C strand exists as a conserved feature of all eukaryotic chromosomes, although the length of this overhang varies between species and organisms (Henderson & Blackburn 1989; Zahler & Prescott 1989; Wellinger *et al.* 1996). In humans, this overhang is several hundred nucleotides in length and plays an important role in the formation of the T-loop (see 1.3.1.3: Three dimensional structures) which seems to provide many of the protective roles conveyed by telomeres (Huffman *et al.* 2000; Wright *et al.* 1997). Whilst the 3' overhang is important for telomere function, the mechanisms which allow for its existence are not fully known. Following lagging strand synthesis during DNA replication, removal of the last RNA primer is unlikely to leave an overhang of the required length. It is generally believed, therefore, that maintenance of 3' protrusions in dividing cells requires an active exonucleolytic process at the C strand (de Lange 2002). Certainly, C-strand processing is required at the ends generated by leading strand DNA synthesis, since synthesis of the leading strand would be expected to produce a blunt end.

1.3.1.2 Telomere-associated proteins

A large number of proteins have been identified which either bind to telomeric sequence directly or can be co-purified with telomeric sequence. Although the function of some of these proteins has been partially elucidated, the function of several has not. These are summarised in Table 1.2 and will be discussed further in section 1.3.1.4: Assembling the telomere.

Table 1.2: Telomere-associated proteins identified in mammalian cells

Name	Role at Telomere
POT1	Binds single-stranded TTAGGG repeats
	Necessary for telomere length maintenance and protection
TRF1	Present in T loops
	Binds double-stranded TTAGGG repeats
	Negative regulator of telomere length (dependent on telomerase)
TRF2	Present in T loops
	Binds double stranded TTAGGG repeats
	Negative regulator of telomere length (independent of telomerase)
	Loss of function causes end-to-end fusions
	Loss of function leads to apoptosis and telomere-induced senescence
TANK1	Telomere-associated poly(ADP-ribose) polymerase 1
	Ribosylates TRF1
	Positive regulator of telomere length
TANK2	Telomere-associated poly(ADP-ribose) polymerase 2
	Ribosylates TRF1
	Positive regulator of telomere length
TIN2	TRF1-binding protein
	Regulates telomere length
RAD50	DNA-repair complex that binds TRF2
NBS1	Possible role in T loop formation
MRE11	Negative role in telomere length regulation
Ku86	Negative regulator of telomere length
	Role in telomere capping
DNA-PKcs	Role in telomere capping
	Putative role in post-replicative processing of telomeres

1.3.1.3 Three dimensional structures

Considerable attention has focused on the three dimensional arrangements assumed by the telomere, since it is thought that many of its functions are related to properties conveyed by these physical structures. The T-loop model has been shown to exist in vitro, whilst the G-quadruplex model has largely been proposed using theoretical modelling techniques. However, as will be discussed in Chapter 4, the G-quadruplex model has important implications for the clinical treatment of cancer.

1.3.1.3.1 The T-loop model

When the telomere-binding protein TRF2 is inhibited, telomeric DNA remains largely intact, yet there is a rapid and catastrophic loss of telomere function. Since TRF2 only binds duplex, and not single-stranded TTAGGG sequence, it would seem unlikely that loss of protection of the 3' overhang by TRF2 is responsible for this. A neat solution to this puzzle was provided by Griffith and colleagues, who showed that TRF2 is capable of remodelling telomeric DNA into a structure known as the T loop (Griffith *et al.* 1999). When they provided the TRF2 protein with a model telomeric DNA substrate consisting of a stretch of 0.5 kb duplex TTAGGG repeats and a short 3' overhang, they found that loops of approximately 0.5 kb in size and containing a large amount of TRF2 at their base were formed. When the DNA was then treated to generate interstrand crosslinks with psoralen and UV irradiation and the TRF2 subsequently removed, these loops were stable, showing that formation involved the invasion of the duplex strand by the 3' overhang (Griffith *et al.* 1999).

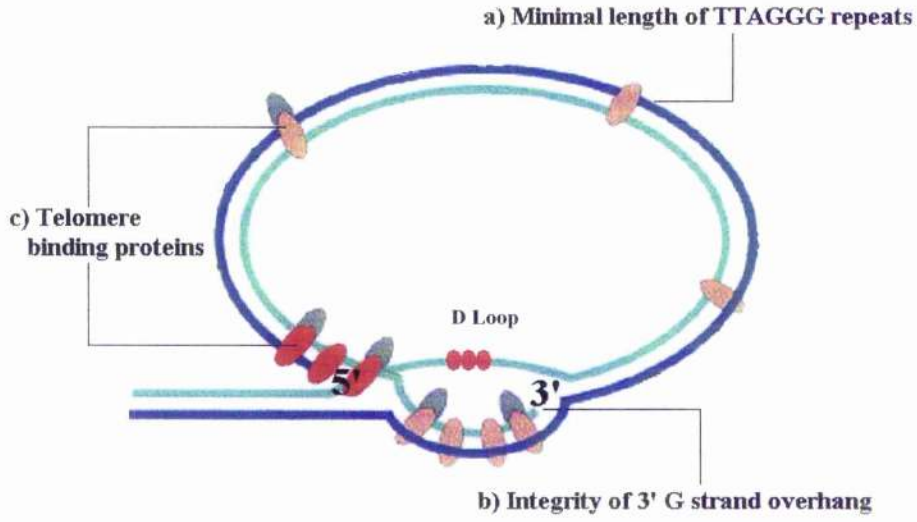
T-loops are large duplex telomeric loops that are formed when the 3' overhang enters the duplex part of the telomere repeat sequence (see Figure 1.2) (Griffith *et al.* 1999). The size of the loop can range from 0.3 kb in trypanosomes up to 30 kb in mice, and in some cases encompasses the whole telomere. Whilst the exact structure of the base of the T loop is not known, the crucial feature appears to be invasion of the double-stranded telomeric DNA by the telomere terminus, which in turn displaces a short segment of single-stranded DNA, composed of TTAGGG repeats, in a D-loop. This displaced segment is in the order of a few hundred nucleotides in many of the T loops (Griffith *et al.* 1999).

The T-loop would provide a neat architectural solution to the problem of telomere protection. By sequestering the 3' overhang, the telomere terminus is prevented from being mistaken for a site of DNA damage (de Lange 2002). The T-loop structure resembles a DNA recombination

intermediate as it occurs during reinitiation of a stalled replication fork or during homologous recombination (de Lange & Petrini 2000; Griffith *et al.* 1999).

In addition, the T-loop solution to telomere capping relies on the most conserved feature of eukaryotic telomeres – the presence of tandem telomeric repeats ending in a single stranded overhang (de Lange 2002).

Figure 1.2: T-loop Structure. T-loop function requires at least 3 factors: a) Minimal length of TTAGGG repeats b) Integrity of 3' G strand overhang c) Telomere-binding proteins



1.3.1.3.2 The G-quadruplex model

In 1987, Oka and Thomas reported an unusual cohesive structure formed by telomeric DNA purified from the protozoan *Oxytricha* (Oka & Thomas, Jr. 1987). Further investigation revealed that telomeric G-rich sequences were able to form higher-order structures such as G-G base pairs and four-stranded structures called G-quadruplexes, G-quartets or G4-DNA (Williamson *et al.* 1989). Although an extensive body of literature has reported on the properties of some of these structures and proposed indirect evidence for their importance of telomere biology, they have not been reported to form under physiological salt and temperature conditions *in vitro* (Phan & Mergny 2002).

The potential formation of quadruplex complexes by telomeric G-strand sequences was first deduced from gel-mobility shift assays and chemical protection data (Sundquist & Klug 1989; Williamson *et al.* 1988). The quadruplex is formed by the chelation of a monovalent cation (either sodium or potassium) between two adjacent guanine quartets, each of which is formed by base-pairing between guanine residues. Two broad classes of structure, 'parallel' and 'anti-parallel'-stranded quadruplexes were found to be formed, dependent on which cation is chelated. All of the proposed G-quadruplexes show three important properties: 1) structural polymorphism, 2) unusual interactions with specific monovalent and divalent cations, and 3) high thermodynamic stability with extraordinarily slow kinetics of formation and unravelling.

The possible existence of G-quadruplexes has led to several theories explaining their functions *in vivo*. Firstly, the forming of G-G bonds within the quadruplex, rather than conventional Watson-Crick base-pairing, may represent a recognition of self. In this way, two or four identical single stranded DNA molecules can recognise one another and bind specifically via their G-rich termini (Sen & Gilbert 1988; Sundquist & Klug 1989). This self-recognition of telomeres may occur at the onset of meiotic pairing of chromosomes. Secondly, the formation of an alternative DNA structure, such as telomeric G-quadruplexes, at the late-S/G2 phase might serve as a signal for the completion of chromosome replication, and the cell cycle to progress normally without being recognised as a double stranded DNA break. Finally, G-quadruplexes, since they are an alternative form of DNA, are not a substrate for elongation by telomerase. This is due both to the inability of the telomerase primers to access the telomere in this form, and to limited extension of primers by telomerase after binding. In this way, the formation of G-quadruplexes might regulate telomere replication (Wellinger & Sen 1997).

Although telomeric DNA does not apparently exist as a G-quadruplex under physiological conditions, strong evidence for the importance of this structure in telomere biology has come from

the activity of a class of telomerase-inhibiting agents. These agents, which include drugs such as telomestatin and TMPyP4, bind and stabilise G-quadruplexes (Kim *et al.* 2003a; Seenisamy *et al.* 2005) and efficiently inhibit telomerase activity (reviewed in greater detail in Chapter 4). Interestingly, these agents require 5-7 days for complete inhibition of telomerase activity in human cells, possibly reflecting the time required for conversion of all of the telomeric DNA into G-quadruplexes (Shammas *et al.* 2004). Following the application of these agents to telomerase-positive cells, telomere shortening and ultimately cell death occurs, after a lag phase of 10-14 days. This would confirm the important role that the G-quadruplex plays in regulating telomerase access to the telomere and thus regulating telomere extension.

1.3.1.4 Assembling the telomere

The interaction of the large number of proteins that bind telomeres is complex, and is likely to regulate both telomere length and function at several different levels. In yeast, telomere lengths appear to be regulated by a mechanism that involves counting the number of proteins bound to telomere ends. It has been suggested that a similar mechanism exists for mammalian telomeres, possibly through counting of the telomere-binding proteins TRF1 and TRF2, since overexpression of both proteins induces gradual shortening of telomeres, probably associated with accumulation of TRF1 and TRF2 molecules at telomeres (Smogorzewska *et al.* 2000).

It would seem that formation of the T-loop requires the action of both TRF1 and TRF2, linked by TIN2. TRF1 binds to the duplex telomeric DNA and is capable of looping and pairing duplex DNA *in vitro* (Bianchi *et al.* 1997; Bianchi *et al.* 1999). Since TRF2 promotes T-loop formation *in vitro* and localises to the junction where the 3' overhang enters the duplex tract, it seems likely that TRF2 aids invasion of the strand into the duplex, either through partially unwinding the duplex telomeric repeat array, or stabilising the displacement loop (Griffith *et al.* 1999) (Figure 1.2).

Taken together, these findings suggest that TRF1 and TRF2 at least partly regulate telomere length through their ability to form T-loops. At the same time, these proteins are intimately involved in the physical capping of telomeres and the processes which would cause end to end fusions and double strand break repair. When the T-loop is formed, the major telomere-elongating processes in mammalian cells are unable to access the telomere and elongate it. Alternatively, when TRF1 and TRF2 are not bound in sufficient quantities the T-loop is not formed, and the telomere assumes an 'open' configuration. In this state, the exposure of the 3' G-strand overhang leads to access by DNA-PK and the MRE11 complex, as well as TANK1 and TANK2. In turn this leads to NHEJ, homologous recombination and end-to-end fusions.

1.3.1.5 Telomere lengths in humans and animals

In humans, telomeric DNA (excluding the subtelomeric regions) comprises on average between 5 and 15 kb (Moyzis *et al.* 1988). In comparison, mice telomeres are between 20 and 150 kb in length and are highly polymorphic between inbred strains (Kipling & Cooke 1990; Starling *et al* 1990). This large difference between human and murine telomeres initially complicated research into telomere function, since first generation $mTR^{-/-}$ knockout mice, which were engineered in order to study the effects of telomere shortening, failed to show any ill-effects associated with telomere dysfunction (Blasco *et al.* 1997). It was only after four generations of these mice had been bred that accumulated telomere shortening allowed the appearance of an early-ageing phenotype, consistent with proposed theories of telomere function (see below).

In another small rodent, the Chinese hamster, telomere lengths are also considerably longer than in humans, at approximately 38 kb, although the telomere length varies between individual chromosomes (Slijepcevic & Hande 1999). In birds, considerable variation in telomere length has been reported, with telomere lengths ranging from 0.5 to 40 kb in length (Delany *et al.* 2000; Hall *et al.* 2004). In addition arrays of telomeric repeats several megabases in length are often found. In pigs, telomere lengths vary significantly between tissues, as does telomerase activity, but in general appear to be shorter than mouse telomeres, with most tissues carrying telomeres between 10 and 30 kb in length (Fradiani *et al.* 2004). Another large mammal, the cow, seems to possess telomeres of similar size to those found in humans, ranging between approximately 12 and 20 kb in length (Miyashita *et al.* 2002). Along with the cow, perhaps the dog possesses telomeres most similar in length to human telomeres, ranging between 11 and 23 kb (Nasir *et al.* 2001; Yazawa *et al.* 2001).

1.3.2 Telomere Function

Specific functions attributed to telomeres include a role in cellular ageing, a gene regulatory role through suppression of genes in the subtelomeric regions, a role in nuclear organisation (as seen in their ability to attach to the nuclear matrix) and the prevention of chromosomes from illegitimate recombination and fusion. (Carroll *et al.* 1999). However, as shall be discussed below, it is likely that we do not yet know all of the functions for which telomeres are responsible. A brief review of the major functions of telomeres will be provided here.

1.3.2.1 Telomeres, senescence and crisis

If human dermal fibroblasts are taken and cultured in the laboratory, the result is a cell strain which continues to divide typically to 50 population doublings (Hayfflick 1965; Hayflick & Moorhead 1961). At the end of this period, the cells enter a viable state known as senescence. The mechanisms involved in replicative senescence have been explained through a series of checkpoints known as the M1/M2 (mortality 1, mortality 2) checkpoints. Continued cellular proliferation occurs up to a certain point, at which cell division ceases and cells become arrested in a G0 or G1-like stage. This is the onset of cellular senescence, also known as mortality stage 1 (M1). At this point, the 'replicometer' informs the cell that a certain number of cell divisions have elapsed, and thus triggers a senescent phenotype.

Those cells that are able to successfully avoid the functions of the p53/p16/Rb tumour suppressor pathways proteins, either naturally or following transfection with the DNA tumour virus genes SV40T or HPVE6/7, can continue to proliferate until they reach another checkpoint of growth arrest known as mortality stage 2 (M2 – also known as crisis) (Wynford-Thomas 1999). Crisis differs from senescence in that growth arrest is due to an increase in cell death rate rather than a decrease in cell proliferation. Other features of M2 include chromosomal abnormalities, particularly end-to-end fusions, and widespread apoptosis. M2 is thought to be induced by critically shortened telomeres that have lost telomeric repeats to the extent that genomic instability and cell death ensue. Bypassing M2 can occur rarely as a spontaneous event (at a frequency of around 1 in 10^7 cell divisions in human fibroblasts), but requires the stabilisation of telomere length, the most common method being through the activity of the enzyme telomerase (Sallinen *et al.* 1997; Carroll *et al.* 1999)

It is now known that, at least in fibroblasts, the function of the replicometer is served by telomeres. However, other mechanisms may serve this function in other species or cell types (Wynford-Thomas 1999), and other potential candidates include changes in gene methylation observed in senescing cultures and the accumulation of random DNA damage (Holliday 1996; von Zglinicki *et al.* 1995). Three important observations all helped support the theory that telomeres are the replicometer in somatic cells, at least in humans. Firstly, human somatic cells were found to have shorter telomeres than sperm from the same individuals, suggesting that human telomeres shorten during development (de Lange *et al.* 1990; Hastie *et al.* 1990). Secondly, telomere lengths in fibroblasts and leukocytes from older individuals are shorter on average when compared with telomere lengths in somatic cells from younger people (Hastie *et al.* 1990; Harley *et al.* 1990). Thirdly, telomeres shorten during the passage of fibroblasts in culture (Hastie *et al.* 1990; Harley *et al.* 1990). All of these findings taken together suggest that telomere-shortening, ageing and

senescence are inextricably linked, confirming the theory that telomeres function as the replicometer in human cells.

Induction of senescence occurs through a number of pathways. One of the most important of these involves p53 tumour suppressor gene function (Wynford-Thomas 1999). Whilst early studies using DNA tumour virus genes that allow fibroblasts to escape senescence suggested that loss of both the p53 and the pRb tumour suppressor genes was required to escape senescence, more recent studies have shown that loss of p53 is sufficient (Bond *et al.* 1994; Rogan *et al.* 1995), showing that p53 forms part of an essential senescence-inducing pathway. The details of the mechanism by which p53 is activated, however, remain to be fully elucidated. Activation of p53 in turn leads to up-regulation of the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1}$, which is involved with direct inhibition of the cell cycle machinery and senescence, illustrated by the close correlation between p21 expression and decreasing cell proliferation (Noda *et al.* 1994; Brown *et al.* 1997).

The onset of senescence in fibroblasts also correlates with up-regulation of $p16^{INK4a}$, another CDK inhibitor (Alcorta *et al.* 1996; Hara *et al.* 1996). This provides a p53-independent pathway to cell cycle inhibition. However, both p16 and p21 converge on pRb, suggesting that the common endpoint for both pathways is the up-regulation of pRb expression. In fibroblasts, these two pathways represent a backup plan in the event of uncontrolled growth. If p53 function is lost, for instance through the expression of the HPV16E6 or mp53 genes, but p16/Rb function retained, cells escape senescence but undergo growth arrest before reaching the M2 checkpoint (Bond *et al.* 1999). At this growth checkpoint, cell morphology is indistinguishable from M1. Thus, in fibroblasts the p16/Rb pathway provides an alternative means of inducing senescence if p53 function is lost.

Other cell types studied seem to utilise the p53 and p16 pathways in the induction of M1. However, in other cells, the order in which these pathways are induced varies. For example, when primary breast epithelial cells are cultured, a subpopulation arises which is able to undergo 30-40 population doublings before the onset of M1 (Wynford-Thomas 1997). Initial studies showed that abrogation of p53 alone through the expression of HPVE6 in these cells allows further proliferation up to M2 (Shay *et al.* 1993). Although these results apparently suggest that the p16 pathway is non-functional in these cells, later work revealed that, in fact, the p16 pathway had been suppressed prior to the onset of M1, through methylation of the p16 promoter (Foster *et al.* 1998; Brenner *et al.* 1998). Therefore, these cells utilise both pathways, but rely mainly on activation of p53 as a barrier to proliferation, having abrogated p16 early in life. However, the end result in both examples is the upregulation of pRb and the onset of cellular senescence.

1.3.2.2 Telomeres distinguish chromosome ends from DNA strand breaks

The identification of telomeres began with the work of Hermann J Muller in 1938 (Muller 1962). In irradiating Drosopholia with X-rays to produce mutations, Muller noticed that while ends of fragments produced by radiation could be rejoined with other broken ends to produce chromosomal rearrangements, the natural ends of chromosomes did not fuse to other fragment ends (Muller 1962). Therefore, he proposed, a structure existed at the terminus of the chromosome which identified the chromosome end and effectively 'sealed' it from fusion with other chromosomes. This work was then augmented by Barbara McClintock, working with maize (McClintock 1941). She engineered the formation of dicentric chromosomes by allowing rearranged chromosomes to recombine during meiosis. In doing so, she showed that the movement of the two centromeres to opposite spindle poles during cell division broke the dicentric chromosome at a variable location. The ends of these broken chromosomes, which were inherited by the daughter cells, could fuse with the ends of their broken sister chromatids prior to mitosis, producing a breakage-fusion-bridge cycle that resulted in either gene amplification or deletion. McClintock proposed that telomeres protected normal chromosome ends from this process, further showing that telomeres are structures that are distinct from DNA breaks and which provide protection from processes that fuse broken chromosomes.

This theory has been supported by findings in knockout mice deficient in the RNA component of telomerase. *mTR*$^{-/-}$ mice, unlike their wild-type counterparts, lack telomerase activity in somatic tissues. As a result, telomeres shorten in tissues with progressive cell divisions. Although mice possess considerably larger telomeres than several other vertebrates studied, continued breeding of *mTR*$^{-/-}$ mice eventually results in a phenotype with very short telomeres. In these late generation mice, there is a considerable increase in the number of signal-free ends (telomeres without detectable telomere repeats when analysed by FISH) and in end-to-end chromosome fusions (Blasco *et al.* 1997; Hemann *et al.* 2001). Chromosome ends that most frequently lack a FISH signal preferentially participate in end-to-end fusions, confirming that short or absent telomeres result in end-to-end fusions or non-homologous end joining (NHEJ).

1.3.2.3 Exceptions to the rule: problems with the current theories concerning telomere function

Whilst there is a considerable body of circumstantial evidence that supports the functions of telomeres as protecting chromosome ends and providing a mechanism for cell ageing, several observations do not easily fit this picture and suggest other functions as yet not fully established:

1. Many eukaryotic cell types devote more of their resources to their chromosome ends than should be required simply to stop the loss of a few nucleotides at each replication. For examples, human chromosomes have up to 15 kb of telomeric DNA on each chromosome end – considerably more than should be necessary to keep ahead of sequence loss.
2. The total amount of telomeric DNA is regulated in species-specific and cell type-specific ways, suggesting that the length of telomeric DNA is important, possibly for different reasons in different cell types.
3. Whilst telomeric repeats are very simple sequences, apparently minor changes in these sequences can have disastrous consequences. For example, the sequence which forms telomeres in *Oxytrichia* (G_4T_4/C_4A_4), when added to chromosome ends in *Tetrahymena*, caused chromosome division failure, despite the similarity of the sequence (G_4T_2/C_4A_2). If telomeric sequence was merely a DNA buffer, these two very similar sequences should be interchangeable.
4. Linear viruses that live in eukaryotic cells and which also have to address the end replication problem, do so in a variety of ways, all of which seem to be simpler than the chromosomal solution. Despite this, RNA-dependent replication by telomerase is used by most, if not all, animals, plants and single-celled eukaryotes. It would appear that this solution offers organisms some advantage that viral solutions do not.

1.4 Telomerase: structure and function

The end replication problem, its consequences for telomere length and the concept that telomeric attrition limits cell proliferation all raise an important question: how do immortal organisms, germline cells of higher organisms and cancer cells maintain the integrity of their telomeres and therefore avoid senescence? The solution for this problem arose through studies performed on the *Tetrahymena* organism by Carol Greider and Elizabeth Blackburn in 1985, who discovered an enzyme that could synthesise and elongate telomeres (Greider & Blackburn 1985). They called this enzyme telomerase. The structure and function of this holoenzyme complex will be reviewed here as background for further discussion and for the studies which will follow.

1.4.1 Telomerase structure

The progressive loss of TTAGGG repeats from telomeres can be prevented through the activity of telomerase, a cellular reverse transcriptase. Telomerase comprises telomerase reverse transcriptase (TERT), the main catalytic subunit, and an mRNA template (known as TERC, TRT or TR) together with a number of associated binding proteins, and functions to add TTAGGG repeats onto pre-existing telomeres.

1.4.1.1 Telomerase reverse transcriptase catalytic subunit (TERT)

Following the discovery of the telomerase enzyme in *Tetrahymena* by Carol Greider in 1985, several studies examined the components that go to make up the holoenzyme. Purification of telomerase from the ciliate *Euplotes aediculatus* revealed two proteins of 43 and 123 Kd in size. p123 was found to be a homologue of the protein Est2, which in *Saccharomyces cerevisiae* is essential for telomere maintenance and telomerase activity (Lendvay *et al.* 1996; Counter *et al.* 1997; Lingner *et al.* 1997). Est2 and p123 contain six of seven conserved reverse transcriptase (RT) motifs, and mutation of amino acids within the conserved RT motifs results in abrogation of telomerase activity, suggesting that both p123 and Est2 are proteins that catalyse the RNA-dependent synthesis of telomeric sequence. In 1997, Harrington *et al* cloned and sequenced a human gene encoding a protein they termed TP2 (telomerase-associated protein 2) (Harrington *et al.* 1997). TP2 was found to share 30% amino acid identity with p123. BLAST searching revealed that TP2 was similar to several other reverse transcriptases, and homology with p123 was highest in the region that contained RT motifs similar to other RTs. TP2 was found to localise to the nucleus of telomerase-positive HeLa cells and was associated with telomerase activity (along with another telomerase-associated protein, TP1). Importantly, mutation of aspartate residues in a region highly conserved across all RTs significantly reduced telomerase activity. Thus, TP2 fulfilled all the criteria required of the reverse transcriptase catalytic subunit of telomerase.

The same protein was found by other groups at around the same time, who called their respective proteins TRT1 and hEST2 (Meyerson *et al.* 1997; Nakamura *et al.* 1997). Finally, the protein was renamed hTERT (human Telomerase Reverse Transcriptase) according to the HUGO Nomenclature Committee of the Genome Database (Counter *et al.* 1998a).

1.4.1.1.1 Organisation of the TERT gene in humans and dogs

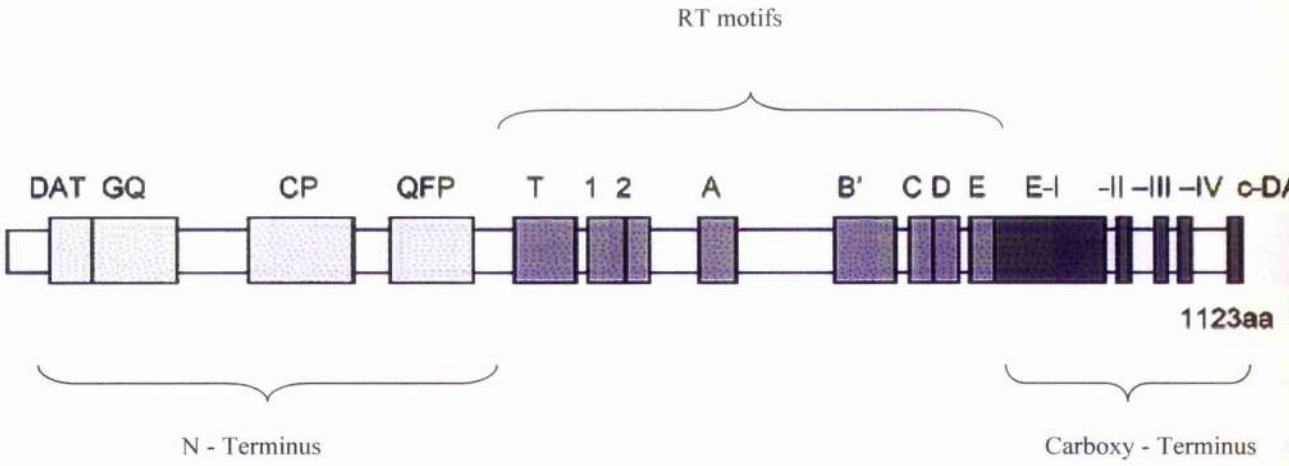
The hTERT gene has been characterised by several authors through a combination of cDNA library screening and genomic walking (Wick *et al.* 1999; Cong *et al.* 1999). hTERT is most likely encoded by a single copy gene and is composed of 16 exons and 15 introns. Exons range from 62 to 1354 bp in size, while introns vary in length from 104 to 8616 bp. The TGA translation stop codon is located within exon 16, together with a 3'-untranslated region. All exon/intron splice junctions contain GT/AG consensus sequences with the exception of intron 15 (Cong, *et al.* 1999). No putative polyadenylation signal has been identified either within exon 16 or in the following 3' flanking region. The canine TERT gene appears to be organised in a similar way to the human TERT gene (Nasir *et al.* 2004).

1.4.1.1.2 Structure of the TERT protein in humans and dogs

The TERT protein contains seven RT motifs that are also found in other reverse transcriptases, designated 1, 2, A, B', C, D and E. In addition, TERT contains a telomerase-specific motif known as the T motif (Nakamura, *et al.* 1997). The T motif is located on exon 3, RT motifs 1 and 2 on exon 4, RT motif A on exon 9, RT motif B' on exon 10 and RT motifs C and D on exon 11. RT motif A is the only motif to be encoded by two exons – exons 5 and 6. These are shown in Figure 1.3 and in Figure 5.1, Chapter 5. The protein in vertebrates varies in size from 1191 to 1346 amino acids and has a predicted mass of 127 – 155 kDa. In dogs, the TERT molecule is comprised of 1123 amino acids and has a predicted molecular mass of 124 kDa (Nasir *et al.* 2004). Homology between the TERT molecules of different vertebrate species varies significantly, but is more tightly conserved within these motifs, with up to 75% of amino acids in some motifs conserved across a range of species including dog, chicken, *Xenopus*, human, mouse, rat and hamster TERT (Nasir *et al.* 2004; Delany & Daniels 2004; Nakamura *et al.* 1997; Kuramoto *et al.* 2001; Santarius *et al.* 1997). In addition, TERT contains several conserved regions both in the N-terminal and C-terminal regions of the protein that are conserved across several species. These have been termed regions v-I, v-II, v-III and v-IV in the N-terminal region, and v-V, v-VI and v-VII in the C-terminal region (Delany & Daniels 2004; Kuramoto, Ohsumi, Kishimoto, & Ishikawa 2001). Between the v-I and v-II regions of the TERT protein there exists a flexible linker region which varies considerably between species in length and amino acid content. Of the species so far examined, the chicken TERT possess the longest flexible linker, with 298 amino acids, compared with 199, 154, 158 and 161 for *Xenopus*, human, mouse, rat and hamster respectively (Delany & Daniels 2004).

The N-terminal region of TERT is essential for telomerase activity (Bachand *et al.* 2001). Within this area are three functional regions that have been termed motif GQ, motif CP and motif QFP (see Figure 1.3) (Xia *et al.* 2000). These regions overlap with the v-I – v-IV domains (motif GQ corresponding to v-I and motif QFP corresponding to v-IV) which in yeast have been shown to be essential for viability, with v-III necessary for RNA binding (Friedman & Cech 1999; Xia *et al.* 2000). Similarly, abolition of amino acids 1-350 of the hTERT molecule abolishes telomerase activity, and a 287-residue fragment of the N-terminal region has been shown to bind hTR, suggesting that this region is also important in vertebrates for binding RNA and allowing telomerase activity (Bachand & Autexier 2001). In addition to the motifs mentioned above, a region called the DAT domain (Dissociates Activities of Telomerase) within the N-terminal region has been shown to be necessary for *in vivo* telomere elongation. In humans this domain has been mapped to residues 69-134 aa of hTERT (Armbruster *et al.* 2001). Other functions ascribed to the N-terminal region of TERT include telomerase RNP assembly, catalysis and interaction with p23 (Bachand *et al.* 2001; Bachand & Autexier 2001; Friedman & Cech 1999).

Figure 1.3: Diagram of conserved motifs in the human and canine TERT proteins. Reproduced from (Nasir, Gault, Campbell, Veeramalai, Gilbert, McFarlane, Munro, & Argyle 2004)

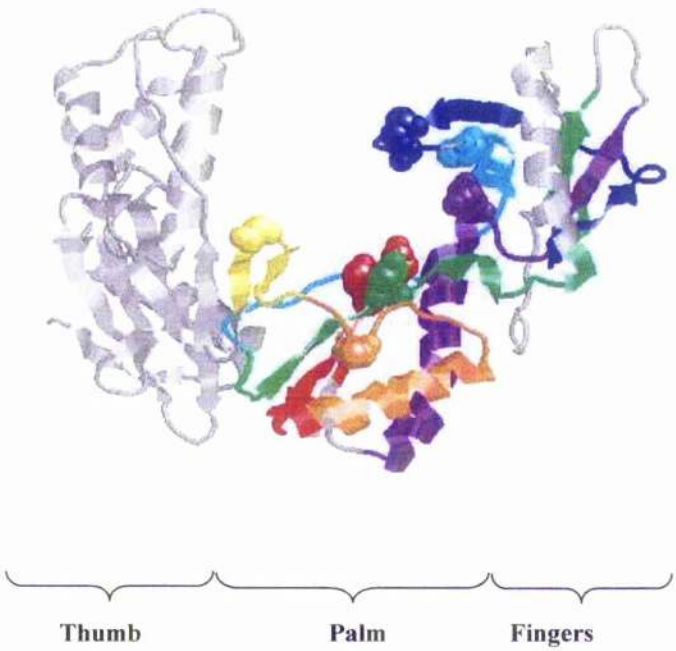


The reverse transcriptase and T motifs are important for catalytic function of the TERT subunit. Motif E is known as the 'primer grip' in reverse transcriptases, and mutations to this region affect RNA priming (Jacobo-Molina *et al.* 1993; Powell *et al.* 1997). It is likely, therefore that the TERT uses this region to bind the telomere as a DNA template and then catalytically copies the template sequence of the tightly bound RNA subunit in a repetitive manner (Nakamura *et al.* 1997).

The carboxy terminal region of TERT is less well characterised than the N-terminal region. Two important functions ascribed to this region include enzyme processivity and multimerisation with other TERT molecules (Beattie *et al.* 2001). The hTERT molecule C-terminus contains a CRM1 and a 14-3-3 binding site, both of which appear to regulate nuclear export (Seimiya *et al.* 2000). Banik and colleagues also identified four regions in human TERT downstream of the E motif which are essential for *in vivo* function, termed E-I, E-II, E-III and E-IV (Banik *et al.* 2002). E-I forms a continuous stretch of sequence with motif E, and E-II is reported to bind 14-3-3 protein (Seimiya *et al.* 2000). In addition, deletion of a three amino acid residue DAT domain in the C-terminus abolishes *in vivo* function of telomerase but retains *in vitro* activity (Banik *et al.* 2002). Several studies show that in several species, the functional telomerase holoenzyme includes a dimer formed by two TERT molecules, and results are consistent with a model whereby dimerisation occurs through N- to C- terminal interactions. It appears that the C-terminal extension and a section of the RT domain are essential for multimer formation to occur, but the region of the N-terminus involved in these interactions remains to be elucidated (Beattie *et al.* 2001). Other functions conveyed by the C-terminus include competence for telomere recruitment, nucleolar localisation, primer binding and processive elongation (Banik *et al.* 2002; Counter *et al.* 1998b; Peng *et al.* 2001).

The tertiary structure of the TERT molecule is similar to that of other reverse transcriptases, and has been compared to being similar to a human right hand, with a 'thumb', 'palm' (composed of motifs A, B', C D and E) and 'fingers' (composed of motifs 1 and 2 - Figure 1.4) (Nakamura *et al.* 1997).

Figure 1.4: Conserved tertiary structure of reverse transcriptase enzymes showing 'thumb', 'palm' and 'finger' regions. Reproduced with permission from (Nakamura, Morin, Chapman, Weinrich, Andrews, Lingner, Harley, & Cech 1997).



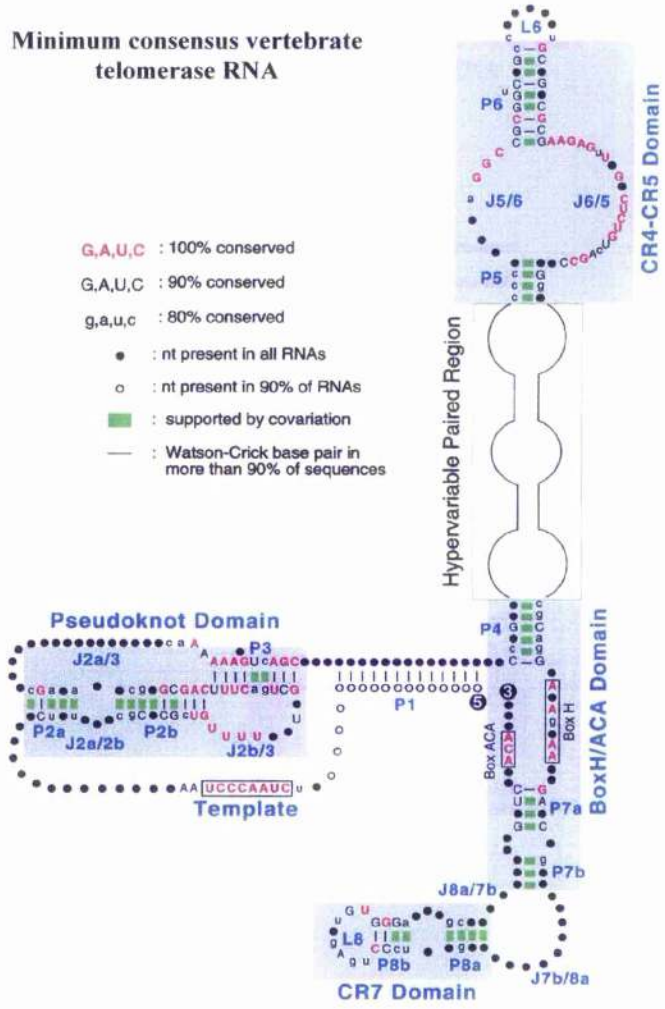
Overall these results produce a picture of an enzyme which evolved from reverse transcriptases in an ancestor of present day eukaryotes. The reverse transcriptase domains have been conserved, together with a telomerase-specific T motif, across phylogenetically diverse species. The N- and C- terminal regions, however, have been less well conserved. Despite this, and unlike other reverse transcriptases, TERT relies on the N- and C-terminal regions for catalytic activity in addition to the conserved RT motifs. The N- and C- terminal regions are also required for a number of other important functions, including binding of the telomerase RNA component, binding telomerase-associated proteins, forming multimers and localising the enzyme within the nucleus.

1.4.1.2 Telomerase RNA (TR)

The RNA subunit of telomerase was the first component of the telomerase enzyme to be cloned, by Carol Greider and Elizabeth Blackburn in 1989 (Greider & Blackburn 1989). Its size and sequence varies dramatically amongst mammals, ranging from 382 to 559 nucleotides in length (Chen *et al.* 2000). Yeast and ciliate RNA size and sequence also vary in length, from 148 to 209 nucleotides in length in the case of ciliates, and up to approximately 1300 nucleotides in length in yeast (Singer & Gottschling 1994; Chen & Greider 2003). Whilst the RNA sequence shows some variation, the secondary structure of the RNA component seems to be conserved across species. Indeed, this conservation extends to the point where the telomerase RNA of several ciliates is functionally interchangeable *in vivo* (Bhattacharyya & Blackburn 1997). Phylogenetic comparison and mutational analysis of vertebrate telomerase RNAs suggests that the secondary structure has also been highly conserved across vertebrate species (Chen *et al.* 2000).

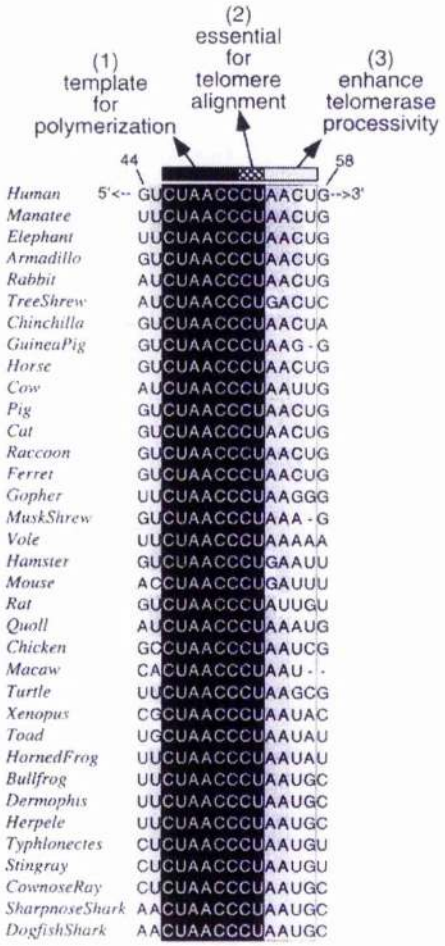
Sequence analysis of the telomerase RNA gene from birds, mammals, reptiles and amphibians has revealed a highly conserved core structure with ten conserved helical regions formed by nucleotide pairing that are present in all the vertebrate RNAs so far examined (Chen *et al.* 2000). These ten helices constitute four distinct structural domains: the 'pseudoknot' fragment, located at the 5' end of the molecule, the CR4-CR5 fragment, the BoxH/ACA domain and the CR7 domain (see Figure 1.5) (Chen *et al.* 2000). The exact location of these domains within the molecule varies slightly between species. For example, in humans the pseudoknot fragment extends from nucleotides 44 - 184, whilst in the mouse the fragment starts at nucleotide 1 and extends through to nucleotide 145.

Figure 1.5: Minimum consensus Vertebrate Telomerase RNA. Reproduced with permission from (Chen, Blasco, & Greider 2000). Regions denoted by P represent helical regions formed by base pairing. Regions denoted by J represent junctions between helices. Numbers 5 and 3 in black circles represent the 5' and 3' ends of the molecule respectively. Note that not all species possess the P1 paired region. These include mouse, rat and hamster.



The pseudoknot region contains the RNA template located at its 5' end (extending from nucleotide 44 in humans and nucleotide 1 in mice) that binds the telomere and serves as a primer for further elongation (Figure 1.5). This potential template region varies across vertebrate species from 8-11 nucleotides in length. Eight of these nucleotides are absolutely conserved across all of the vertebrate species so far examined, including sharks, amphibians and mammals (Figure 1.6) (Chen *et al.* 2000). Within this region, the first six nucleotides (5'-CUAACC-3') serve as the actual template for DNA polymerisation, while the last two nucleotides (5'-CU-3') are required for primer alignment during translocation of the telomerase complex. The four bases extending in the 3' direction from this region are conserved to varying degrees across species. Importantly, when these nucleotides are complementary to telomere sequence, they cumulatively enhance the processivity of the telomerase holoenzyme (the number of nucleotides synthesised before the enzyme dissociates)(Greider 1991; Hammond & Cech 1997). The observation that humans have a significantly more processive enzyme when compared with the mouse is therefore explained by the fact that human telomerase possesses a TR molecule with 11 nucleotides complementary to the telomere as opposed to the mouse TR molecule with only 8 complementary nucleotides (Prowse *et al.* 1993; Greider 1991). In addition, reconstituting mouse telomerase by adding the human pseudoknot fragment to the mouse TERT protein results in an enzyme with significantly greater processivity than the original murine telomerase holoenzyme (Chen & Greider 2003).

Figure 1.6: Sequence conservation in the template region of the pseudoknot domain of vertebrate telomerase RNA. The sequence alignment and adjacent region of 35 vertebrate telomerase RNAs is shown. Nucleotides conserved in all species are shown in black. Nucleotides that are moderately conserved are shown in grey. Reproduced with permission from (Chen & Greider 2003)



Within the pseudoknot fragment, several secondary and tertiary structures that have also been conserved across species. P1, P2 and P3 are the three first three paired (P) helices, with P2 and P3 forming the stem and loop of the pseudoknot respectively (Figure 1.5). Mutations of nucleotides in the single-stranded regions flanking the P2a-1 helix or near the P3 loop in human TR produce an enzyme that is significantly less functional than wild-type telomerase, suggesting that these areas are important for a functional holoenzyme (Chen & Greider 2003). The P3 loop of the RNA molecule allows for individual RNA molecules to form a dimer under physiological conditions through a reaction that requires interstrand complementarity at this location (Figure 1.5) (Ly *et al.* 2003). Ly and colleagues suggested that this dimerisation is required for enzymatic function of the telomerase molecule without interfering with binding of TR to TERT, since mutations in this area result in a monomeric molecule that is still able to coimmunoprecipitate with Flag-epitope-tagged TERT but which is catalytically inactive (Ly *et al.* 2003). However, recently Theimer and colleagues have suggested that mutations within the P3 helix cause telomerase defects by altering or disrupting the ability of TR to form a pseudoknot, regardless of whether the pseudoknot is formed by one (monomer) or two (dimer) molecules (Theimer *et al.* 2005).

The CR4-CR5 fragment contains a high affinity site for contact with TERT that is essential for catalytic function (Bachand *et al.* 2001). The pseudoknot and CR4-CR5 fragments can bind independently to TERT and the presence of both is required to reconstitute telomerase activity *in vitro* and *in vivo* (Chen *et al.* 2002).

1.4.1.3 Telomerase-associated proteins

Whilst the structure of TR and TERT have been remarkably conserved across a range of phylogenetically diverse species, the proteins associated with them have not. As yet there exists no conserved set of proteins universally associated with telomerase in all organisms (Nugent & Lundblad 1998). The telomerase holoenzyme complex varies between different organisms and under different developmental stages, ranging from 230 to greater than 5000kDa in size (Wenz *et al.* 2001; Harrington 2003).

In mammals, the protein TEP1 has been cloned as a telomerase RNA-binding protein associated with telomerase activity in cell extracts (Nakayama *et al.* 1997). This was found to be a mammalian homolog of p80, a protein of 80 kDa first discovered in *Tetrahymena* following its co-purification with telomerase activity (Collins *et al.* 1995). TEP1 interacts with TERT through the RT domain, but it is not known whether this interaction is direct or indirect (Beattie *et al.* 2000). TEP1 also binds an RNA component, vRNA, which is present within the cytoplasmic vault complex (Kickhoefer *et al.* 1999). Loss of TEP1 in telomerase-positive cells leads to greatly

reduced vRNA levels, together with a loss of TEP1 and vRNA from vault caps, but does not affect either telomerase activity or telomere length (Kickhoefer *et al.* 2001). Thus, TEP1, whilst binding other RNA species, does not appear to be essential for telomerase function.

The mammalian protein La has also been found to associate with telomerase activity (Ford *et al.* 2001). La is a protein important for RNA Polymerase II transcript maturation (Aigner *et al.* 2000), and a homolog, p43, has also been identified that co-purifies with telomerase activity in the ciliate *Euplotes aediculatus* (Lingner & Cech 1996). Although telomerase RNA is a product of RNA Polymerase II, the overexpression of La leads to gradual telomere shortening in human cells. Other proteins associated with telomerase RNA include proteins that bind the H-ACA motif (including dyskerin, GAR1, NHP2 and Nop10 – see 1.4.1.4: Assembling the telomerase holoenzyme), the hnRNP proteins 1, C1/C2 and D (Ford *et al.* 2002), mammalian Staufen (Stau) and L22 (Le *et al.* 2000). Recently, the product of the survival motor neuron gene (SMN), a protein implicated in RNP biogenesis, has also been identified as a human telomerase associated protein (Bachand *et al.* 2002).

Proteins known to interact either directly or indirectly with TERT include 14-3-3, p23, p90, phosphatase 2A, Akt, cTb1, p53, PARP and PinX1 (Kharbanda *et al.* 2000; Cao *et al.* 2002; Kang *et al.* 1999; Li *et al.* 1997). The 14-3-3 proteins are involved in signalling pathways and as molecular chaperones, and consistent with this a mutant version of 14-3-3 which is still able to bind TERT results in a holoenzyme restricted to the cell cytoplasm and which is unable to translocate to the nucleus (Seimiya *et al.* 2000). The p23 and p90 proteins are foldasomes that stably associate with hTERT and which may promote maintenance of an active conformation of the telomerase RNP (Holt *et al.* 1999). Phosphatase 2A, Akt, cAb1, p53 and PARP have all been implicated in the post-translational modification and regulation of TERT and telomerase activity (Li *et al.* 1999; Zhou & Lu 2001). Finally PinX1 is a nucleolar protein which inhibits telomerase activity by competitively binding TERT at a domain which overlaps with the RNA-binding domain (Zhou & Lu 2001). As a result, PinX1 may act as a tumour suppressor by sequestering TERT in a non-active state within the nucleolus (Blackburn 2005). The roles of all these proteins as so far described are summarised in Table 1.3.

Table 1.3: Proteins associated with telomerase activity in mammalian species

Protein	Role in telomerase function	Other roles?
TEP1	Association with but not required for telomerase activity	Vault RNA (vRNA) stability
Stau	Binds TR	Nucleocytoplasmic transport
La	Associated with telomerase activity	Involved with TR transcription?
L22	Binds TR	Ribosome component
Dyskerin/GAR1/NHP2/ Nop10	Bind TR	Telomerase RNP processing
p23/p90	Bind N-terminus of TERT	Foldasomes/chaperones
hnRNPs (A1, C1/C2, D)	Associated with telomerase activity; affect telomere length	RNP assembly/RNA splicing
SMN	Associated with telomerase activity	RNP synthesis
Phosphatase 2A, Akt, cAbl, P53, PARP	Post-translational modification	Regulation of TERT and telomerase activity
PinX1	Inhibition of telomerase activity	Competitive binding to RNA-binding motif of TERT

1.4.1.4 Assembling the telomerase holoenzyme

The mammalian telomerase holoenzyme is a multimeric complex of 1000 kDa or more, whose composition is not fully known (Nakamura *et al.* 1997). However, after weakly interacting proteins are removed from the complex by salt-stripping, the smallest telomerase complex that retains enzymatic activity in humans has a mass of approximately 600 kDa. This is consistent with a molecule containing two copies each of TR and TERT (Wenz *et al.* 2001). This multimerisation has been confirmed *in vivo* in yeast and humans (Prescott & Blackburn 1997; Ly *et al.* 2003; Wenz *et al.* 2001) and involves the pseudoknot domain of the TR molecule and the N-terminal region of TERT (Moriarty *et al.* 2002; Ly *et al.* 2003). Formation of a dimer appears to be important for telomerase activity to occur, but it is possible that the three-dimensional structure of the resultant enzyme is responsible for function, rather than the number of molecules involved (Theimer *et al.* 2005).

The RNA component of all vertebrates and of yeasts is produced by RNA polymerase II (Chen *et al.* 2000). The accumulation of most cellular non-coding RNA requires active processing of a primary transcript, including events such as precursor cleavage, base and sugar modifications and directed assembly of proteins that protect the RNA from nuclease digestion (Collins & Mitchell 2002). In the first part of this process, RNA polymerase II transcription of telomerase RNA beyond the mature RNA 3' end creates a precursor that must be then cleaved to generate a final product. Whilst creation of the mature product is critical in the formation of functional telomerase RNA in all species, it is accomplished by different organisms in different ways. For example, in budding yeast, the RNA precursor is polyadenylated before being processed in association with Sm proteins (Chapon *et al.* 1997; Seto *et al.* 1999). Whilst these details are not known in humans, no evidence for a polyadenylated precursor RNA molecule has been found, suggesting that transcription termination without adenylation may occur (Collins & Mitchell 2002).

Once the mature version of telomerase RNA has been synthesised, it accumulates within the nucleus of telomerase-positive cells together with TERT. In humans localisation has been shown to occur within intranuclear structures known as Cajal bodies (Zhu *et al.* 2004), with a small fraction (approximately 7%) found in nucleoli. In humans and in mice TERT also accumulates in the nucleus, in a punctate or speckled pattern (Martin-Rivera *et al.* 1998; Harrington *et al.* 1997). In *Xenopus* oocytes, telomerase has also been shown to accumulate in Cajal bodies, and within the nucleolus (Lukowiak *et al.* 2001; Narayanan *et al.* 1999). Localisation requires the H/ACA RNA motif composed of primary and secondary structure elements in an overall hairpin-Hinge-hairpin-ACA configuration located towards the 3' end of the RNA molecule (Figure 1.5) (Mitchell &

Collins 2000). Alterations to the stem and loop structures within the H/ACA domain either prevent telomerase RNA from accumulating, or prevent correct folding of the molecule, resulting in a molecule that is able to accumulate but which does not convey telomerase activity (Mitchell & Collins 2000). The H/ACA motif associates with four proteins, in humans known as dyskerin (homologue of Cbf5p in yeast), NHP2, NOP10 and GAR1 (Collins & Mitchell 2002). Alteration to at least one of these proteins, dyskerin, also results in dysfunctional telomerase, due to a decrease in the rate of accumulation and processing of telomerase RNA, through interaction at the H/ACA motif. This demonstrates that several associated proteins are required for synthesis of TR within a stable ribonucleoprotein (RNP) complex. The telomerase enzyme accumulates within the nucleus, and is at least partially distributed to the nucleolus, consistent with the finding that most other H/ACA motif RNA species also localise to the nucleolus (Mitchell & Collins 2000).

The synthesis and characterisation of TERT is less well known. Once TERT has been synthesised, it appears that it associates with TR that has been assembled into a stable RNP, and that binding occurs via the pseudoknot and CR4/CR5 domains of TR (see Figure 1.5). Although the exact binding sites have not been defined, one hypothesis that fits the accumulated data is that binding occurs at several core domain elements (including the pseudoknot and CR4/5 domains) but that each individual interaction is weak. This would then allow for temporary association and disassociation of the two molecules required for the translocation involved in telomere synthesis (Theimer *et al.* 2005).

In cells that possess telomerase activity but which have not been transformed, the TERT subunit has been shown to localise to the nucleolus (Wong *et al.* 2002). Telomerase is then released to the nucleoplasm at the expected time of telomere elongation. By contrast, in tumour cells and cells transformed by SV40, telomerase seems to be mainly excluded from the nucleolus and remains largely confined to the remainder of the nucleoplasm. However, DNA damage due to ionising radiation is sufficient to induce reassociation of telomerase with the nucleolus (Wong *et al.* 2002).

1.4.2 Telomerase function

Telomerase activity is not present in sufficient quantities to prevent telomere shortening in most normal adult somatic cells. However, telomerase activity is present in adult germ cells and a sub-population of adult stem cells. In addition, telomerase activity is present in a high proportion of all human cancers. A more detailed review of telomerase activity in relation to cancer will be provided in Chapter 3. However, a broad review of the other functions of telomerase will be provided here.

1.4.2.1 Telomerase activity

1.4.2.1.1 Telomerase activity is reconstituted by TR and TERT

Although several telomerase-associated proteins have been identified in various organisms, it has been shown in humans that in fact the minimal components required to restore telomerase activity are TR and TERT. Weinrich *et al* showed that the *in vitro* transcription and translation of hTERT, when either mixed or co-synthesised with hTR, conveyed the enzymatic properties of native telomerase (Weinrich *et al.* 1997). Enzymatic activity could be restored irrespective of whether the hTERT was synthesised in conjunction with or separate to hTR. In addition, mutations to the hTERT sequence abrogated this activity, confirming that hTERT is the reverse catalytic subunit of the holoenzyme. This important work was the first step in later research examining the importance of TERT as the major determinant of telomerase activity.

1.4.2.1.2 The reverse transcriptase catalytic subunit is the rate-limiting factor in telomerase activity

Although TR and TERT are minimally required to generate telomerase activity *in vitro*, several findings suggest that in fact the presence of TERT is the prime determinant of telomerase activity *in vivo*. Work by Counter *et al* showed that ectopic expression of hTERT in telomerase-negative human fibroblasts is sufficient to restore telomerase activity to levels comparable with immortal telomerase-positive cells (Counter *et al.* 1998a). Two other findings support this idea. Firstly, TR has been shown to be widely expressed in telomerase-negative somatic tissues (Feng *et al.* 1995). Secondly, hTERT mRNA can be found in telomerase-positive tissues, cancer cell lines and tumours, but not in cells or tissues known to lack enzymatic activity (Kilian *et al.* 1997; Meyerson *et al.* 1997; Nakamura *et al.* 1997). Together these findings suggest that hTERT is the primary determinant of telomerase activity in telomerase-positive cells.

1.4.2.1.3 Telomerase activity during development

In humans, telomerase activity is detectable at the blastocyst stage but is lost from almost all embryonic tissues by 20 weeks of gestation. As well as being present at high levels in the blastocyst, telomerase is seen in essentially all human tissues tested during the first trimester. As gestation proceeds, telomerase activity continues in a subset of fetal somatic tissues (liver, kidney, intestine, lung, skin, muscle and adrenal glands) but is no longer detectable in brain or bone extracts after 16 weeks of gestation, despite ongoing cell division in those tissues (Wright *et al.* 1996).

The mechanism or mechanisms by which telomerase is switched off during development is unknown. However, regulation would appear, at least in some tissues, to involve alternative splicing of hTERT mRNA (Forsyth *et al.* 2002). Using hTR levels as a surrogate marker for telomerase activity during development, some researchers suggested telomerase activity was present in tissues up to and beyond week 21 of gestation (Yashima *et al.* 1998). However, a second study revealed that while fetal heart, liver and kidney tissues displayed the presence of hTR at all gestational ages tested (from 8-21 weeks), telomerase activity disappeared from heart after 13 weeks, and from kidney after 17 weeks. In heart tissue, hTERT mRNA was no longer detectable after 13 weeks of gestation, but from the 17th week, the transcript was present in kidney as an inactive alternative splice variant (Ulaner *et al.* 1998).

1.4.2.1.4 Telomerase activity in stem cells

In multicellular organisms, the requirement for telomerase to maintain telomere length depends on species, cell type and stage of development. This may in part be due to the considerably longer telomeres found in multicellular as compared to unicellular organisms, where telomeres are measured in kilobases rather than hundreds of bases respectively. In the majority of animals, this large reserve could be expected to cope with the shortening associated with the limited number of cell divisions required by somatic cells. It would be reasonable to assume, then, that within a multi-cellular organism, those cells with a capacity for self-renewal might parallel the situation found in single-celled organisms which are by their very nature also self-renewing. In particular, this situation might apply to stem cells, which are required to undergo a much greater number of divisions over the lifetime of the individual. In fact, this is partly true. In humans and mice, at least, many stem cells with the ability to generate cells of several lineages do possess telomerase activity. However, telomerase activity is absent from some cell types, including mesenchymal and stromal cells derived from human bone marrow (which differentiate to form skeletal tissues) and from haemopoietic stem cells derived from umbilical cord blood (Greenwood & Lansdorp 2003; Banfi *et al.* 2002; Gronthos *et al.* 2003; Parsch *et al.* 2004; Zimmermann *et al.* 2003; Samper *et al.* 2002; Allsopp *et al.* 2003). As expected, telomeres in many of these cells progressively shorten with time, although this has not been proven in all. Stem cells and progenitor cells in humans that do possess telomerase activity include neuronal stem cells, haematopoietic stem cells and embryonic stem cells (Harrington 2004).

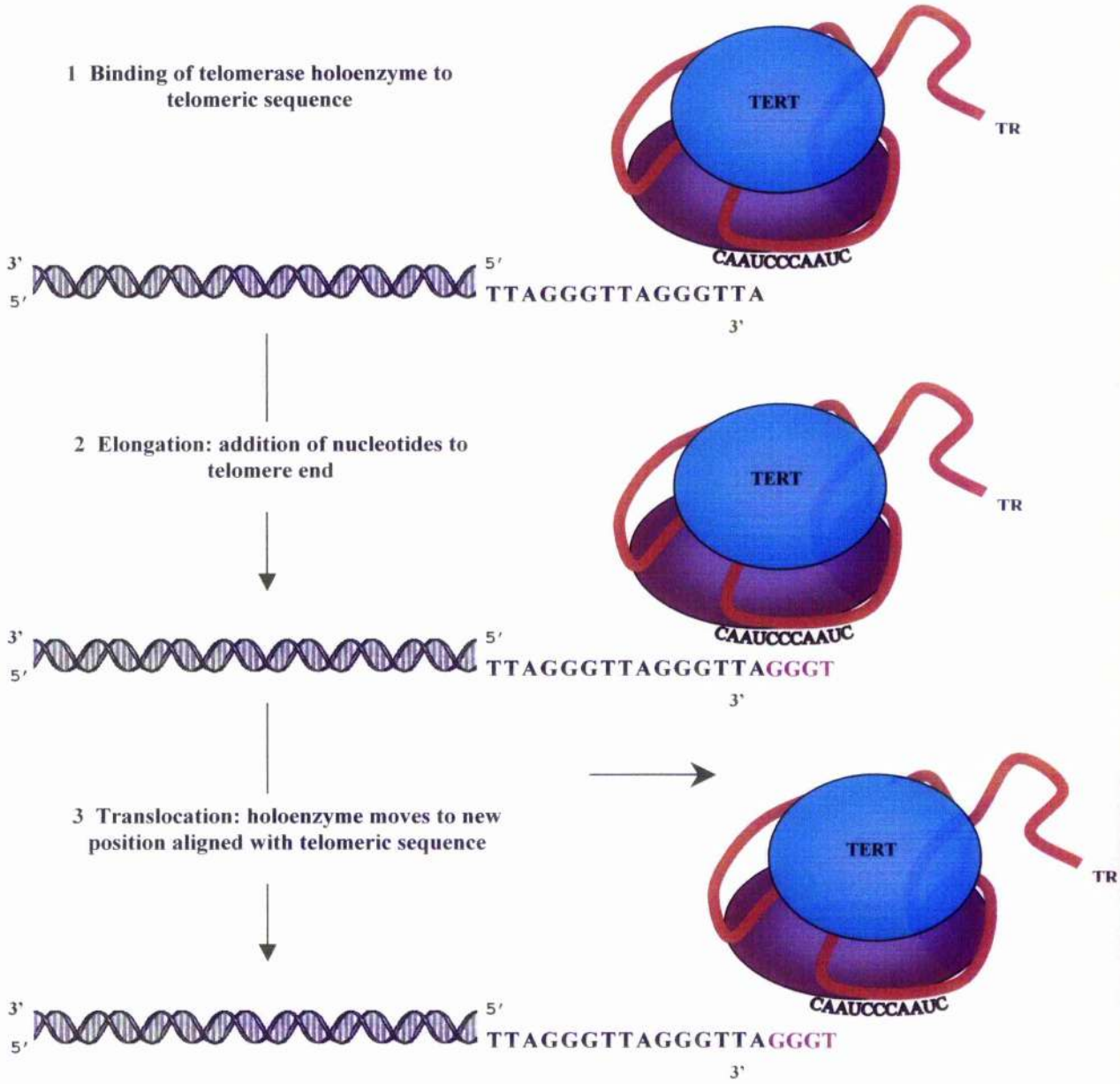
In addition to these cells, low levels of telomerase activity have been detected in other stem and stem-like cell populations in adult humans. This lower level of activity is apparently sufficient to slow, but not prevent, telomere shortening, since the progeny of these cells exhibit telomere shortening as a function of ageing. Examples of these cells include basal keratinocytes in skin, crypt cells in the intestines, basal cells of the trachea and bronchi, kidney collecting ducts and

urothelium, glandular prostate tissue, germinal centres and parafollicular areas of lymphoid tissue, and squamous epithelial basal cells and hair follicle bulbs (Forsyth *et al.* 2002). It is possible that some stem cell types do not need to maintain telomere length simply because limited telomere attrition can be tolerated in those stem cells only undergoing a small number of population doublings during the lifetime of the organism. The progeny of these cells, however, may represent rapidly dividing progenitor cells, and thus it may be that a more important factor is the activation of telomerase activity in these stem cell successors (Harrington 2004). Examples of this have been demonstrated in blood and skin (Hiyama *et al.* 1995; Weng *et al.* 1996; Bickenbach *et al.* 1998).

1.4.2.2 Telomere elongation by telomerase

The principal effects of telomerase relate to the ability of the enzyme to add nucleotide repeats to the end of telomeres. Telomere elongation can be divided into three steps: binding, elongation and translocation. These are shown pictorially in Figure 1.7. In the first step, the telomerase holoenzyme binds to the telomere through alignment of the telomerase RNA template region with TTAGGG repeats. During the second step, the TERT subunit functions to extend the telomere by adding nucleotides to the end of the telomere using the TR template as a guide. In the third step, the holoenzyme dissociates from the telomere, translocates to the end of the telomeric sequence, and re-binds the telomere via the RNA template, ready for another round of elongation.

Figure 1.7: Schematic representation of the steps involved in telomerase elongation of telomeres



Before the telomerase is able to extend shortened telomeres, the holoenzyme must find and extend a chromosome 3' end. The extent to which telomerase interacts with the telomere by direct binding to single-stranded DNA rather than through protein-protein interactions is unclear. For most of the cell cycle, telomeres are packaged into the chromatin structure responsible for end protection (Collins & Mitchell 2002). The T-loop formed by the folding back of the 3' overhang back into the duplex repeat region of the same chromosome seems likely to restrict elongation of the telomere in this state. During the S phase of the cell cycle there would appear to be an interval when T-loops are disassembled by the DNA replication machinery, allowing access to telomeric DNA by telomerase (Ray *et al.* 2002). In addition, POT1 may mediate binding of telomerase to the telomere during the S phase by stabilising telomeres in the 'open' configuration (Baumann *et al.* 2002). However, the relative scarcity of telomerase would suggest that active recruitment is also required (Collins & Mitchell 2002). Proteins that could possibly be involved in this role in mammals include the hnRNP proteins A1 and C (Collins & Mitchell 2002). Recombinant hnRNP A1 can simultaneously bind TR and a telomeric DNA substrate, and overexpression of hnRNP A1 in a hnRNP A1-deficient mouse strain results in telomere elongation (Fiset & Chabot 2001). Similarly, recombinant hTR can also interact with hnRNP C, and antibodies against hnRNP C immunopurify endogenous telomerase but not telomerase assembled from C-terminally epitope-tagged TERT *in vitro* (Ford *et al.* 2000).

Following recruitment of telomerase to the telomere, one mode of substrate recognition involves hybridization of the telomerase RNA to the substrate, telomeric DNA (Lingner & Cech 1996). This does not seem to require absolute complementarity at the 3' end of the RNA template, depending on the organism involved. Substrate recognition also involves an 'anchor site', found within the TERT subunit. G-rich DNA at the 5' end of the oligonucleotide is recognised distal to the catalytic centre, at this anchor point (Hammond *et al.* 1997).

Following substrate recognition, telomerase RNA then directs the next nucleotide to be added to the telomeric DNA substrate, depending on its alignment with the telomere. In adding nucleotides, the affinity of TERT to different nucleotides varies, with highest affinity for dGTP, although other dNTPs, rGTP and ddNTP can all be incorporated by most telomerases (Collins & Greider 1995). Interestingly, as well as being required for telomere synthesis, dGTP appears to affect TERT activity, since the concentration of dGTP influences the ability of telomerase to processively elongate DNA substrates (Bryan *et al.* 2000). In addition, elongation may involve a dGTP binding site separate from the dNTP binding site utilised during polymerisation (Hammond & Cech 1997). During the elongation step, telomerase RNA does not form base pairs with all nucleotides in the template, and RNA-DNA bonds break at the distal end of the template at the same time as new bonds are formed at the proximal end (Hammond & Cech 1998).

1.4.2.3 Telomerase is involved in bypassing senescence

Given the ability of telomerase to extend telomeres, it would be reasonable to expect that expressing telomerase in normal cells extends lifespan past the point at which replicative senescence or crisis would be expected to occur. Several researchers have confirmed this hypothesis. Condon *et al* showed that expression of hTERT in human myometrial cells led to a cell line capable of division continuously in culture, yet which retained almost all the phenotypic characteristics of normal smooth muscle cells *in vitro* (Condon *et al.* 2002). Jiang *et al* showed similar findings in fibroblasts, and Bodnar *et al* confirmed this in retinal pigment epithelial (RPE) cells (Jiang *et al.* 1999; Bodnar *et al.* 1998). Importantly, telomerase increases the proliferative capacity of cells, but is not in itself an oncogene, as the immortalisation process is not associated with the development of a malignant phenotype (such as the ability to grow in low-serum conditions or in soft agar) (Jiang *et al.* 1999; Morales *et al.* 1999).

These initial findings suggested that the presence of telomerase activity alone is all that is required for the immortalisation of cells. However, when some groups attempted to immortalise other cell lines, they found that telomerase on its own was not sufficient. Human foreskin keratinocytes (HFKs), human mammary epithelial cells (HMECs) and human adenoidal epithelial cells (HAKs) all required the additional abrogation of the Retinoblastoma (Rb) pathway, either through expression of human papilloma virus E7 protein, or inhibition of $p16^{INK4a}$ (Kiyono *et al.* 1998; Farwell *et al.* 2000). The HFK and HAK cell lines examined in more detail by Farwell *et al* all possessed hypermethylated Rb, either following methylation of the $p16^{INK4a}$ promoter, large scale deletion of chromosome 9p (the location of the $p16^{INK4a}$ gene), or with complete down-regulation of $p14^{ARF}$ (Farwell *et al.* 2000). Thus, the processes involved in immortalisation would appear to vary between cell types, although telomerase activity seems to be an essential requirement for those cell lines with telomerase-dependent telomere-elongating mechanisms.

It is not always the case that such findings *in vitro* are paralleled *in vivo*. However, confirmation that reconstitution of telomerase activity in telomerase-deficient mice results in preferential elongation of short telomeres, preventing senescence and end-to-end fusions was provided by Hemann and colleagues in 2001 (Hemann *et al.* 2001). This finding added further weight to the theory that telomerase is involved in immortalisation and that this holds true in several mammalian species. Interestingly, restitution of telomerase activity in mTERT-/- mice (lacking telomerase activity) does not necessarily restore telomere length to the original, wild-type state but maintains telomeres at a level which is sufficient to escape senescence but shorter than that of normal cells (Blasco 2002). This occurs in several cancer cell lines as well which possess high levels of telomerase activity but generally short telomeres (Hastie *et al.* 1990; de Lange *et al.* 1990).

The elongation of short telomeres by telomerase is required to allow immortal growth of cells with short telomeres. Telomerase is often activated at early stages of tumour progression in human cancers, probably reflecting the proliferative or differentiation status of the tumour. This activation may be the result of transcriptional or post-transcriptional activation by various oncogenic alterations. However, the implications for tumour development of early telomerase activation are not known (Blasco 2002).

1.4.2.4 Telomerase activity allows cells to escape from crisis

Cells which have undergone transformation such that they are able bypass replicative senescence are not, in themselves, immortal. Halvorsen *et al* showed that human pancreatic cell lines transformed with SV40 large T antigen proliferate beyond M1 but then show slowed growth, altered cell morphology, chromosomal instability and high rates of cell death associated with crisis. However, adding hTERT to these cells allows them to continue proliferating indefinitely (Halvorsen *et al.* 1999). Counter *et al* reported very similar findings in human embryonic kidney (HEK) cells, with telomere length maintained at approximately 9Kb, and Davies *et al* with human ovarian surface epithelial cells (Counter *et al.* 2003; Davies *et al.* 2003). Thus, whilst expression of oncogenes in many cell lines allows the first mortality checkpoint to be overcome, immortalisation of these cells and bypassing crisis requires telomerase activity.

Whilst the findings of Counter *et al* suggested that telomere length maintenance was vital for the avoidance of crisis, the findings of Zhu *et al* appeared, initially at least, to contradict this. They showed that following immortalisation with SV40 and hTERT, human fibroblasts contained telomeres that continued to shorten for a further 50 population doublings before stabilising at approximately 5-6 Kb in length (Zhu *et al.* 1999). These cells bypassed the crisis undergone by control cells which showed large numbers of dicentric and abnormal chromosomes associated with M2. Initially these findings were suggested to be due to a physical role played by telomerase in capping the ends of short telomeres, such that telomerase activity, in addition to immortalising cells, allowed the resetting of the minimal critical telomere length compatible with continued cell division (Zhu *et al.* 1999). Previously, Counter *et al* had shown that modification of hTERT to include an influenza virus haemagglutinin (HA) epitope tag at the carboxyl terminus produces a mutant form of TERT that conveys soluble telomerase activity but does not prevent telomere shortening (Counter *et al.* 1998b). Cells containing this construct, despite being telomerase positive, continue to undergo telomere shortening and enter crisis when telomere length reaches approximately 4 Kb suggesting that physical interaction between hTERT and telomeres via the carboxy-terminal end of the reverse transcriptase subunit is important for telomere elongation to

occur. This would be consistent with the proposed role for telomerase in capping the end of short telomeres suggested by Zhu *et al.* 1999.

However, Ouellette *et al* proposed an alternative theory for this finding. They found that when pre-senescent BJ foreskin fibroblasts that had been immortalised with hTERT were then transfected with a plasmid expressing an inactive hTERT mutant, a further 20 population doublings elapsed with associated shortening of telomeres before the onset of senescence (Ouellette *et al.* 2000). Since this mutant varied in sequence from wild type hTERT by only a single amino acid, it is unlikely that the proposed role for capping short telomeres would be lost – only that telomere length maintenance would have ceased in these cells. If the capping of short telomeres by hTERT was important for division to continue at these critically short lengths, it was suggested, then replacement of catalytically active hTERT at telomere ends with the catalytically-inactive mutant should have resulted in complete cessation of cell division. Thus, these researchers reasoned that rather than telomerase playing a physical role in capping critically-short telomeres, factors that recruit telomerase to the shortest telomeres must be involved. It must be remembered, though, that the work of Ouellette *et al* was performed in cells that were bypassing the M1 checkpoint rather than the M2 checkpoint, and that it is possible different roles for telomerase may be played at different checkpoints.

1.4.2.5 Telomerase and cell proliferation

The ability of cells to bypass senescence and crisis, and thereby become immortal, is an important step in the development of a cancerous phenotype. However, whilst substantial evidence has accumulated from tissue culture systems to suggest that telomerase is able to immortalise cells, evidence from mice suggests that telomerase may play other roles in the development of tumours. *mTR/* knockout mice lack the telomerase RNA component and are consequently unable to synthesise telomerase. Gonzalez-suarez and colleagues engineered *mTR/* mice that constitutively expressed the catalytic subunit of telomerase specifically in basal keratinocytes in the skin. These mice were found to be viable and showed histologically normal epithelium. However, when the carcinogenic phenol esters DMBA and TPA were applied to their skin, these mice displayed significantly more papillomas compared with wild type mice. In addition, these mice showed greater mortality as a result of the development of other tumours not seen in wild type littermates (Gonzalez-Suarez *et al.* 2001). In addition, the skin surrounding these papillomas displayed a marked hyperplasia not seen in wild-type littermates, suggesting an increased proliferative response to treatment with mitogens. Whilst the increased skin proliferation in response to mitogenic stimulation resulted in tumours, a more positive finding was that wound healing following the creation of circular lesions was significantly faster in these mice than in wild-type

littermates. All of these findings suggest that telomerase, when expressed constitutively *in vivo*, conveys a proliferative advantage to cells.

1.4.2.6 Telomerase and oncogenesis

The ability of telomerase to rescue shortened telomeres raises an intriguing paradox. It could be that the genetic instability induced by telomere shortening is an important part of tumorigenesis, allowing the genetic mutations to arise that bypass mechanisms that would normally prevent a cell becoming malignant. Thus, telomerase, it could be argued, prevents the genetic instability required for cells to develop a cancerous phenotype. However, the presence of telomerase, in allowing cells to bypass the normal checkpoints of replicative senescence and crisis, provides for unlimited potential to divide, a hallmark of all malignant tumours. Depending on which angle one takes, telomerase could be seen to be both a tumour suppressor and a tumour promoter.

Support for the theory that telomere shortening contributes to genetic instability comes from the *mTR*$^{-/-}$ telomerase knockout mouse. In these mice there is a 4-6-fold increase in spontaneous tumours in late generations with short telomeres, and the age of tumour onset is younger than in wild-type mice (Rudolph *et al.* 1999). Tumours arise particularly from highly proliferative tissues, and therefore those tissues that have the shortest telomeres. Importantly, *mTR*$^{-/-}$ tumours have a 3-18-fold increase in chromosome fusions and a twofold increase in aneuploidy compared to tumours of *mTR*$^{+/+}$ mice (Rudolph *et al.* 1999). Thus, the absence of telomerase activity allows telomere shortening and consequently an increase in genetic instability which promotes tumour formation, consistent with the telomerase as tumour suppressor theory.

Support for the theory that telomere shortening inhibits tumour formation is harder to come by. In primary human cell culture systems, the model of replicative senescence induced by telomere shortening following continued cell division is well established (Harley *et al.* 1990). That bypassing senescence leads to crisis and death of the majority of cells in culture has also been well established, suggesting that telomere shortening in culture systems provides a barrier to tumour growth. Consistent with this is the finding that telomeres are shorter in human tumour cells compared to surrounding normal tissue (de Lange *et al.* 1990). However, it has not yet been definitively demonstrated that telomere shortening limits the formation of tumours *in vivo*. Perhaps the best evidence that telomere shortening does inhibit tumour formation comes from human cancer cell lines in which telomerase activity is abrogated. Transfection of telomerase-positive cancer cells with a dominant negative mutant of TERT results in loss of telomerase activity, telomeric shortening and loss of capacity for growth following a lag period (Hahn *et al.* 1999a; Zhang *et al.* 1999). Similarly, dominant negative TERT inhibition of telomerase activity in these cells eliminates their ability to form tumours in nude mice.

In addition, evidence from mouse models have suggested a significant role for telomerase in tumour formation. In the *mTR*/ telomerase knockout mouse model, early generation mice with long telomeres show a 33% lower incidence of papillomas when compared with wild-type mice (Gonzalez-Suarez *et al.* 2000). In the β-actin-mTERT transgenic mouse model, TERT expression is driven by the β-actin constitutive promoter (Artandi *et al.* 2002). Overexpression of TERT is found in most tissues in these mice, which show an increased incidence of mammary tumours as they age, compared with wild-type mice, despite the fact that they have very long telomeres. Taken together, these findings from both humans and mice suggest that telomere shortening does in fact inhibit the formation of tumours, and that telomerase is a tumour promoter.

Reconciling these dual functions of telomerase could potentially be explained by the timing of telomerase activation. The *in vitro* findings of senescence, crisis and immortalisation in primary cells suggest a model in which the activation of telomerase expression occurs as a discrete event that permits passage through crisis and cellular immortalisation (Kolquist *et al.* 1998). This, together with the fact that many human cancers express telomerase whilst having short telomeres, has been taken as evidence that the activation of telomerase typically occurs late in tumour progression, after many rounds of cellular proliferation have resulted in significant telomere shortening (Kolquist *et al.* 1998). In contrast, chromosomal and genetic instability are thought to arise early in tumorigenesis (Shih *et al.* 2001). Thus, telomerase activity arising after the initiation of genetic instability prevents further instability by stabilising chromosome ends at the same time as it provides for unlimited tumour growth (DePinho 2000; Rudolph *et al.* 2001). However, some researchers have found that this may be an oversimplification of telomerase activation *in vivo*. Kolquist and colleagues reported that TERT expression (and consequently telomerase activity) was present in lesions that were early precursors to breast carcinogenesis, and that TERT expression increased during the progression from pre-neoplastic to malignant lesions (Kolquist *et al.* 1998). Their findings suggest that *in vivo*, telomerase activation may not occur as a simple step, and that cells might be continuously selected for incrementally higher levels of telomerase activity as they proliferate and acquire the genetic changes associated with invasive cancer.

1.4.2.7 Telomerase maintains telomere structure in normal cells

The observation that telomere attrition in normal fibroblasts occurs with progressive cell divisions led to the proposal that telomere shortening in normal cells occurs due to the strong repression of telomerase in those cells (Harley *et al.* 1990). However, some researchers have found that hTERT expression is detectable in some somatic cells (Broccoli *et al.* 1995; Masutomi *et al.* 2003). Moreover, telomere length does not always correlate with the onset of replicative senescence

(Karlseder *et al.* 2002). Together, these findings suggest that the relationship between telomere length, telomerase expression and proliferative lifespan are more complex than previously thought.

Further evidence suggesting that telomerase and telomeres interact in normal cells came from Masutomi *et al*, who found that in fact TERT is transiently expressed in normal human fibroblasts during the S phase of the cell cycle (Masutomi *et al.* 2003). This regulated expression produces functional TERT activity. Disruption of this expression slows proliferation, with delayed entry into the S phase and exit from the G2/M phases. Disruption of telomerase expression also results in a reduction in the proliferative lifespan of these cells and accelerated entry into senescence, although overall shortening of telomeres is not accelerated in normal fibroblasts with hTERT inhibition. However, the 3' overhang in these cells is significantly shorter than those of controls (Masutomi *et al.* 2003).

Taken together, these findings suggest that telomerase expression is involved with transit of normal human cells through the cell cycle, and that inhibition of TERT function affects lifespan by accelerating entry into senescence. This may be because disruption of telomerase function in the S phase prevents telomerase from maintaining the integrity of the normal telomeric cap.

1.4.2.8 Other functions of telomerase

There is growing evidence to suggest that in addition to the functions mentioned above, telomerase may play other roles in cells.

In the brain, it would appear that the telomerase plays a role in cell survival in the face of apoptosis. Suppression of TERT in developing hippocampal neurons from mice results in an increase in apoptosis, whilst overexpression of TERT conveys a protective effect to the same cells when exposed to a number of situations known to induce apoptosis, including withdrawal of trophic factors, DNA damage and exposure to β-amyloid (Fu *et al.* 2000; Lu *et al.* 2001; Zhu *et al.* 2000). This ability of TERT to influence cell death and apoptosis could have an important role to play in the developing brain. Natural neuron death occurs in most regions of the nervous system at late embryonic or early postnatal stage together with synaptogenesis. At the same time, TERT expression is decreasing in the developing mammalian brain. Since competition for a limited supply of trophic factors is thought to be an important determinant of which cells live and which die during this period of organogenesis, telomerase may play an important role during brain formation (Mattson *et al.* 2001).

As well as playing a possible role in brain development, telomerase may also play a role in response to injury in mature neurons. Following occlusion of the middle cerebral artery (MCAO)

in mice, levels of TERT RNA rise significantly in the cerebral cortex, suggesting an upregulation of TERT expression. In addition, transgenic mice overexpressing TERT show significantly reduced areas of cortical infarction and neuronal death following MCAO when compared to wild-type controls (Kang *et al.* 2004). Interestingly, TERT seems to protect neurons from death induced by exictotoxic neurotransmitters (eg NMDA), but has no significant effect on apoptosis or death due to reactive oxygen species.

Telomerase also appears to promote cell survival in other organs. The α-MHC-mTERT transgenic mouse was engineered to target TERT expression to the heart using the α myosin heavy chain (MHC) promoter to investigate whether upregulation of TERT could prevent ventricular myocytes from exiting the cell cycle and so prevent heart failure with age (Oh *et al.* 2001). Unexpectedly, TERT expression in myocytes from these mice seems to confer protection from apoptosis. Similar findings have been reported in human mammary epithelial cells exposed to TGF-α (Stampfer *et al.* 2001).

All of these findings suggest that telomerase promotes cell survival in a range of situations. It has been proposed that the presence of telomerase activity in mammalian cells could occur as a signal to cells that 'it is safe to divide', incurring the activity of survival and growth signals that are independent of telomere length but dependent on telomerase activity (Blasco 2002). The mechanisms behind this are currently unknown. It could be that physical interaction between telomerase and the telomere provides a signal for cell survival. Alternatively, telomerase activity may be required for a complete telomeric cap which could signal cell proliferation and survival independent of telomere length. Alternatively, active telomerase could have a role in suppressing or processing DNA damage in the genome, thereby favouring cell survival and proliferation (Blasco 2002).

1.4.3 Telomerase activity: species variation

Whilst the primary biological function of telomerase in all organisms so far examined is to add nucleotides to the ends of telomeres, the importance of telomere maintenance varies between species. Single celled organisms with a capacity for self-renewal, such as ciliates and yeast maintain, and in some instances lengthen, their telomeres. For example, in the yeast *Saccharomyces cerevisisae* the average telomere length in cells is maintained at 350 +/- 50 bp. This length is maintained by the balance between the opposing activities of telomerase and telomere-shortening processes, such as the mechanisms of lagging-strand synthesis and possible exonuclease activity (Wellinger *et al.* 1996). Eliminating telomerase activity from these cells either through disrupting the telomerase RNA or reverse transcriptase subunits allows the telomere-shortening processes to dominate, and consequently leads to progressive telomere erosion

with an eventual increase in chromosome instability and infertility until the population becomes 'senesecent' (Lendvay *et al.* 1994; McEachern & Blackburn 1995; Miller & Collins 2000). Thus, in these organisms for which self renewal is an integral part of day-to-day function, and for which ageing does not play an important survival-limiting role, telomerase activity is maintained continually.

In vertebrates, continual telomerase activity can also be found in multiple tissues in some species. In particular, the laboratory mouse, *Mus musculus*, was found early on to possess telomerase activity in multiple tissues including skin, lung, liver, colon and uterus((Prowse & Greider 1995) and reviewed in (Kipling 1997)). In fact, this discovery, together with the finding that mouse telomeres are considerably larger than those found in humans, initially proved difficult to reconcile with the growing circumstantial evidence supporting the theory of telomere-driven ageing which could be overcome through telomerase activity. However, the mouse has proved not to be the only vertebrate to possess telomerase activity in tissues composed of terminally differentiated somatic cells. Telomerase activity has also been found to be present in spleen, lymph node, lung and kidney of the pig (Fradiani *et al.* 2004).

By way of contrast, surveys of telomerase activity in humans have shown that telomerase activity is absent from the vast majority of somatic tissue. Exceptions include those tissues containing stem cells, or cells with high proliferative potential, including bone marrow, skin, hair follicles, gastrointestinal tract and endometrium (reviewed in (Collins & Mitchell 2002)). Dogs seem to regulate telomerase activity in a similar way to humans, with telomerase activity being absent from all tissues examined with the exception of testis, gastrointestinal tract and mammary gland (Nasir *et al.* 2001; Yazawa *et al.* 2001). Birds would appear to regulate telomerase activity in a manner more similar to humans and dogs than mice. During organogenesis in chickens, all tissues display strong telomerase activity (Taylor & Delany 2000), after which organ-specific variability emerges. Most tissues retain telomerase activity at hatching, with the exception of pancreas and brain, but by adulthood, telomerase activity is only present in testes, kidney and spleen (Venkatesan & Price 1998). Thus, although telomerase activity is present in most tissues for slightly longer than is seen in humans, ultimately it is undetectable in the majority of somatic tissues in the adult bird.

1.5 Alternative Lengthening of Telomeres (ALT)

Although the majority of immortalised mammalian cell lines maintain their telomere length through the activity of the telomerase holoenzyme, a substantial minority do not. These cell lines maintain telomere length through the activity of a different mechanism, known as the alternative lengthening of telomeres (ALT) pathway. To date, approximately 10% of cell lines have been reported to possess the ALT mechanism (Henson *et al.* 2002). In addition, a number of tumours

have also been reported to possess the ALT pathway within cells, including osteosarcomas, soft tissue sarcomas, glioblastoma multiforme, renal cell carcinoma, adrenocortical carcinoma, breast carcinoma, non-small cell carcinoma of the lung and ovarian carcinoma (Reddel 2003). In the glioblastomas examined, the proportion of tumours possessing the ALT pathway is higher than the incidence seen in other malignant cell types, with 25% of these tumours using the ALT mechanism to maintain telomere length (Hakin-Smith *et al.* 2003).

The mechanisms underlying the ALT pathway remain to be fully characterised. Four mechanisms have been proposed, all of which are consistent with a model in which telomeres use other telomeres or extrachromosomal telomeric DNA as copy template, following invasion by the 3' overhang of the telomere being elongated. In some cases, activation of the ALT pathway can also use TTAGGG repeats in the subtelomeric region of critically-shortened telomeres to prime further repeats, leading to replacement of the most distal variant subtelomeric repeats with TTAGGG sequences (Varley *et al.* 2002). These mechanisms are reviewed more fully in the article by Henson *et al.* (Henson *et al.* 2002). In addition, some forms of ALT exist in which polymerase-mediated extension is unnecessary, with telomere-lengthening being dependent on recombinational chromosome exchange mechanisms (Londono-Vallejo *et al.* 2004; Bailey *et al.* 2004). Consistent with all these mechanisms, examination of telomere lengths in cells utilising the ALT pathway reveals a wide variation in telomere length between cells. Telomere lengths in human cells gradually shorten to approximately 5-8 kb *in vitro* before the onset of replicative senescence, and tumour cell lines possess telomeres with a mean telomere length less than 10 kb. In contrast, telomeres in cells that have activated the ALT pathway have a very wide length distribution with a mean of approximately 20 kb and a range of between 3 kb and 50 kb (Bryan & Reddel 1997). Examination of individual chromosomes within a single cell using FISH reveals that some cells have undetectable telomeres whilst others have extremely strong telomeric signals (Perrem *et al.* 2001). The dynamics of telomere length in ALT-positive cells also supports a recombinational mechanism for telomere-lengthening. Prior to the activation of ALT, the telomeres of some cells shorten to less than 200 bp, before a rapid and heterogeneous increase in length occurs (sometimes >20 kb) (Henson *et al.* 2002). In other cells, such increases can also occur without prior shortening.

The implications of the ALT pathway potentially impose limitations on strategies targeting telomerase as part of cancer therapy. In some tumours, both ALT and telomerase have been shown to be activated in different cells (Reddel & Bryan 2003). Treatment of such tumours with either telomerase inhibitors or telomerase-targeting strategies is likely to select for the population of cells that maintain telomere length through other mechanisms, leading to a 'resistant' phenotype. This important possibility will need to be addressed before such strategies are tested in clinical settings. However, some findings suggest that in fact selection of ALT-positive cells may not necessarily be

a negative outcome. For example, glioblastomas positive for ALT have a significantly better outcome than those tumours which are positive for telomerase activity (Hakin-Smith *et al.* 2003). Evidence that telomerase contributes more to a malignant phenotype than simply the extension of lifespan suggests that removing the population of cells within a tumour that is positive for telomerase may slow cell division and tumour growth (Stewart *et al.* 2002). Additionally, one class of telomerase-inhibitors, the G-quadruplex ligands, has also been shown to cause apoptosis and cell death in ALT-positive cell lines (Kim *et al.* 2003a). For this reason, the existence of ALT mechanisms in a minority of tumours should not prevent the investigation of telomerase-targeting techniques as novel cancer treatment strategies.

1.6 Telomeres, Telomerase and Cancer

The functions of telomerase raise an important question – is telomerase potentially a useful target in the treatment of cancer? Several strands of evidence point towards telomerase activity being a central part of the acquisition of a malignant phenotype by cancer cells. Firstly, the ability of telomerase to circumvent pathways triggering both senescence and crisis suggests that acquiring telomerase activity would allow tumour cells to proliferate without limit, and thus acquire immortality. In addition, telomere shortening can be viewed as a means for generating the genetic mutations that underpin the evolution of cancer, with the activation of telomerase a final step in rescuing cells from end-to-end chromosome fusion and crisis. Secondly, the strong links between telomerase activity and the cell cycle (see 1.4.2.5: Telomerase and cell proliferation) suggest that telomerase is also intimately involved in mechanisms of proliferation. Together these two findings point to the importance of telomerase in cancer cells. Following on from this, a large body of evidence has now accumulated reviewing the extent to which telomerase can be detected in cancers in humans, and confirms that the majority of cancers and cancer-derived cell lines now in use possess telomerase activity. In fact, Shay and Bachetti, in reviewing a large number of tumours of different origin have reported that in excess of 80% of all human cancers are positive for telomerase activity (Shay & Bacchetti 1997). This will be discussed in greater detail in Chapter 3. Although less is known about telomerase activity in dogs, the reports published to date have suggested that a similar percentage of tumours in this species are also positive for telomerase (Argyle & Nasir 2003). Despite the significant effort applied to finding new molecular targets over the past three decades, few other candidates appear to be as ubiquitous in cancer as does telomerase. Thus, telomerase would seem to represent a potentially valuable target in developing new anti-cancer strategies.

1.7 Dogs as a model for cancer research in humans

Over the past two decades or so, major advances in molecular biology and genetics have increased our understanding of the biology of cancer in all species enormously. However, these advances, perhaps unsurprisingly, have outstripped our ability to apply this new knowledge to clinical applications. A number of factors have contributed to the lag phase between the acquisition and application of this knowledge, but undoubtedly one of the most important must be the lack of suitable *in vivo* cancer models in which these advances can be tested. Whilst a number of mouse models have been used in the preclinical testing of new treatment strategies designed for humans, few of these are characterised by spontaneous cancer development in immune competent and syngeneic hosts. Dogs provide several advantages in this respect: Firstly, dogs share many environmental risk factors with their human owners, suggesting that they might provide useful sentinels of disease (Kelsey *et al.* 1998). Secondly, the strong genetic similarities between dogs and man have meant that dogs with spontaneous cancers can be used in the identification of cancer-associated genes (Khanna *et al.* 2004; Lingaas *et al.* 2003). This has recently been facilitated by the completion of the canine genome project and the publication of several studies which have provided a high quality map of the canine genome at a resolution of 1 Mb and also mapped canine genes to the human genome (Kirkness *et al.* 2003; Guyon *et al.* 2003; Breen *et al.* 2001). Furthermore, the canine genome has now been shown to share more similarities with the human genome than either do with the mouse genome (Cooper *et al.* 2003). Thirdly, canine cancers share aspects of tumour biology, prevalence and behaviour with human cancer, and in many cases have identical histological appearances and response rates to conventional chemotherapies (Withrow & MacEwan 2001). Recent studies also suggest that these similarities extend to the level of the genome (Thomson *et al.* 2003). Fourthly, in most cases the prevalence of many canine cancers, including brain tumours, is sufficient to allow preclinical trials and biological studies to be performed, although numbers are generally smaller than is the case with human tumours. Their size also allows relatively easy (and affordable) translation of dose regimens from humans. Furthermore, due to the lack of well-defined 'gold standards' of therapy, early and ethical testing of novel therapies allow clinical trials to be performed, which generally are completed more rapidly than in humans. Hansen and Khanna have provided a comprehensive comparison of important aspects of tumours in humans and dogs, to which the reader is referred for further information (Hansen & Khanna 2004).

Given the importance of telomerase as a target for anticancer strategies, and the potential usefulness of dogs as a clinical model for human cancer, the principal aim of this thesis was to examine a number of ways in which telomerase could be used as a diagnostic and therapeutic tool in dogs.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Materials in regular use, such as equipment, general reagents and solutions are detailed in this section.

2.1.1 Cell culture materials

2.1.1.1 Plasticware

Tissue culture flasks, 6-well plates, Falcon conical centrifuge tubes (15 and 50ml) and pipettes (5, 10, 25, 50ml) were supplied by Greiner (Gloucestershire, UK).

2.1.1.2 Solutions, media and supplements

All solutions and media for cell culture were supplied by Invitrogen, UK, unless otherwise stated.

2.1.1.3 Media

All media were supplied as sterile solutions and stored at 4° C.

Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax-I with sodium pyruvate, glucose and pyridoxine was supplied by Invitrogen, UK.

Dulbecco's Modified Eagle's Medium (EMEM) with glucose and sodium bicarbonate, without L-Glutamine was supplied by Sigma-Aldrich, UK

Minimal Essential Media (MEM-α) was supplied by Invitrogen, UK.

RPMI 1640 was supplied by Invitrogen, UK

2.1.1.4 Supplements

Foetal Calf Serum (FCS): virus and mycoplasma screened. FCS was heat inactivated at 56° C for 30 minutes, then stored in 50ml aliquots at -20° C.

L-glutamine: supplied as 200mM (100x) stock solution and stored at -20° C in 5 ml aliquots.

Penicillin/streptomycin (P/S): supplied as a 100x stock solution of 10,000 units penicillin and 10,000 units streptomycin per ml and stored in 5 ml aliquots at -20° C.

Fungizone: supplied as a 100x stock solution and stored in 5 ml aliquots at -20° C.

Trypsin-EDTA: supplied as a 100x stock solution and stored in 5 ml aliquots at -20° C.

Sodium pyruvate: supplied as a 100mM stock solution and stored at 4° C.

Non Essential Amino Acids (NEAA): supplied as a 100x stock solution and stored at 4° C.

Epithelial Growth Factor: supplied as 200 mM stock solution and stored at -20° C in 5 ml aliquots

CML 10, D17, MDCK, A72 and GHK cell lines were cultured in DMEM (Invitrogen, UK) supplemented with 10% fetal calf serum.

CMT3, CMT7 and CMT8 cell lines were cultured in RPMI (Invitrogen, UK) supplemented with 10% fetal calf serum and 2mM L-glutamine.

Canine primary skin fibroblasts were cultured in MEM-α (Invitrogen, UK) supplemented with 10% fetal calf serum 2mM L-glutamine and epithelial growth factor.

U-87 MG cells were cultured in EMEM supplemented with 10% fetal calf serum, 2mM Glutamine, 1% Non Essential Amino Acids (NEAA) and 1mM Sodium Pyruvate.

2.1.1.5 Freeze Medium

Medium for cryopreservation of cells was prepared by mixing 9ml growth medium (appropriate to the cell line being stored) with 9ml fetal calf serum and 2ml DMSO (45% medium/45% fetal calf serum/10% DMSO).

2.1.1.6 Cell lines

MDCK, CML10, D-17 and **A-72 cells** were all supplied by the American Type Culture Collection (ATCC), Manassas, VA.

GHK cells (canine kidney)

CMT3, CMT7 and **CMT8 cells** were donated by G. Ruttemann, Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, University of Utrecht, PO Box 80.154, 3508 TD Utrecht, The Netherlands.

K9SF cells (canine primary skin fibroblast) cultures were generated and maintained by Mrs Elizabeth Gault from post mortem skin biopsy samples taken with informed owner consent at Glasgow University Veterinary School..

U87-MG cells (human glioblastoma) were supplied by the European Collection of Cell Cultures (ECACC), Porton Down, Wiltshire, UK.

2.1.2 General chemicals

Chemicals used were of analytic, ultrapure or molecular grade quality and were supplied by a range of biotech companies.

2.1.3 Complete kits

QIAquick® PCR Purification Kit (QIAGEN, UK)

QIAquick® Gel Extraction Kit (QIAGEN, UK)

QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK)

EndoFree® Plasmid Maxi Kit (QIAGEN, UK)

GeneRacer™ Kit (Invitrogen, UK)

Universal GenomeWalker Kit (Clontech, UK)

TOPO TA Cloning® (Invitrogen)

Dual-Luciferase® Reporter Assay System (Promega, UK)

ECL-PLUS Western Botting Detection Reagents (Amersham Pharmacia Biotech, UK)

DNA Sequencing Kit Big Dye™ Terminator Version 3.0 Cycle Sequencing Ready Reaction (ABI Applied Biosystems, UK)

TeloTAGGG Telomerase PCR ELISA (Roche Applied Science, UK)

2.1.4 Bacterial strains

2.1.4.1 E. coli One Shot® TOP10

Chemically Competent E.coli Cells (>1 x 10^9 cfu/μg) (Invitrogen, UK). Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC)ϕ80 lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG.

2.1.5 DNA

Plasmid, molecular weight markers and oligonucleotide DNAs were stored at -20°C.

2.1.5.1 Plasmid vectors

2.1.5.1.1 pCR®2.1-TOPO®

pCR®2.1-TOPO® (3900 bp), supplied by Invitrogen, UK, contains lacZ+, Beta-lactamase (ampr) and Kanamycin resistance (Kanr) genes. This plasmid is designed for directly cloning PCR products with 3' deoxyadenosine residues (A overhangs), generated by the non-template dependent activity of Taq polymerase. The vector is supplied as linearised DNA with single 3' deoxythymidine (T) residues and topoisomerase attached, allowing for efficient ligation of the

target sequence to the vector. T7 promoter and M13 forward and reverse primer binding sites are also present to allow sequencing of cloned products.

2.1.5.1.2 pCINeo

The pCI-Neo mammalian expression vector (5472 bp) was supplied by Promega and contains the neomycin phosphotransferase selectable marker under the regulation of the SV40 enhancer and early promoter region. In addition, pCI-Neo carries the human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells, and a multiple cloning region containing restriction enzyme sites. Expression of the neomycin phosphotransferase gene derived from Tn5 confers resistance to the antibiotic G-418, an aminoglycoside antibiotic produced by streptomycetes that induces cytotoxicity by blocking translation. Neomycin phosphotransferase inactivates G-418 through phosphorylation, thereby blocking its toxic effects. The pCI-Neo vector allows for stable expression of desired genes by selecting transfected cells with the antibiotic G-418.

2.1.5.1.3 pBABEpuro

The pBABEpuro vector was kindly supplied by RA Weinberg of the Whitehead Institute. pBABEpuro is a plasmid 5169bp in length that contains both ampicillin and puromycin resistance genes, together with a multiple cloning site.

2.1.5.1.4 pGL3-basic Vector

pGL3-basic Vector (4818 bp), supplied by Promega, UK, lacks eukaryotic promoter and enhancer sequences, allowing cloning of putative regulatory sequences into the multiple cloning site (MCS). The coding region for firefly (Photinus pyralis) luciferase gene (luc+) is followed by a SV40 poly(A) signal. This vector also contains Beta-lactamase gene (ampr) and RV3, RV4 and GL2 primer binding sites.

2.1.5.1.5 pGL3-control Vector

pGL3-control Vector (5249 bp), supplied by Promega, UK, contains a SV40 promoter, enhancer, late and up-stream poly(A) sequences resulting in strong expression of firefly (Photinus pyralis) luciferase gene (luc+) in many types of mammalian cells. Beta-lactamase gene (ampr) and RV3, RV4 and GL2 primer binding sites are also present.

2.1.5.1.6 pRL-CMV Vector[9]

pRL-CMV Vector[10] (4079 bp), supplied by Promega, UK, contains the CMV immediate-early enhancer/promoter region providing strong constitutive expression of Renilla (*Renilla reniformis*) [sea pansy] luciferase in a variety of cell types. The Beta-lactamase gene (*amp*^{r}) and SV40 late polyadenylation signal are also present.

2.1.5.2 Molecular Size Standards

All markers were supplied by Invitrogen, UK and include 1 kb DNA Ladder (size range: 75-12,216 bp), 1 Kb Plus DNA Ladder™ (size range: 100- 12,000 bp), and 100 bp DNA Ladder (size range: 100-2072 bp).

2.1.5.3 Oligonucleotide primers

Oligonucleotides for use in polymerase chain reactions (PCR) and cycle sequencing were synthesised by MWG Biotech and Sigma-Genosys. Primers were either reverse phase, desalted or Poly Acrylamide Gel electrophoresis (PAGE) purified and supplied as lyophilised DNA. Primers were reconstituted in dH_2O and stored at –20°C in 500 µl aliquots at 10µM.

2.1.6 Enzymes

All enzymes were stored at –20°C, being removed immediately before use.

2.1.6.1 Restriction enzymes

All restriction enzymes and their associated buffers were supplied by Promega, with the exception of HinfI and RsaI, which were supplied by Roche Applied Science. These are detailed in Table 2.1.

Table 2.1: Restriction Enzymes

Enzyme	Cutting Site [5' – 3']	Buffer	% Activity	Temperature (°C)
EcoR 1	[G | AATTC]	Buffer H	100%	37
		Buffer D	50-75%	37
		Multi-Core™	100%	37
Xho 1	[GGTAC | C]	Buffer D	100%	37
		Multi-Core™	10-25%	37
Sma 1	[CCC | GGG]	Buffer J	100%	25
		Multi-Core™	100%	25
Xba 1	[T | CTAGA]	Buffer D	100%	37
		Multi-Core™	100%	37
Sac 1	[GAGCT | C]	Multi-Core™	100%	37
Sal 1	[G | TCGAC]	Buffer D	100%	37
		Multi-Core™	<10%	37
Hinf1	[G | ANTC]	React®2	100%	37
Rsa1	[GT | AC]	React®3	100%	37
Stu 1	[AGG | CCT]	Buffer B	100%	37
Dra 1	[TTT | AAA]	Buffer B	100%	37
Pvu II	[CAG | CTG]	Buffer B	100%	37
EcoR V	[GAT | ATC]	Multi-Core™	100%	37

2.1.6.2 T4 DNA Ligase

T4 DNA ligase was provided by Promega with ligation buffer (used at a final concentration of 30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP) to catalyse the joining of two strands of DNA between the 5'-phosphate and the 3'- hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration.

2.1.6.3 Taq DNA polymerase

Taq DNA polymerase was provided by Invitrogen and has 5' to 3' exonuclease activity and non-template dependant activity with the addition of 3' deoxyadenosine (A) residues to PCR products for TA cloning.

2.1.6.4 Platinum® Taq DNA Polymerase High Fidelity

Platinum® *Taq* DNA polymerase high fidelity, provided by Invitrogen, is an enzyme mixture composed of recombinant *Taq* DNA polymerase, *Pyrococcus species* GB-D polymerase, and Platinum® *Taq* antibody. *Pyrococcus species* GB-D polymerase possesses a proofreading ability by virtue of its 3' to 5' exonuclease activity. Mixture of the proofreading enzyme with *Taq* DNA polymerase increases fidelity approximately six times over that of *Taq* DNA polymerase alone and allows amplification of simple and complex DNA templates over a large range of target sizes. The enzyme mixture is provided with an optimised buffer that improves enzyme fidelity and amplification of difficult templates. An anti-*Taq* DNA polymerase antibody complexes with and inhibits polymerase activity. Activity is restored after the denaturation step in PCR cycling at 94°C, thereby providing an automatic "hot start" for *Taq* DNA polymerase in PCR, which provides increased sensitivity, specificity and yield while allowing assembly of reactions at room temperature.

2.1.6.5 ThermalAce DNA Polymerase

ThermalAce™ DNA Polymerase, supplied by Invitrogen, is a thermostable enzyme from a proprietary archebacterium that is specifically designed for high-yield PCR amplification of GC-rich templates (>65% GC content). The enzyme retains full activity after incubation at 95°C for 4 hours and has approximately five times greater processivity than Taq DNA polymerase.

2.1.6.6 Taq PCRx DNA Polymerase

*Taq*PCRx DNA Polymerase is provided by Invitrogen and contains standard *Taq* DNA Polymerase together with an optimised buffer system consisting of Amplification buffer, 50mM $MgSO_4$ and PCRx Enhancer solution. Enhancer solution (the co-solvent) is a novel PCR co-solvent that facilitates efficient amplification of GC-rich sequences and problematic templates. Titration of the Enhancer solution to various concentrations ranging from 0x through to 4x in the final mixture allows rapid optimisation of appropriate conditions for amplification.

2.1.6.7 KOD HotStartTaq

KOD Hot Start DNA Polymerase, provided by Novagen, is a premixed complex of KOD HiFi DNA Polymerase, derived from *Thermococcus kodakaraensis* and two monoclonal antibodies that inhibit the DNA polymerase and 3' to 5' exonuclease activities during assembly of the PCR reaction at ambient temperatures. KOD HiFi DNA Polymerase acts with high fidelity, fast extension and high processivity, enabling amplification of targets up to 20 Kbp and efficient amplification of GC-rich targets. The antibodies allow a hot start to be performed by reducing non-specific amplification due to mispriming events during set up and initial temperature increase.

2.1.6.8 HotStartTaq®

HotStarTaq DNA Polymerase, provided by Qiagen, is a modified form of a recombinant 94-kDa DNA polymerase originally isolated from *Thermus aquaticus*, cloned in *E.coli*. The Polymerase is provided in an inactive state with no polymerase activity at ambient temperatures in order to prevent the formation of misprimed products and primer-dimers at ambient temperatures. Activation of the polymerase occurs following a 15 minute 95°C incubation step. In addition, HotStarTaq DNA Polymerase is provided with Q-solution, a PCR additive that facilitates amplification of difficult templates containing GC-rich sequences or a high degree of secondary structure by modifying the melting behaviour of DNA.

2.1.6.9 Failsafe™ PCR System

The Failsafe™ PCR premix selection kit is provided by Cambio and contains a blend of thermostable DNA polymerases together with twelve reaction premixes. The Failsafe PCR enzyme mix provides proofreading fidelity approximately three times greater than taq DNA polymerase alone. The twelve premixes contain a buffered salt solution together with dNTPs and various amounts of $MgCl_2$ and Failsafe PCR Enhancer which contains betaine and is designed to rapidly optimise amplification of long (up to 20 Kbp) and difficult templates. The presence of beatine improves the yield and specificity of amplification of many target sequences, especially those

containing a high GC content or secondary structure. Betaine may also enhance PCR by protecting DNA polymerases from thermal degradation.

2.1.6.10 Advantage® Genomic Polymerase Mix

Advantage® Genomic Polymerase was provided by Clontech and provides amplification of genomic DNA templates using a combination of the primary *Tth* DNA polymerase (thermostable DNA polymerase from *Thermus thermophilus*), a minor amount of a second DNA polymerase to provide 3′ to 5′ proof reading activity ($Vent_R$) and TthStart™ Antibody to provide automatic 'hot-start' PCR. This antibody reduces non-specific amplification products and primer-dimer artifacts created prior to the onset of thermal cycling. The antibody inhibits enzymatic activity during PCR reaction set-up at ambient temperatures. Polymerase activity is restored at the onset of thermo-cycling because the antibody is denatured at high temperatures. The loss of inhibition is complete and irreversible, so the polymerase regains full activity for PCR.

2.1.6.11 Ready-To-Go™ PCR beads (Amersham, Pharmacia)

Ready-To-Go™PCR beads (Amersham, Pharmacia) are designed as pre-mixed, pre-dispensed reactions for performing PCR amplifications. When brought to a final volume of 25 µl, each reaction contains *Taq* DNA Polymerase (1.5 units), 10 mM Tris-HCL, (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200µM of each dNTP and Bovine Serum Albumin (BSA).

2.1.6.12 Murine Moloney Virus Reverse Transcriptase Enzyme

Murine Moloney Leukaemia Virus Reverse Transcriptase (MMLV-RT) enzyme, provided by Invitrogen, uses single stranded RNA in the presence of a primer to synthesise a complementary DNA strand. The enzyme is isolated from *E.Coli* expressing a portion of the *pol* gene of the M-MLV on a plasmid.

2.1.6.13 Avian Myeloblastosis Virus Reverse Transcriptase (AML-RT)

Avian Myeloblastosis Virus Reverse Transcriptase functions in a similar manner to MMLV-RT.

2.1.6.14 Superscript III Reverse Transcriptase

Superscript III™-RT, provided by invitrogen, is a version of MMLV-RT that has been engineered to reduce RNase H activity and provide increased thermal stability.

2.1.6.15 RNaseOUT™ Recombinant Ribonuclease Inhibitor

RNaseOUT™ Recombinant Ribonuclease Inhibitor, provided by Invitrogen, is an acidic protein with a very high binding affinity for ribonucleases such as RNase A, B, C and is a non-competitive inhibitor.

2.1.6.16 Alkaline Phosphatase

Calf Intestinal alkaline phosphatase provided by Promega catalyses the hydrolysis of 5'- phosphate groups from DNA.

2.1.6.17 DNase I: DNA-free™

DNase I, provided by Ambion, is a deoxyribonuclease I enzyme that cleaves double-stranded or single stranded DNA. Cleavage preferentially occurs adjacent to pyrimidine (C or T) residues, and the enzyme is therefore an endonuclease. Major products are 5'-phosphorylated di, tri and tetranucleotides. In the presence of magnesium ions, DNase I hydrolyzes each strand of duplex DNA independently, generating random cleavages. In the presence of manganese ions, the enzyme cleaves both strands of DNA at approximately the same site, producing blunt ends or fragments with 1-2 base overhangs. DNase I does not cleave RNA.

2.1.7 Equipment

2.1.7.1 Major Equipment

Benchtop centrifuge: CPR Centrifuge (Beckman)
Microcentrifuges: Centrifuge 5402, 5415R and Minispin (eppendorf)
Water baths: Sub 36, and W6 (Grant)
Spectrophotometer: GeneQuant *pro* RNA/DNA calculator
Automatic Sequencing Apparatus: ABI 3100
Pipettes: Finnipipette Techpette (0.5-10, 5-40, 40-200, 200-1000 µl)
Ultraviolet trans-illuminator: T2201 (Sigma Chemical Company)
Gel documentation system: Uvi tec (Thistle Scientific)
Luminometer: Dynex MLX
Horizontal orbital shaker: 4628-1CE Labline Instruments inc (IL)
Incubator: B5042 (Heraeus)
Gel systems: Hoefer HE 33 Mini Horizontal Submarine Unit
Power packs: PAB 35-0.2 (Kikusui electronics corp)
Balance: Precisa 100A-300M (Precisa Balances Ltd, Bucks, UK)

Stirrer: Magnetic Stirrer Hotplate (Stuart Scientific, UK)

PCR Machines: GeneAmp PCR System 2400, 2700 and DNA Thermal Cycler 480 (Perkin Elmer)

Flow Cytometer: Epics XL Flow Sorter (Beckman-Coulter Inc., Miami, FL)

2.1.7.2 Consumables

Syringe top filters (0.2μm pore size) were supplied by Nalgene™, (NY, USA) for sterilisation of ampicillin and tissue culture reagents.

Ependorf tubes: screw top 1.5ml and 0.5ml and 1.5ml flip top tubes were supplied by Thermo Life Sciences.

Pipette tips were supplied by Greiner.

Filter tip pipette tips (10, 100, 200, 1000μl) were supplied by Finnitip (Thermo Lab Systems)

Petri dishes were supplied by Sterilin (Staffs, UK)

Bijoux were supplied by Greiner

Universals were supplied by Greiner

Scalpel blades were supplied by Swan-Morton (Sheffield)

Parafilm was supplied by Sigma

2.1.8 Buffers, solutions and growth media

2.1.8.1 Water

Sterile water for procedures involving recombinant DNA, PCR etc was supplied by Baxter. Vivendi Water systems (USF ELGA) was used to supply water for preparation of general solutions and media.

2.1.8.2 Antibiotics

Ampicillin: Reconstituted with dH_2O to 10mg/ml, filter sterilised and stored in 1 ml aliquots at -20°C until use.

G-418: Supplied by promega in 50 mg/ml solution and stored at –20°C.

2.1.8.3 Buffers and solutions

Ammonium persulphate: 10% (w/v) stock solution in dH_2O

50 x TAE Buffer Solution: Tris base 484.5g, NaOAc 272.15 g, NaCl 116.8 g, Na_2EDTA 74.45 g. pH adjusted to 8.15 with glacial acetic acid and made up to 2 L volume.

TBE Buffer Solution: 0.09M Tris Borate, and 0.002M EDTA

Lysis Buffer: 25mM Tris HCL pH 8.0 with glacial acetic acid and made up to 2 L volume

Lysis Buffer for Western Blotting: 0.5% NP40, 150mM NaCl, 50mM TrisHCL adjusted to pH 8.0, protease inhibitor tablet (1 per 10 ml of lysis buffer)

1M Tris HCL: 121g Tris base, 800ml dH_2O. Adjusted to the desired pH with concentrated HCL and made up to 1L.

TE Buffer: 10mM Tris-HCl. (pH 8.0), 1mM EDTA

1 x PBS: 140mM NaCl, 2.7mM KCL, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3)

0.2M (2x) Phosphate Buffered Saline (PBS): 0.2M Na_2HPO_4, 0.2M NaH_2PO4, 1.8% w/v NaCl, pH 7.4

10x DNA Gel Loading Buffer: 20% w/v Ficoll 400, 0.1M Na_2EDTA, pH 8, 1.0% w/v sodium dodecyl sulphate, 0.25% bromophenol blue, 0.25% xylene cyanol. Stored at room temperature and used at a 1:10 dilution.

Ethidium bromide: made to a working dilution of 10mg/ml with dH_2O in a fume cupboard. Stored away from light.

X-Gal solution: (5-bromo-4-chloro-3-indole-b-galactoside) prepared as 50 mg/ml stock in dimethylformamide; stored at –20°C in the dark.

Transfast™ Transfection Reagent: (Promega) Supplied as dried lipid film that forms multi-lamellar vesicles upon hydration with nuclease-free water (400µl) and stored at - 20oC.

DEPC water: 0.5ml of Diethylpyrocarbonate in 500ml water, overnight at room temperature in the fume cupboard and autoclaved.

Lysis mix: 0.32M sucrose, 10mM Tris, 5mM MgCl, 1% Triton X

Nuclei lysis mix: 10mM Tris, 0.4 NaCl, 2mM EDTA

TRIS-EDTA: (10/1 pH 7.4)

TRIS-EDTA: (10/0.1 pH7.5)

NU-PAGE Transfer Buffer: supplied by Invitrogen and diluted 1:20

NU-PAGE Running Buffer: supplied by Invitrogen and diluted 1:20

10x Ponceau's Red Stain: 2 g Ponceau S, 30 g Trichloroacetic acid (TCA), 30 g Sulphopalicylic acid. Made up to 100 ml by addition of dH_2O

TBS-Tween: 150 mM NaCl (8.77 g), 50 mM Tris (6.06 g) and 0.05% Tween (0.5 ml). Made up to 1L by addition of dH_2O

Blocking Buffer: 1 g non-fat skimmed milk powder in 20 ml TBS-Tween

Antibody diluent: 0.2 g non-fat skimmed milk powder in 20 ml TBS-Tween.

2.1.8.4 Bacteriological Media

Media was sterilised by autoclaving at 121°C for 15 minutes, unless stated otherwise

LB Medium: 20g tryptone, 20g NaCl, 10g Yeast Extract to 2L with dH2O, pH adjusted to 7.0 with NaOH.

SOC Medium: 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose.

2.2 Methods

Common methods used throughout the thesis are described in this chapter, whilst techniques specific to one area are described in later chapters. Many of the methods described are based on standard techniques, which are detailed in Current Protocols in Molecular Biology Volumes 1 & 2 (Ausubel *et al.* 1994).

2.2.1 Growth and manipulation of mammalian cells

2.2.1.1 Basic techniques

Techniques used in the culture and manipulation of mammalian cells were performed under aseptic procedures. Where possible all procedures were performed in a laminar flow hood.

2.2.1.1.1 Cell cryopreservation and storage

For long term storage of cell line stocks, cells were stored in liquid nitrogen. Prior to storage, cells were grown to mid-log phase and trypsinised with trypsin-EDTA at a concentration of 0.25% (w/v) and transferred to a sterile 15 ml centrifuge tube. The cells were centrifuged at 1200g for 5 minutes, the supernatant discarded and cells re-suspended in freezing medium (appropriate culture medium supplemented with fetal calf serum at 45% and DMSO at 10%) to a concentration of 2-4 x 10^6 cells/ml. The cell suspension was transferred in 1 ml aliquots to labelled cryovials (NALGENE™) and brought to -70°C at a controlled rate (-1°C/min) using a NALGENE™ Cryo 1°C Freezing Container (NALGENE, USA) transferred to a –70°C freezer overnight. Finally, vials were transferred to a liquid nitrogen freezer for long term storage. Cell stocks were revived by rapid thawing in a 37°C water bath and subsequently used following standard techniques as described below.

2.2.1.1.2 Cell Counting

Cells were counted using a haemocytometer. Following trypsinisation or revival from frozen stocks, cells were transferred to a 15 ml centrifuge tube and centrifuged at 1200g for 5 minutes. Supernatant was discarded and cells were resuspended in 5 ml of complete medium. A 25 µl sample was withdrawn and added to 25 µl trypan blue (Sigma) resulting in a two-fold dilution. A sample of this mixture was then introduced to the haemocytometer chamber and counted using an inverted microscope at 40x magnification. Cells lying on the top and right hand perimeter of each large (1 ml) square were included whilst those on the bottom and left hand perimeter were excluded. Cell concentrations (cells/ml) were calculated by multiplying the mean number of cells per large square by 10^4 and 2 to correct for the dilution factor. Where an estimation of live cell numbers was required the cell suspension was allowed to incubate for five minutes at room

temperature prior to counting. Non-viable cells stained blue and were counted as above and expressed as a percentage of the total cell count.

2.2.1.1.3 Preparation of cell pellets

Cells obtained from tissue culture were used in a number of applications including genomic DNA extraction, TRAP assays, RNA extraction and protein extraction. Following trypsinisation and counting as described above, cells were transferred in either 2 x 10^5 (for use in TRAP assays) or 2 x 10^6 (for all other applications) aliquots in 1.5 ml eppendorf tubes. Cells were centrifuged at 4°C at 3000 rpm for 5 minutes and growth medium discarded. Cells were then washed in 500 µl phosphate buffered saline (PBS) and centrifuged again at 4°C at 3000 rpm for 5 minutes and saline decanted. A final wash in 500 µl PBS was repeated before final centrifugation and decanting of saline. Cells were then rapidly frozen in liquid nitrogen before being stored at –80°C prior to use.

2.2.1.2 Cell lines

All cell lines were grown as an adherent monolayer in culture, in either 25 or 75 cm^2 tissue culture flasks stored in an incubator at 37°C with 5% CO_2 unless stated otherwise.

A72 Cells

The A72 cell line is an immortal canine cell line derived from an 8 year old female dog, with cells possessing a fibroblast morphology. Cells were maintained in DMEM with gluatamax-1 supplemented with 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cultures were trypsinised and reseeded at an approximate ratio of 1:5 every 3-4 days.

CML10 Cells

CML10 cells were obtained following biopsy of melanoma in a 10 year old female dogs. Cells possessed an epithelial morphology and were maintained in DMEM with glutamax-1 supplemented with 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cultures were trypsinised and reseeded, typically 1:5 every 3-4 days when sub-confluent.

CMT3 Cells

The CMT3 cell line is an immortal cell line derived from a canine mammary tumour. CMT3 cells were grown in RPMI medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cells were trypsinised as standard every 3-4 days and reseeded at an approximate ratio of 1:5.

CMT7 Cells

The CMT7 cell line is an immortal canine mammary tumour cell line. Culture conditions were as for CMT3 cells.

CMT8 Cells

The CMT8 cell line is an immortal canine mammary tumour cell line. Culture conditions were as for CMT3 cells.

D17 Cells

The D17 cell line was derived from an osteosarcoma obtained from an 11 year old female poodle. Cells were maintained in DMEM with glutamax-I supplemented with 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin and 1.25 µg/ml fungizone in 75 cm^2 tissue culture flasks. Cells were typically trypsinised and reseeded at a ratio of 1:5 every 3-4 days.

GHK Cells

The GHK immortal canine cell line was derived from renal epithelial cells taken from a greyhound of unknown age and gender at GUVS. Cells were maintained in DMEM with glutamax-I supplemented with 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin and 1.25 µg/ml fungizone.

K9SF Cells

The K9SF canine skin fibroblast cell line was obtained following skin biopsy taken from an 8 year old female newfoundland at GUVS. Cells were maintained in MEM-alpha medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin, 1.25 ug/ml fungizone and 2 mM L-glutamine. Cells were passaged routinely after 5-7 days. Confirmation of the species of origin was performed using RT-PCR and sequence analysis for the canine cyclophilin gene. Cultures were trypsinised and reseeded at an approximate ratio of 1:5 when confluent.

MDCK Cells

The MDCK cell line was derived from a kidney of an apparently normal female cocker spaniel in September 1958 by S.H. Madin and N.B. Darby. Cells were cultured in DMEM with glutamax-I supplemented with 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin and 1.25 µg/ml fungizone. Cultures were split, typically 1:9 every 3-4 days when sub-confluent and were trypsinised and seeded into new flasks.

U87-MG Cells

The U-87 immortal human cancer cell line is derived from a malignant glioma from a female patient by explant technique. It is reported to produce a malignant tumour consistent with glioblastoma in nude mice. Cells grow in an adherent monolayer with epithelial morphology and

were cultured in EMEM supplemented with 10% FCS, 2 mM L-glutamine, 1% Non Essential Amino Acids (NEAA), 1mM Sodium Pyruvate (NaP), 100 IU/ml penicillin, 100 IU/ml streptomycin and 1.25 ug/ml fungizone. Cells were trypsinised and reseeded at an approximate ratio of 1:5 every 5-7 days.

2.2.2 Preparation of canine genomic DNA from peripheral blood mononuclear cells

Since genomic DNA is identical in every cell from a given individual, any canine cell could be used for isolating canine gDNA for use as a template in PCR reactions. For ease of obtaining samples, peripheral blood mononuclear cells (PBMCs) were selected. Canine whole blood samples (5 ml) were mixed with lysis mix (45 ml) and incubated on ice for 10 mins. The mixture was then centrifuged at 2800g for 10 mins at 4° C and the pellet re-suspended in nuclei lysis mix (3 ml). After the addition of 10% SDS (200µl) and 75µl of proteinase K (10mg/ml) the mixture was incubated at 55° C for 2 hours or at 37 C overnight. Two extractions with 3ml phenol/chloroform (50:50) were then performed, each centrifuged for 10 mins at 2800g at room temperature. The upper aqueous phase was then transferred to a fresh tube and the DNA precipitated by adding 3 volumes of 100% ethanol. The DNA was then re-suspended in 250µl of TE Buffer and the quantity and quality of gDNA was assessed using UV spectrophotometry and agarose gel electrophoresis.

2.2.3 Recombinant DNA techniques

2.2.3.1 Growth and storage of plasmid-containing E. coli

Plasmids were maintained in the One Shot® TOP10 strain of *E.coli*. Storage of transformed bacteria containing the desired plasmid was carried out following preparation of glycerol stocks. The desired bacterial culture was streaked onto a 1.5% agar plate (1.5% agar in LB medium). In cases where the plasmid contained a selectable ampicillin-resistance gene, medium was supplemented with 50-100µg/ml ampicillin. The plate was incubated overnight at 37°C and the next day single colonies were picked using a pipette tip into approximately 5 ml LB medium (supplemented with 50-100µg/ml ampicillin) in a sterile universal tube. The resultant cultures were incubated at 37°C overnight in a horizontal orbital incubator at 225 rpm. In order to confirm that the culture was derived from bacteria containing the desired plasmid, DNA extraction and restriction digestion were performed (cross ref).

For long term storage, glycerol stocks (15%) were prepared by the addition of 90µl of glycerol (diluted 50% in distilled water and sterilised) to 210µl of culture, and the resultant stock stored at

-20° C. For subsequent work requiring these stocks, a sterile pipette tip was used to scratch the surface of the stock, following which it was streaked onto an agar plate as described above.

2.2.3.2 Extraction and purification of plasmid DNA

Plasmid DNA was isolated using a modification of the alkali lysis technique (Birnboim & Doly 1979).

2.2.3.2.1 Large Scale Plasmid Preparations

For techniques such as sequencing or transient transfections, large quantities of high quality endotoxin-free plasmid DNA were obtained using the EndoFree® Plasmid Maxi Kit (QIAGEN, UK). A starter culture (5 ml) of the desired transformant was grown as described above and used to seed a larger culture by diluting 1/500 to 1/1000 into 250 ml of LB medium containing 50-100μg/ml ampicillin. This was then grown overnight at 37° C with horizontal shaking at 225 rpm. The culture was then aliquoted into 50 ml Falcon tubes and spun at 3000 rpm for 30 minutes at 4° C for harvesting of bacterial cells. The supernatant was then discarded and the remainder of the protocol performed according to the manufacturer's instructions. DNA was stored at -20°C.

2.2.3.2.2 Small Scale Plasmid Preparations

For other purposes such as restriction endonuclease digestion or sequencing, smaller quantities of high quality plasmid DNA were purified from 5 ml of an overnight culture of transformed bacteria using the QIAprep® PCR Spin Miniprep Kit (QIAGEN, UK). In order to harvest the bacteria, the culture was spun at 3000 rpm for 10 minutes and the supernatant removed. DNA was then purified from the pelleted cells according to the manufacturer's instructions. Lysis of the cells was performed in this procedure in order to release the plasmid DNA, which was separated from bacterial cell debris by centrifugation and then attached to a filter and washed with a buffer before being eluted in sterile water (50μl). DNA was stored at -20°C.

2.2.3.3 Determination of (ribo)nucleic acid concentration

2.2.3.3.1 Determination by spectrophotometry

DNA and RNA samples were diluted 1:50 by addition of 2 μl of sample to 98 μl of dH_2O. The optical density was measured at 260 nm and 280 nm, in comparison to a reference of dH_2O. An optical density (OD) reading of 1.0 at 260 nm corresponds to an approximate concentration of 50 μg/ml for double stranded DNA, 40 μg/ml for RNA, or 33 μg/ml for single stranded

oligonucleotides. The ratio of the OD readings at 260 nm and 280 nm (OD_{260}/OD_{280}) was used to estimate sample purity, with pure preparations of DNA and RNA producing an OD_{260}/OD_{280} of 1.8 and 2.0, respectively. A lower value suggests possible protein or phenol contamination of the sample.

2.2.3.3.2 Estimation of double stranded DNA concentration via gel electrophoresis

In some instances, insufficient concentration of (ribo)nucleic acid or verification of the purity of DNA fragments of a particular size did not allow quantification via spectrophotometry. In these cases, the concentration of double stranded DNA was determined by running the sample on a 1% agarose gel along with a marker of known quantity and size. The intensity of the sample fluorescence was compared to that of a mass marker (Low DNA Mass™ Ladder) following staining with ethidium bromide and visualisation by UV transillumination.

2.2.3.4 Restriction endonuclease digestion

Typically, 1-2 µg of DNA were digested in a 20 µl reaction mix containing the appropriate buffer and 5 to 10 units of desired restriction enzyme. The reactions were incubated at the recommended temperature (typically 37° C) for a minimum of one hour. Where the isolation of restriction fragments was required, larger quantities of DNA, generally 5-10 µg, were digested, with the reaction volume and components being increased proportionally.

Recombinant plasmids contain a variety of restriction enzyme sites. One or more appropriate restriction enzymes can be incubated with DNA constructs at 37° C in the correct buffer, as determined by the manufacturer's recommendations, to cut at specific positions and produce fragments of different sizes. The sizes of these fragments could be pre-determined and then viewed by gel electrophoresis.

2.2.3.5 Electrophoresis of DNA

DNA fragments of 0.1-10 kb were separated and identified by agarose gel electrophoresis using a Hoefer HE 33 Mini Submarine Electrophoresis Unit (Amersham Pharmacia Biotech, San Francisco, CA). Typically, 0.5 – 0.1 g of agarose was added to 50 ml of 0.5x TAE buffer, melted in the microwave (medium for 1.5 minutes), and mixed to produce a 1 to 2% gel. Once the gel mix had cooled to 55°C, 1.5µl of ethidium bromide (100mg/ml) was added and the gel poured into a gel support (100 x 65 mm) in its casting tray and an appropriate gel comb (twelve or twenty well) inserted. The gel was allowed to solidify before transferring to an electrophoresis tank; the gel was immersed 0.5x TAE buffer and the comb carefully removed. DNA samples were prepared by the addition of an appropriate volume of 10x gel loading buffer. Molecular size standard DNA was

prepared similarly and the samples loaded into the wells using a micropipette. Gels were run at 100 volts for 20 to 30 minutes depending on the agarose percentage, then removed from the gel apparatus and visualised on a UV transilluminator (UVi tec, Thistle Scientific) and photographed using a Mitsubishi P91 photographic unit.

2.2.3.6 Purification of restriction enzyme fragments

Where purification of DNA fragments was required for construction of recombinant plasmids, DNA was purified from agarose gels using the QIAquick® Gel Extraction Kit (QIAGEN). Following electrophoresis, DNA fragments of interest were excised from agarose gels using a clean scalpel and the remainder of the procedure was performed according to the manufacturer's instructions.

2.2.3.7 Ligation of restriction digested DNA fragments

Fragments of DNA generated by restriction digestion were ligated into approximately 50-100 ng of vector using T4 DNA ligase (Promega) according to the manufacturer's instructions. Using a known (eg 100 ng) quantity of vector, the quantity of insert DNA was calculated using the following equation, with ligation performed at either a 1:1 or 1:3 vector:insert ratio:

$$\frac{\text{X ng vector x Y kb}}{\text{Z kb vector insert}} \text{ x insert : vector molar ratio } = \text{ ng of insert required}$$

Vector and insert were mixed with ligation buffer (1x) and DNA ligase (1 unit), in a total volume of 10 µl. Ligation reactions were allowed to proceed at 16°C overnight.

2.2.3.8 Ligation using TA cloning methods

Ligations of DNA fragments generated by *Taq* Polymerase during PCR were carried out according to the manufacturer's instructions with the pCR2.1®TOPO TA Cloning Kit (Invitrogen). Briefly, freshly purified PCR products (15 ng) were directly cloned into the pCR®2.1-TOPO plasmid vector (0.5µl), incubated for 5 minutes at room temperature. Topoisomerase I is covalently bound to the ends of the linearised vector to facilitate the cloning procedure.

All ligations were stored at -20°C if not used immediately. A negative control ligation, omitting the DNA insert, was generally set up in parallel to the above, in order to check for 'background' when performing subsequent transformations.

2.2.3.9 Transformation of bacteria with plasmid DNA

In addition to the transformation with recombinant plasmid, the bacteria were also transformed with a control plasmid (as a positive control) and a ligation reaction from which the insert DNA had been omitted (as a negative control).

2.2.3.10 Transformation of TOP10 cells

Cells were thawed on wet ice, gently mixed and 25-50 µl of cells was transferred to chilled microcentrifuge tubes for each transformation as required. Ligation reaction (0.5-5µl) or control plasmid (pUC18) was added to cells (1-20 ng DNA), moving the pipette through the cells while dispensing in order to facilitate mixing. Cells were left on ice for 30 minutes, then heat shocked in a 42° C water bath for 30 seconds. After heat shocking, the cells were placed on ice for 2 minutes, 200 µl of SOC medium was added and the tubes were incubated at 37° C for 1 hour with shaking at 225 rpm. Variable volumes of cells were plated onto LB plates containing ampicillin (100 mg/ml) and X-Gal stock (35 µl) solution was spread on the plate one hour prior to use if blue-white colour selection was used and all plates incubated overnight at 37° C.

2.2.3.11 Screening of transformants for desired recombinant plasmids

Most plasmids used in this project conferred ampicillin resistance upon the host bacteria, allowing selection and maintenance of transformed bacteria with ampicillin supplemented media.

2.2.3.11.1 α- complementation

The pCR2.1®TOPO plasmids contain genes encoding the *lacZ*α fragment of the β-galactosidase and the *lac* promoter and are therefore capable of α-complementation with the φ fragment encoded by the *E.coli* host strains DH5α, JM109 and TOP10 One Shot™ giving active β-galactosidase. The incorporation of X-gal into LB agar plates allows the selection of transformants based on blue-white screening. Disruption of the *lacZ*α expression occurs with the cloning of fragments into the MCS of the vector, hence recombinants with plasmid containing insert DNA appear white whilst non-recombinants, expressing a functional β-galactosidase, appear blue.

2.2.3.11.2 Restriction analysis of small-scale plasmid preparations

Plasmid DNA, isolated as described in 2.2.3.2: Extraction and purification of plasmid DNA, was subjected to restriction digestion with the appropriate enzyme(s) (Table 2.1), and the resulting products of digestion were run on an agarose gel. Bacteria with plasmids containing inserts of the

desired size were stored as glycerol stocks as detailed in section 2.2.3.1: Growth and storage of plasmid-containing E. coli.

2.2.3.11.3 PCR amplification of broth or small-scale plasmid preparations

A sample of bacterial culture (50 µl) was removed and heated at 95°C for 6 minutes and centrifuged at 12,000 rpm for 6 minutes. Aliquots of the supernatants (10µl) were then used as template in PCR reactions.

2.2.4 RNA techniques

Clean, full-length RNA is essential as the starting material for molecular techniques, hence when isolating the RNA it was necessary to ensure that all the preparation material is free from ribonuclease (RNAse) activity. This is a very stable, active and ubiquitous enzyme that degrades RNA requiring no cofactors for function. Inhibition of this enzyme was instigated by wiping all equipment with RNase ERASE (ICN Biomedicals, Inc, Ohio) followed by a thorough rinse with DEPC treated water. All plastic-ware was treated by soaking overnight in DEPC treated water followed by autoclaving and drying. All solutions were prepared using DEPC treated water. Gloves were worn and changed frequently.

2.2.4.1 Extraction and Purification of RNA

2.2.4.1.1 RNA extraction using RNAwiz™

Various methods have been used for the isolation of undegraded RNA (Chirgwin *et al.* 1979). Several procedures have now been elucidated which isolate RNA isolation in a single-step procedure (Chomczynski & Sacchi 1987). The RNAzol™ B methods promote formation of complexes of RNA with guanidinium and water molecules and abolishes hydrophilic interactions of DNA and proteins. In effect, the DNA and proteins are efficiently removed from the aqueous phase with the RNA remaining in this phase during the sample extraction.

In this project, RNA was extracted from a variety of sources: from tissues snap-frozen in liquid nitrogen and stored at –80° C, from cells grown as a monolayer in tissue culture flasks, and from cell pellets stored at –80°C. In order to extract RNA from frozen tissue, tissue was ground in liquid nitrogen using a mortar and pestle to create a powder to which was added RNAwiz™ at a ratio of 1ml per 100 µg of tissue. RNA extracted directly from cells grown as a monolayer was performed using RNAwiz™ at a ratio of 1 ml per 10cm^2 of tissue culture flask area. RNA was also extracted by cell pellets containing 2 x 10^6 cells stored at –80°C (see 2.2.1.1.3: Preparation of cell pellets) using RNAwiz™ at a ratio of 0.2 ml per cell pellet.

Following the addition of RNAwiz™ solution, cells/tissue were homogenised by pipetting up and down vigorously several times. The homogenate was then incubated at room temperature for 5 minutes to allow dissociation of nucleoproteins from nucleic acids. Then, chloroform (without isoamyl alcohol or other additives) was added at a ratio of 0.2x the starting volume of RNAwiz™ used and the mixture agitated by shaking vigorously for approximately 20 seconds. Samples were then centrifuged at 13,000 rpm at 4° C for 15 minutes in order to separate the homogenate into 3 phases: an upper aqueous phase (containing RNA), a semi-solid interphase (containing most of the DNA), and a lower organic phase. The upper phase was carefully removed by pipetting and transferred to a clean, DEPC-treated 1.5 ml eppendorf. RNAse-free (DEPC-treated) water was added at a ratio of 0.5x the starting volume of RNAwiz™ mixed thoroughly. Isopropanol was then added (using the same volume as the starting volume of RNAwiz™), the sample was mixed, and then incubated at room temperature for 10 minutes. The sample was then centrifuged at 13,000 rpm at 4° C for 15 minutes in order to pellet the RNA. The supernatant was decanted carefully and the RNA pellet washed in ice-cold 75% ethanol before centrifugation at 13,000 rpm at 4° C for 5 minutes. Following this final centrifugation step, ethanol was decanted and the RNA pellet dried for no longer than 10 minutes (overdrying makes resuspension difficult) before being resuspended in 30 µl of DEPC-treated water. Samples were stored at –80°C prior to use.

2.2.4.1.2 DNase Treatment of RNA

Contaminating DNA was removed from RNA samples using DNA-free™ (Ambion) methods. Briefly, 0.1 volume of 10x DNase I buffer (final concentration: 10 mM Tris-HCl pH7.5, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$) and 2 units of DNase I were added directly to the RNA samples, mixed and incubated at 37° C for approximately 60 minutes. The enzyme reaction was stopped by the addition of DNase Inactivation Reagent (5µl) for two minutes at room temperature Because of the nature of the DNase Inactivation Reagent, tubes were periodically agitated to ensure resuspension in the RNA sample. Samples were then centrifuged at 10,000g for one and a half minutes to pellet the Inactivation Reagent and the supernatant containing the pure RNA was then transferred to a clean ependorf tube.

2.2.4.1.3 Assessment of RNA quality using agarose gel electrophoresis

RNA samples were assessed by agarose gel electrophoresis on a 1% agarose TAE gel by comparing the bands to a 100 bp molecular weight standard (INVITROGEN, UK). The quality of the RNA samples were assessed by examining the integrity of the 18S and 28S ribosomal RNA bands. The 18S ribosomal unit runs at approximately 4.5 Kb and the 28S at approximately 1.2 Kb.

The 18S subunit should appear at twice the intensity of the 28S subunit. mRNA appears as a smear from 0.5 through to 12 Kb.

2.2.5 First strand cDNA synthesis

The synthesis of complementary DNA (cDNA) requires the Reverse Transcriptase enzyme, initially identified as the enzyme responsible for forming DNA-RNA hybrids in retrovirus replication. Reverse transcriptase utilises an RNA template to synthesise a complementary DNA strand from a primer base-paired to the RNA that contains a free hydroxyl group at the 3' end. Primers that can be used are either random primers, designed to prime at multiple sites throughout the RNA, gene specific primers (GSPs) designed to prime at the gene of interest, or oligo-dT primers which pair with the poly-A sequence at the 3' end of most eukaryotic mRNA molecules. All instigate the synthesis of cDNA strands in the presence of the four dNTPs. The RNA-DNA hybrid is subsequently hydrolysed by raising the pH (unlike RNA, DNA is resistant to alkaline hydrolysis) or by using a ribonuclease. The 3'- end of the newly-synthesised DNA strand then forms a hairpin loop which enables priming of the synthesis of the opposite DNA strand. S1 nuclease, which recognises unpaired nucleotides, then removes the hairpin loop.

Superscript III™ reverse transcriptase was used for first strand cDNA synthesis. Superscript III™ is a version of MMLV-RT that has been engineered to reduce RNase H activity and provide increased thermal stability. Briefly, total RNA samples (up to 5 µg total RNA) were added to 1ul of primer (either oligo(dT), GSP or random primers) and 1µl of 10mM dNTPs, and DEPC water was added to 10µl total volume. This mixture was then denatured to remove any RNA secondary structure by heating for 5 minutes at 65° C and before quenching on ice. First strand synthesis reactions were performed using the heat-treated RNA in a reaction mix containing reverse transcription buffer (10x), reverse transcriptase (200 units), DTT (0.1 M), RNase inhibitor (RNaseOUT™ 40 units) and MgCl2 (25 mM). The reaction was incubated at 50° C for 50 minutes if oligo(dT) primer was used, or 25° C for 10 minutes followed by 50° C for 50 minutes if other primers were used, and was incubated at 85-95° C for 5 minutes to terminate the reaction before being placed on ice. Finally, RnaseH (1µl) was added and the mixture incubated at 37° C for 20 minutes to remove the RNA template from the DNA-RNA hybrid. A Perkin-Elmer (PE) thermal cycler 480 was used for the reaction. cDNA was stored at 4° C before immediate use or –20° C for long term storage.

2.2.6 Amplification of DNA by the polymerase chain reaction

The polymerase chain reaction (PCR) is a powerful technique for amplification of specific DNA sequences from a complex mixture of DNA. The procedure was developed by Mullis and co-workers in the mid 1980's (Mullis *et al.* 1986; Mullis & Faloona 1987) enabling large amounts of a single copy gene to be generated from genomic (Saiki *et al.* 1988) or viral DNA (Kwok *et al.* 1987). The initial method used the Klenow fragment of DNA polymerase I, which had to be replenished during each cycle as it is readily denatured by the amplification conditions used. The substitution of the thermostable *Taq* polymerase, isolated from *Thermus aquaticus*, circumvented this problem and allowed the automation of thermal cycling (Saiki *et al.* 1988).

PCR enables the amplification of any unknown DNA sequence by the simultaneous extension of primer pairs, flanking the unknown sequence, each complimentary to opposite strands of DNA. The uses of PCR are many although it has been superseded by more conventional molecular biological methods in many areas, including sequencing (Innis *et al.* 1988), cloning (Scharf 1990) and detection and analysis of RNA. An extensive overview of PCR and its applications is available (Innis & Gelfand 1990) but an overview of the procedure is given below, with more detail in the appropriate chapters.

2.2.6.1 Primer design

Primer design was aided by some basic guidelines (Innis & Gelfand 1990). An optimal primer pair should hybridise efficiently to the sequence of interest with negligible hybridisation to other sequences present in the sample. The distance between primers is rather flexible, ranging up to 10 kbp, however the PCR reaction is considerably less efficient with distances > 3kbp (Jeffreys *et al.* 1988). Smaller distances between primers lessen the ability to obtain much sequence information and to reamplify with nested internal oligonucleotides, if required. For any given pair, the annealing temperatures (Tm) and GC % were balanced.

For many applications, primers are designed to be exactly complementary to the template. For others, however, such as engineering for mutagenesis or new restriction endonuclease sites or for efforts to clone or detect gene homologues where sequence information is lacking, base-pair mismatches will be intentionally or unavoidably created. It is best to have mismatches, such as in a restriction endonuclease linker, at the 5′ end of the primer; the closer to the 3′ end of the primer, the more likely a mismatch will prevent extension.

The annealing portion of primers should generally be 18-30 nucleotides in length; it is unlikely that longer primers will help to increase specificity significantly. Additional sequences such as restriction enzyme sites, epitope tags or other desired motifs can be added to the 5′ end of the primer, and may effect specificity of primer binding at low temperatures on complex templates so

these primers are successfully used when amplifying from a single template vector. Primers should be designed with a GC content similar to that of the template, avoiding unusual sequence distributions such as stretches of polypurines or polypyrimidines if possible. The formation of a secondary structure such as hairpin loops greatly effects efficiency of annealing.

Primer-dimers are a common artifact most frequently observed when small amounts of template are taken through many amplification cycles. They form when the 3′ end of one primer anneals to the 3′ end of the other primer and polymerase then extends each primer to the end of the other. Therefore, these can be avoided by using primers without complementary sequences, especially in the 3′ end (this can be minimised by optimising the $MgCl_2$ concentration).

2.2.6.2 Preparation of PCR reactions

As PCR is such a sensitive procedure it is essential to take stringent precautions to avoid PCR contamination from tube to tube or carry over of PCR products (Saiki, *et al.* 1988). All PCR reactions were set up in a designated separate room, at the side of the main laboratory where PCR products were handled. A set of micropipettes was kept for the sole purpose of setting up PCR reactions. Filter tip pipette tips were used to decrease the risk of reaction components passing from one tube to the next. A bulk reaction mix was used in order to minimise the number of pipetting steps. Reaction components (including primers) were aliquoted prior to use and stored at –20°C.

2.2.6.3 Reaction conditions

The use of high quality reagents is essential for the success of PCR; to facilitate this a number of PCR amplification kits, each containing the necessary reagents, were used following the manufacturers' instructions. Typically reaction mixes were set up in either 50 µl or 25 µl volumes using thin walled ependorf tubes (0.5 ml or 0.2 ml respectively). Variable concentrations of primers (µM), dATPs, dCTP, dTTP and dGTP (mM), $MgCl_2$ (mmol/L), DNA polymerase (Units), 1x PCR Buffer containing Tris-HCl (mM), KCl (mM) and gelatin was mixed with an appropriate volume of DNA or cDNA template (ng). Thermal cycling was carried out using DNA thermal cyclers (Perkin Elmer). Two PCR machines were generally used, a Perkin Elmer (PE) 480 which required overlaying the reaction mix with mineral oil in 0.5 ml ependorf tubes, and a Perkin Elmer (PE) 2400 size for the 0.2 ml ependorf tubes requiring no mineral oil. Cycles varied between reactions but most commonly consisted of an initial denaturation of 94° C for five minutes, followed by 25-45 cycles of the following; denaturation at 94° C for one minute; annealing at 55-72° C for one minute; extension at 72° C for one minute; with a final extension step at 72° C for 4 – 30 minutes. Reaction products were visualised by agarose gel electrophoresis, generally using 5-10 µl of reaction product per well.

2.2.6.4 Purification and assessment of PCR products

Single PCR products were purified following the QIAquick®PCR purification kit protocol, (QIAGEN). Briefly, the DNA adhered to the filter within the column, separating it from all other components of the PCR reaction which were washed away with various buffer solutions. The PCR products were finally eluted in 30-50µl of sterile water, 4µl of which were assessed by gel electrophoresis on a 1% agarose TAE gel by comparing the bands created to a 100 bp molecular weight standard (INVITROGEN, UK)

2.2.7 DNA sequence analysis

2.2.7.1 Automated sequencing

Sanger dideoxy DNA sequencing is the most commonly used method for DNA sequencing, particularly in large-scale genomic sequencing (Sanger *et al.* 1977). A variation of automated DNA sequencing using dye-labelled terminators, in which the dyes are attached to the terminating dideoxynucleoside triphosphates (Prober *et al.* 1987) has been used in this project. Primers used for automated sequencing are shown in Table 2.2.

Table 2.2: Primers commonly used for DNA sequencing

Primer name	Primer sequence (5′ to 3′)	Tm (°C) & GC (%) content
M13 Reverse (M13F)	CAGGAAACAGCTATGAC	68.0 °C : 55.6 %
M13Forward (-20) (M13R)	GTAAAACGACGGCCAG	70.9 °C : 58.6 %
GL2	CTTTATGTTTTTGGCGTCTTCCA	57.1 °C : 39.1 %
RV3	CTAGCAAAATAGGCTGTCCC	57.3 °C : 50.0 %
RV4	GACGATAGTCATGCCCCGCGCCCACCGGAA	87.0 °C : 66.7 %

2.2.7.2 Sample preparation

During sample preparation, DNA fragments in a sample are chemically labelled with fluorescent dyes. The dyes facilitate the detection and identification of the DNA. Typically each DNA molecule is labelled with one dye molecule, but up to five dyes can be used to label the DNA sample. More specifically, PCR reactions were performed using plasmid DNA samples (200-500ng) in a total volume of 20µl containing 0.5µM of primers, 40mM Tris-HCl, 1mmol/L $MgCl_2$ and 4µl of Big Dye™ Terminator Cycle Sequencing Ready Reaction (ABI Prism). Samples were prepared in the PE 2400 thermal cycler incorporating 25 cycles of amplification, each cycle consisting of a denaturation step 96° C for 10 seconds followed by an annealing temperature of 50° C for 5 seconds, and an elongation step of 60° C for four minutes. DNA was then purified by precipitation methods using 3M sodium acetate (Sigma) and ethanol (95%), room temperature for 15 minutes. Pelleted DNA (14,000 rpm for 20 minutes) was washed in ethanol (70%) and re-pelleted (14,000 rpm for 5 minutes) before all ethanol was removed and dried at 90° C for 1 minute. Template Suppression Reagent (25µl) was then added and the mixture heated to 95° C for 5 minutes and chilled before transfer to genetic analyser sample tubes.

2.2.7.3 Sample sequencing

Samples were finally loaded and run on the ABI PRISM® 3100 Genetic Analyzer (PE Applied Biosystems, UK) under standard sequencing conditions for generation of automated sequence data. The ABI PRISM® 3100 Genetic Analyzer is a multi-colour fluorescence-based DNA analysis system using the proven technology of capillary electrophoresis with 16 capillaries operating in parallel. The 3100 Genetic Analyzer is fully automated from sample loading to data analysis.

2.2.7.4 Sequence evaluation

The length of read is 750 bases at the 98.5% base calling accuracy with less than 2% ambiguity. The output is in the form of a chromas file. A series of different computational software programs were utilised for sequence analysis including the 'Blast' search engine contained within the NCBI database, VectorNTI (Invitrogen), ClustalW (http://www.ch.embnet.org/software/ClustalW.html), Transfac and Motif Search.

2.2.8 Transient transfection of mammalian cells

Cationic liposome mediated transfection involves the incubation of cationic lipid-containing liposomes and DNA to associate and compact the nucleic acid (Kabanov & Kabanov 1995;Labat-Moleur *et al.* 1996), presumably from electrostatic interactions between the negatively charged

nucleic acid and the positively charged head group of the synthetic lipid. The liposome/nucleic acid complex is dispersed over the culture, entering cells by the processes of endocytosis, or fusion with the plasma membrane via the lipid moieties of the liposome (Gao & Huang 1995). The Trans™Fast Transfection Reagent (Promega) is comprised of the synthetic cationic lipid, (+)-N,N [bis (2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide and the neutral lipid, DOPE, designed for high efficiency transfer of DNA into mammalian cells and was used in all cell types for this project.

Cells were seeded onto plates at various concentrations one day before the transfection experiment and incubated overnight at 37° C, 5% CO_2. For optimal assay conditions the cells should be approximately 80% confluent on the day of transfection. The TransFast™ Reagent was thawed, warmed to room temperature and mixed thoroughly by vortexing while the serum-free media was pre-warmed to 37° C. DNA to be transfected was re-suspended in TE buffer to a final concentration of 0.1-0.5 µg/µl and the DNA purity checked by determining the ratio of absorbency at 260nm (A_{260}) and 280nm (A_{280}) using a spectrophotometer. Transient transfections were carried out using a 1:1 ratio of Trans™Fast reagent with DNA according to the manufacturer's instructions. More specifically, total DNA (50-100ng) was added to pre-warmed serum free media (40µl) and thoroughly mixed before addition of TransFast™ Reagent (0.3µl) calculated per well using a 96-well plate. The DNA/transfection reagent samples were incubated for 10-15 minutes at room temperature to allow complex formation before the growth medium on the 96-well plates was aspirated and replaced with the transfection samples. The cells were incubated with the complexes for 1 hour at 37° C, 5% CO_2 before pre-warmed complete medium was added for cell recovery overnight at 37° C. Initially an optimisation procedure was performed, testing various amounts of DNA (0.25, 0.5, 0.75, 1.0 µg per well) at charge ratios (TransFast™ Reagent to DNA) of 1:1 and 2:1. For this initial optimisation, serum-free conditions with adherent cells in 24 well plates, using an exposure time of one hour was performed.

2.2.8.1 Genetic reporter systems

Genetic reporter systems are widely used to study eukaryotic gene expression and cellular physiology. Applications include the study of receptor activity, transcription factors and intracellular signalling in this study.

A dual reporter Assay system has been used in this project to improve experimental accuracy, by correcting for transfection efficiency. The term 'dual reporter' refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. In the Dual-Luciferase® Reporter (DLR™) Assay System, the experimental firefly (*Photinus pyralis*) luciferase reporter (de Wet *et al.* 1985) is correlated with the effect of specific experimental conditions, while activity of the co-transfected control *Renilla* (*Renilla reniformis*) luciferase

reporter (Lorenz *et al.* 1991) provides an internal control, which serves as a baseline response. Normalising the activity of the experimental reporter to the activity of the internal control minimises experimental variability caused by differences in cell viability or transfection efficiency. Other sources of variability, such as differences in pipetting volumes, cell lysis efficiency and assay efficiency can be effectively eliminated. Thus dual reporter assays often allow more reliable interpretation of the experimental data by reducing extraneous influences.

Both firefly and *Renilla* luciferase are immediately active upon mRNA translation, and as neither is naturally present in mammalian cells, their assays are not interfered with by endogenous enzymatic activities. As a dual-reporter system these enzymes also offer the advantage of having completely separate evolutionary histories and thus independent biochemistries, allowing their luminescent reactions to be distinguished.

DLR™ assays were performed in triplicate 72 hours post transfection according to the manufacturer's protocol. More specifically, media was removed from the adherent cell mono-layer, washed once with PBS and lysed with Passive Lysis Buffer (PLB) (1x) for 15 minutes at room temperature. Using a Dynex, MLX luminometer, the activities of firefly and *Renilla* luciferase activities were measured sequentially from single samples (per well). The firefly luciferase reporter was measured first by adding Luciferase Assay Reagent II (LAR II) (50µl) to generate a 'glow-type' luminescent signal. After quantifying the firefly luminescence over 12 seconds, the Stop & Glo® Reagent (50µl) was then added to the same well to quench the first reaction (3 seconds) and then quantify the second 'glow-type' signal of *Renilla* luciferase activity which decayed slowly over the course of the 12 seconds measurement. Firefly and *Renilla* luciferases, because of their distinct evolutionary origins, have dissimilar enzyme structures and substrate requirements, enabling the discrimination between their respective bio-luminescent reactions. Firefly and *Renilla* luciferase values were obtained for each well and analysed using an Excel Spread- Sheet.

2.2.9 Protein Analysis

2.2.9.1 Estimating Protein Concentration

2.2.9.1.1 Bicinchonic Assay

The bicinchonic acid (BCA) assay was used to quantify the amount of protein in samples. The principle of the assay is a colour change that occurs when bicinchonic acid forms a complex with reduced copper (Cu^{1+}) to produce a purple colour that is detectable at 562 nm. The reduction of Cu^{2+} (present in copper sulphate solution) to Cu^{1+} occurs through the action of the Biuret complex in alkaline solutions of protein as the result of the interaction of copper and BCA with cysteine,

cystine, tryptophan and tyrosine amino acid residues. The product of this reaction is stable and increases in a proportional fashion over a broad range of increasing protein concentration.

To perform the BCA assay, a solution of $CuSO_4.5H_2O$ (4% w/v, Sigma, UK) was made up in Bicinchonic Acid (BCA, Sigma, UK) using 1 part $CuSO_4$ to 50 parts BCA. For each sample to be tested, 10 µl of a 1/10 dilution of the sample was added to a single well of a 96-well plate. In addition, 10 µl of dH_2O was added to each of 3 extra wells to serve as blanks, and 10 µl of each of 6 known albumin standards (80 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, 1000 µg/ml, 2000 µg/ml) was added to a further 6 wells. To each of the test, blank and standard wells, 200 µl of the $CuSO_4$/BCA developing solution was added. The samples were then incubated at 37° C for 30 – 60 minutes to allow colour change to occur. At the end of the incubation period the plate was then scanned using a microtitre plate reader with a wavelength of 562 nm. The readings for the 6 albumin standards were used to create a standard curve against which the readings of the samples were measured to calculate the protein concentration.

2.2.10 Measurement of Telomerase Activity

2.2.10.1 Telomerase repeat amplification protocol

Telomerase activity was measured using the TeloTAGGG Telomerase PCR ELISA^{PLUS} assay (Roche Molecular Biochemicals, UK), following the manufacturers recommended protocol.

2.2.10.1.1 Sample Preparation

Samples used for telomerase assay analysis included cell pellets from cultured cells (D17, CMT7, K9SF and U87 cell lines) and protein extracts derived from tissue samples. Cells were harvested and counted as described in chapter 2. Aliquots of 2 x 10^5 cells were harvested for each assay. Cells were pelleted at 3000g for 5 minutes at 4°C, the supernatant withdrawn, and following a brief resuspension in 100 µl of PBS the cells were again pelleted and the supernatant carefully removed. Cell pellets were then resuspended in 200 µl of ice-cold lysis reagent (Roche Molecular Biochemicals, UK) and incubated on ice for 30 minutes. The lysate was then centrifuged at 16,000g for 20 minutes at 4° C and the 175 µl supernatant was carefully removed with a micropipette, taking care not to disturb the pellet of cellular debris. Cell extracts were then either used immediately or snap frozen in liquid nitrogen and stored at -80° C.

Samples of normal tissue (brain) were placed in tumour pots and snap frozen immediately after harvesting, before being stored at -80° C. This was to preserve the integrity of telomerase RNA and the catalytic subunit in order to reduce the likelihood of false negative results. Thin slices of

frozen samples were then removed (approximately 5-10 slices being sufficient) using a sterile scalpel blade on a disposable Petri dish. Samples were then immediately transferred to Rnase-free 1.5 ml eppendorf homogenisation tubes and 200 µl of ice-cold lysis reagent (Roche Molecular Biochemicals, UK) was added. Samples were then thoroughly homogenised using a sterile pestle and stored on ice for 30 minutes to achieve thorough lysis. Following lysis, samples were centrifuged at 16,000g for 20 minutes at 4°C and the protein-rich supernatant carefully removed with a micropipette as for cell pellets. Protein concentrations were then measured using the Bradford assay (Sigma UK) as described in Chapter 2. Tissue extracts were either used immediately for the TRAP assay or snap frozen in liquid nitrogen and stored at -80° C for later use.

2.2.10.1.2 Primer Elongation and Amplification

All steps in preparation for the TRAP PCR procedure were carried out on ice, including thawing of samples prior to analysis. Master mixes were made up for all samples and controls consisting of 25 µl of a 2x reaction mixture (Roche Molecular Biochemicals) and 5 µl of the 216 bp internal standard. A 30 µl volume of the mix was placed into a PCR tube for each sample and to this was added 1 µl of the cell extract, or a volume equivalent to 5 µg of total protein. A 1 µl volume of a positive control was used, and 1µl of lysis buffer alone was used as a negative control. Additional negative controls were provided by adding 1 µl of each sample that had been previously heated to 85°C for 10 minutes in order to inactivate telomerase activity. All reactions were then made up to a total volume of 50 µl using Rnase free water (Roche Molecular Biochemicals, UK) and transferred to a DNA thermal cycler (Perkin Elmer) for the combined primer elongation/amplification reaction.

2.2.10.1.3 Primer Elongation/Amplification Reaction Protocol

Cycles used for primer amplification consisted of initial primer elongation at 25°C for 20 minutes followed by a telomerase inactivation cycle of 94°C for 5 minutes. This was then followed by 30 cycles of amplification (94°C for 30 seconds), annealing (50°C for 30 seconds) and extension (72°C for 30 seconds) followed by a final extension step of 72°C for 10 minutes. Finally, samples were held at 4° C until ready for analysis.

2.2.10.1.4 Hybridisation and ELISA

Hybridisation steps were carried out in 0.5ml eppendorf tubes placed in colour-coded racks to differentiate between test, internal standard and negative (including heat inactivated) controls. A 2.5 µl volume of amplification product each sample of the previous step was added to 10 µl of a denaturation reagent (Roche Molecular Biochemicals, UK) in the corresponding eppendorf tube. These mixtures were then incubated at room temperature for 10 minutes before addition of 100 µl of either a test hybridisation buffer (to test and control samples) or an internal standard buffer (to

internal control samples). These buffers contained DIG-labelled detection probes specific for telomeric repeats and the internal standard respectively. Thorough mixing of the contents of all tubes was performed by briefly vortexing before 100 µl of the contents of each sample was removed and placed in a corresponding well of a streptavidin-coated microtitre plate (Roche Molecular Biochemicals, UK) according to a predetermined pipetting scheme. A plate contained 12 modules of 8 wells each, and modules could be removed to facilitate separate analysis such that more than one assay could be performed per kit.

Microtitre plate wells were then clearly labelled, covered with an adhesive strip to prevent dehydration, and incubated at 37° C on a heated plate shaker (Dynatech, UK) rotating at 300 rpm for 2 hours. This allowed immobilisation of the telomerase and internal standard products with their corresponding detection probes to the streptavidin-coated walls of the plate wells, via the biotin label of the kit primer. Following hybridisation the solutions were completely removed from the wells, after which the wells were washed three times for a minimum of 30 seconds per wash with 250 µl of wash buffer (Roche Molecular Biochemicals, UK) to ensure buffer was completely removed from the wells between steps.

The anti-DIG working solution was a polyclonal antibody (Roche Molecular Biochemicals, UK) raised in sheep and conjugated to horseradish peroxidase (HRP), that was diluted to a concentration of 10 mU/ml in a conjugate dilution buffer (Roche Molecular Biochemicals, UK). A 100 µl volume of this anti-DIG-HRP working solution was then added to each of the wells which were then again covered with adhesive film and incubated for 30 minutes at room temperature on a plate shaker rotating at 300 rpm. The working solution was then removed completely from the wells and five 30-second wash steps performed as described previously. Following this, 100 µl of a substrate solution, equilibrated to room temperature and containing the HRP-sensitive compound 3,3', 5,5'-tetramethylbenzidine (TMB) was added to each well and the plate covered and incubated for colour development at room temperature for 10 minutes, whilst rotating at 300 rpm. Without removing the substrate solution from the well, 100 µl of a stop reagent (<5% sulphuric acid, Roche Molecular Biochemicals, UK) was then added. The stop solution was designed to stop the reaction and simultaneously cause a colour change of the reacted HRP substrate from blue to yellow, increasing the sensitivity of the reaction. The absorbance of the samples was then immediately measured at 450 nm with a reference wavelength of 690 nm using a microtitre plate reader (Dynex Technologies, UK). Samples were regarded as telomerase positive if the absorbance was higher than 0.2 arbitrary units (A_{450nm}-A_{690nm}). The absorbance reading obtained with the positive control supplied with the kit was always higher than 2.0 U.

2.2.10.1.5 Quantification of Telomerase Activity

The level of telomerase activity in samples was determined from the absorbance obtained using 1 µl of the positive control template. Low and high activity control templates provided were identical to a telomerase elongation product of 8 telomeric repeats and were at a concentration of 0.001 amol/ml (low activity) and 0.1 amol/ml (high activity). The amplification of both telomerase-specific products and internal standards was semi-competitive since the same primers were used to amplify both products. In most cases, low activity control template was used. The relative telomerase activity (RTA) was determined by normalising the absorbance of the sample to that of the internal standard and expressing this as a proportion of the positive control. The formula used was as follows:

$$RTA = \frac{\Delta A_S / \bar{A}_{S0}}{(A_{TS8} - A_{TS8,0}) / A_{TS8,IS}} \times 100$$

where:

A_S = absorbance of the sample

A_{S0} = absorbance of the sample (heat-inactivated) negative control

$\bar{A}_{S0}$ = mean of all heat inactivated samples

ΔA_S = corrected absorbance of each sample (calculated by subtracting $\bar{A}_{S0}$ from A_S)

A_{TS8} = absorbance of the control template

$A_{TS8,0}$ = absorbance of lysis buffer

$A_{TS8,IS}$ = absorbance of the control template's internal standard

Tests were performed on each sample in duplicate and results only considered valid if less than 10% variation was found between sample results. The values $(A_{TS8} - A_{TS8,0})/A_{TS8,IS}$ obtained using 1 µl of the control template were considered acceptable if within the range of 2.0-4.0 and 0.2-0.5 for high and low activity templates respectively, following 10 minutes of substrate reaction. Test samples with absorbance readings greater than twice the background reading and with $\Delta A_S/A_{S0} > 2.0$ (sample reading greater than twice mean background absorbance) were considered to be telomerase positive. In addition, results were only considered positive if less than 10% variability was found between tests performed in duplicate.

CHAPTER III

IMMUNOHISTOCHEMICAL DETECTION OF TELOMERASE ACTIVITY IN CANINE BRAIN TUMOURS

3.1 Abstract

The ability of telomerase to maintain telomere length represents an important means by which cancer cells can escape the onset of senescence and crisis given their enhanced proliferative capacity. Surveys have shown that 85-90% of all human tumours possess telomerase activity. Brain tumours represent an important subgroup because of the debilitating symptoms associated with their effects and because survival rates for the most malignant remain unchanged despite advances in anticancer treatment strategies over the past thirty years. Their canine counterparts show strikingly similar features both at the histological and genomic levels, and for this reason offer an attractive model for the development of novel therapies. Given the ubiquity of telomerase activity in other tumour types, telomerase activity in human brain tumours has been extensively examined as a potential prognostic indicator. However, there appears to be a wide variation in the reported incidence of telomerase activity in brain tumours which is likely to be due to problems associated with the methods used to date. In this chapter we aimed to investigate the immunohistochemical detection of telomerase activity using a number of antibodies directed at TERT. One antibody, NCL-hTERT, was found to stain telomerase-positive D17 and CMT7 cells and telomerase-positive, formalin-fixed canine testis, at a concentration of 1/200. This antibody was then used to examine the correlation between TERT staining and malignancy in a panel of 93 canine brain tumours and to correlate telomerase activity with proliferation as assayed using the Ki-67 monoclonal antibody in a smaller subgroup. TERT staining was found to correlate significantly with malignancy in brain tumours overall as well as in the meningioma and oligodendroglioma subgroups. When TERT staining was correlated with proliferation, TERT staining was also found to correlate significantly with the MIB-1 labelling index in all tumours, and again in the meningioma and oligodendroglioma subgroups. These results suggest that the NCL-hTERT antibody represents a promising method with which to identify telomerase expression in formalin-fixed, paraffin-embedded material, and that the prevalence of telomerase activity in canine brain tumours is similar to their human counterparts.

3.2 Introduction

The aim of this chapter was to examine telomerase activity in a group of tumours that is seen commonly in the dog – intracranial tumours. This survey was performed for two reasons: firstly, to evaluate telomerase expression as a diagnostic and prognostic tool in canine brain tumours, and secondly as a prelude for the development of telomerase-targeted therapies. An important prerequisite for the development of such therapies in any group of tumours is an accurate knowledge of the incidence of telomerase activity – such therapies would be of little clinical benefit if the incidence of telomerase activity is low. This introduction will review the current knowledge of telomerase activity in brain tumours in humans and will briefly review the biology of their canine counterparts. In addition, it will be of some value to examine the methods currently in use to detect telomerase activity, as a background to determining its value as a prognostic and diagnostic tool.

3.2.1 Brain Tumours in Humans

Brain tumours comprise about 1% of all cancers in humans, but they contribute to 2.5% of overall cancer mortality, and are the third leading cause of death due to cancer in patients between 15 and 34 years of age (Santarius *et al.* 1997). Whilst outcomes for many patients with brain tumours have significantly improved with the development of improved surgical, radiation therapy, and chemotherapeutic techniques, the most malignant subtype, glioblastoma multiforme, remains an intractable problem. Median life expectancy for patients with this diagnosis has remained unchanged at 12 months for the past 30 years, despite the advent of sophisticated therapies such as radiosurgery (Markert 2003). In addition to short survival times, the clinical symptoms of brain tumours, as with other diseases affecting the brain, are often particularly severe and debilitating. For these reasons, considerable attention has been focused on improving the diagnosis and treatment of these tumours.

3.2.1.1 Tumour Biology

Several findings suggest that tumorigenesis in humans is a multistep process, and that these steps reflect genetic alterations that transform normal cells into malignant cells. Many cancers occur with an incidence related to the age of the individual affected, implicating four to seven stochastic events (Hanahan & Weinberg 2000). A large body of work now indicates that the genomes of tumour cells have invariably undergone modification at multiple sites through changes as small as point mutations to changes in chromosome complement (Kinzler & Vogelstein 1996). The

establishment of a cancerous cell line *in vitro* has also been demonstrated to require a multistep process – rodent cells require at least two introduced genetic changes before they acquire tumorigenic competence, whilst human cells are even more difficult to transform (Hahn *et al.* 1999b). Together these findings suggest that tumour development proceeds in a fashion not unlike Darwinian evolution, in which a succession of genetic changes, each conferring a growth or survival advantage, leads to the progressive conversion of normal cells into cancer cells (Hanahan & Weinberg 2000).

Whilst a highly complex body of knowledge has developed from the study of cancer biology, it is likely that the vast range of cancer cell genotypes and phenotypes so far elucidated are all connected by a small number of important functions. It has been suggested that in fact there are only 6 critical physiological functions that dictate cancerous growth (Hanahan & Weinberg 2000). These are:

1) Self-sufficiency in growth signals
2) Insensitivity to anti-growth signals
3) Evasion of apoptotic pathways
4) Limitless replicative potential
5) Sustained angiogenesis
6) Tissue invasion and metastasis

The gain of each of these functions represents the successful breaking down of an anticancer mechanism that is 'hardwired' into the genome of normal cells. Restoring telomerase activity provides a means for acquiring two of these functions: the ability to evade apoptosis and to divide indefinitely. The acquisition of all 6 leads to the development of a cancer that is able to grow indefinitely and metastasise to parts of the body distant from the cell of origin. However, the order in which these functions are acquired may vary with each tumour, and it is possible that not all tumours acquire all 6 functions before causing the death of the host. As an example, brain tumours arising in the brainstem may lead to fatal herniation without becoming very large. Therefore, death of the host may occur before cell numbers expand enough to allow for selection of metastatic ability. In fact, this may be one explanation for the finding that metastasis of primary brain tumours to extracranial locations occurs extremely rarely. For the same reason, the ability to replicate indefinitely may not be crucial for all brain tumours. Interestingly, if one extends this theory by removing the bulk of the tumour and allowing continued growth without the death of the host, this may allow the continued 'evolution' of a cancerous phenotype. If this is the case, one would expect that mechanisms that allow limitless replication may appear in some tumours after initial debulking. Indeed, with some tumours in humans, including pituitary adenomas and meningiomas, that theory appears to hold true (Harada *et al.* 2000a).

These findings suggest that when benign tumours are treated, the evolution of a more malignant phenotype which includes the ability to replicate indefinitely, becomes an important consideration. For this reason, telomerase has been investigated both as a means of diagnosing malignant transformation, and as a target for therapy.

3.2.1.2 Conventional therapies for human brain tumours

The mainstay of treatment for many tumours remains surgical excision in those parts of the brain for which this is feasible. However, some, 'eloquent' parts of the brain are not amenable to this approach. The advent of sophisticated imaging techniques, including functional and intraoperative Magnetic Resonance Imaging (MRI), have allowed refinement of surgical techniques and the ability to resect more tumour tissue whilst sparing important functional areas of the brain. However, even with these techniques complete surgical excision is not always possible (Henson *et al.* 2005).

Radiotherapy, either as adjunctive therapy following surgery, or as the primary treatment for surgically inaccessible tumours, has become another important treatment modality. Since the first demonstration that radiotherapy improved survival in patients with brain tumours in 1976, improvements have led to escalation and refinement of dose administration and developments such as stereotactic radiosurgery (Fiveash & Spencer 2003). Although for some tumours these refinements have increased survival times, this is not the case for patients diagnosed with glioblastoma, for whom the outlook has remained grim.

The use of chemotherapeutic agents has met with success in some tumour types. Oligodendrogliomas have long been known to be sensitive to chemotherapeutic protocols, the most common of which include the use of procarbazine, carmustine (CCNU) and vincristine (PCV) (van den Bent 2004). Cytogenetic studies have revealed that this chemosensitivity is associated with loss of heterozygosity (LOH) on chromosome arms 1p and 19q, and that tumours with mixed oligodendroglial and astrocytic components that possess this genetic makeup are similarly chemosensitive (Bigner *et al.* 1999; Cairncross *et al.* 1998; Engelhard *et al.* 2002). However, other tumours, such as meningiomas and glioblastomas, have proved remarkably resistant to chemotherapeutic approaches (Lusis & Gutmann 2004; Parney & Chang 2003). For these reasons, considerable attention has been directed towards the development of new therapeutic strategies.

3.2.1.3 Prognosis

Whilst the figures for median survival times for most brain tumours are well known, providing a prognosis for individual patients can be challenging. For patients with a diagnosis of glioblastoma multiforme, median survival is a mere 12 months. However, 5% of these patients will survive 5

years or longer, and a small group will survive up to 20 years following initial diagnosis (Senger *et al.* 2003). Similarly, whilst the majority of patients diagnosed with meningioma are cured following gross total resection, up to 19% of patients will suffer a recurrence and have a poorer outlook (Maes *et al.* 2005). Various histopathological features have been associated with aggressive behaviour and recurrence, but there appears to be little agreement on which features are most important to prognosis with the exception of the proportion of cells undergoing proliferation (Carroll *et al.* 1999; Rushing *et al.* 1998). Predicting which patients will respond to therapy and which will go on to suffer relapse, therefore, is of considerable interest. A summary of survival times for the most commonly encountered brain tumours in humans is given in Table 3.1. For a full review, the reader is referred to two excellent sources: (Kleihues & Cavenee 2000) and (Lantos *et al.* 2002)

Table 3.1: WHO classification and survival for the most common human brain tumour types

Tumour type	WHO Classification	5 year survival (Kleihues & Cavenee 2000)
Astrocytomas		
Pilocytic astrocytoma	1	100%[a]
Diffuse astrocytoma	2	b
Anaplastic astrocytoma	3	c
Glioblastoma multiforme	4	<1%[d]
Oligodendrogliomas		
Oligodendroglioma	2	38 – 47%
Anaplastic oligodendroglioma	3	23-41%
Oligoastrocytoma	2	58%
Anaplastic oligoastrocytoma	3	36%
Choroid plexus tumous		
Choroid plexus papilloma	1	100%
Choroid plexus carcinoma	3	40%
Meningiomas		
Benign meningiomas	1	>100%[e]
Atypical meningioma	2	
Anaplastic meningioma	3	

a As a group these tumours, most commonly seen in paediatric patients, are slow growing and may at any point stabilise or even regress and are uncommonly fatal.

b Overall mean survival is approximately 6-8 years. However, survival is mainly determined by the length of time before progression to more malignant phenotype (either anaplastic astrocytoma or glioblastoma), usually approximately 4-5 years.

c Key determinant of survival time is the time interval before progression to glioblastoma, which varies considerably. Mean overall survival is 3 years, with mean of 2 years to development of glioblastoma. Survival time is longer for those tumours with an oligodendroglial component (>7 years).

d Mean survival time for glioblastoma is less than 1 year. Survival at 3 years is 1.8%.

e The major determinant of survival time for patients with meningiomas is time to recurrence, which in turn is largely related to the extent of surgical excision achieved. Benign meningiomas have a recurrence rate of between 7% and 20% For atypical variants (including clear cell and chordoid variants) this figure rises to 29 - 40%, and for anaplastic variants (including papillary and rhabdoid variants) this rises to 50 - 78%.

Conventional predictors of prognosis for patients diagnosed with a brain tumour include age at the onset of disease, clinical status, tumour location and radiological appearance. In addition, the histopathological appearance of many tumours and features such as microvascular proliferation, mitotic figures and necrosis are all important in producing a grading scheme related to prognosis. The development of techniques to analyse the genetic makeup of tumours has also refined some of these predictors, and has even made some redundant (Buckner 2003; Cairncross *et al.* 1998). However, whilst the genetic profile of many tumours is now known, remarkably few mutations are common to the majority of tumours within a given type, perhaps with the exception of LOH of chromosomes 1 and 19 in oligodendrogliomas (Cairncross *et al.* 1998). For instance, glioblastomas are thought to arise either as primary, or de novo, tumours (usually in older patients) or as secondary tumours as a progression from more benign astrocytic tumours (Lantos *et al.* 2002). Mutations or alterations of the EGFR, TP53, MDM2, CDKN2A, RB1, PTEN and DMBT1 ('deleted in malignant brain tumours-1') genes have all been reported in glioblastomas, with some of these found predominantly in primary tumours and others in secondary tumours (Lantos *et al.* 2002). The finding that these tumours arise by one of a number of pathways makes the prediction of prognosis for individual patients difficult.

One of the most consistent predictors of biological behaviour for many tumours has been the degree of proliferation within neoplastic cells, as assessed using a variety of immunohistochemical stains. The proportion of cells within a tumour undergoing proliferation has been shown to correlate well with histological features of malignancy, such as necrosis, neovascularisation and mitotic figures (Wikstrand *et al.* 1998). In addition, separate studies have shown that proliferation is associated with shorter survival times, independent of other characteristics of malignancy. A large body of research has now shown this for most tumour subtypes, including meningiomas, astrocytomas and oligodendrogliomas (Ho *et al.* 1998; Kros *et al.* 1996; Pollack *et al.* 2002; Sallinen *et al.* 2000; Sharma *et al.* 2001; Takahashi *et al.* 2004). Several histological techniques have been employed to assess cell proliferation, including the Ki-67 and Proliferating Cell Nuclear Antigen (PCNA) immunohistochemical stains and Bromodeoxyuridine (BrdU) labelling (Madewell 2001).

3.2.1.3.1 BrdU

BrdU is a thymidine analogue that is incorporated with DNA synthesis during the S-phase of the cell cycles. Once taken up by cells, it can be detected using either radio-labelling or immunohistochemical means. However, BrdU use is limited by several factors. Firstly, BrdU can only be absorbed by living cells, either grown in culture or shortly after sample collection. As a corollary to this fact, cells within biopsies immersed in BrdU for labelling purposes will only be labelled to a depth of 4 mm from the surface due to its inability to penetrate the entire sample

(Zacchetti *et al.* 2003). This therefore precludes its use in archival material. Secondly, BrdU is only taken up by those cells involved in the S-phase of the cell cycle, such that proliferating cells in the G1 and G2 phases of the cell cycle will not be detected (Zacchetti *et al.* 2003). Thirdly, the use of BrdU is complicated by its significant mutagenic potential and hazardous nature (Zacchetti *et al.* 2003).

3.2.1.3.2 Proliferating Cell Nuclear Antigen (PCNA)

PCNA is a subunit of DNA polymerase-delta, with a mass of 36Kd (Madewell 2001). PCNA is essential both for replication of DNA and for DNA repair. Maximal expression occurs in the G1 and S phases and decreases in the G2 and M phases of the cell cycle. Whilst PCNA can be used on archival material, other factors complicate interpretation. As PCNA is involved in DNA repair (as well as replication) and has a relatively long half-life of between 8 and 20 hours (leading to its detection in early G0), overestimation of the number of proliferating cells is possible (Bravo & Macdonald-Bravo 1987; Scott *et al.* 1991; Toschi & Bravo 1988). Finally, titration of the concentration of antibody used must be undertaken with care, as the number of positive-staining cells increases with antibody concentration and with staining time (Madewell 2001).

3.2.1.3.3 Ki-67/MIB-1

The Ki-67 antigen, detected by the monoclonal antibody MIB-1, is a large protein of 345 Kd (Bruno & Darzynkiewicz 1992). Whilst it is present in all stages of the cell cycle, it reaches maximal expression during mitosis, and is rarely detectable in G0 due to its short half-life of approximately 1 hour (Bruno & Darzynkiewicz 1992; Du *et al.* 1991; Gerdes *et al.* 1984). For this reason, it is less likely to overestimate cellular proliferation than PCNA. Additionally, Ki-67 may be used with archival material, unlike BrdU, which makes it an attractive method for the assessment of proliferation.

In humans, Ki-67 has been used in a wide range of tumours, including intracranial neoplasms. In meningiomas, astrocytic tumours, oligodendrogliomas, gangliogliomas and ependymomas, a high MIB-1 LI has been found to correlate well in tumours with aggressiveness, tumour recurrence and short survival times (Engelhard *et al.* 2002; Ho *et al.* 1998; Ho *et al.* 2002; Hsu *et al.* 1998; Konstantinidou *et al.* 2001; Nagai *et al.* 1995; Pollack *et al.* 1998; Sallinen *et al.* 2000; Takahashi *et al.* 2004). This large body of evidence suggests that proliferation, as assessed by Ki-67 staining, is an important predictor of tumour behaviour.

3.2.1.4 Telomerase activity in human brain tumours

Because of the strong link between telomerase activity and oncogenesis, a number of researchers have examined the ability of telomerase expression to provide a prognostic indicator of outcome in human brain tumours. One of the most comprehensive surveys of telomerase activity in human tumours overall was published in 1997 by Shay and Bacchetti (Shay & Bacchetti 1997). This review encompassed almost all tumour types, the vast majority of which were sampled using the Telomerase Repeat Amplification Protocol (TRAP) assay. Another excellent review of telomerase activity in brain tumours specifically was provided by Falchetti *et al*, to which the reader is also directed (Falchetti *et al*. 2002). Overall, the clinical picture suggests that approximately 85-90% of all human cancer specimens express telomerase activity. However, the grade and stage at which it is expressed for each given tumour type is variable, with telomerase expression especially characteristic of high grade neoplasms (Kleinschmidt-DeMasters *et al*. 1998a) Although the overall proportion of tumours positive for telomerase expression was very high in the review by Shay and Bacchetti, within the brain tumours sampled, telomerase activity varied between 10% and 100% of the tumours sampled. Given this wide variation, a more detailed survey is provided here, along with possible explanations for some of this variation.

3.2.1.4.1 Glial tumours

Several studies have examined the activity of telomerase in glial tumours, with varying results. Information on telomerase expression in glial tumours, including astrocytomas, oligodendrogliomas, ependymomas, oligo-astrocytomas, and high grade gliomas, has been limited and somewhat conflicting, with several studies showing that a significant percentage of glioblastomas and anaplastic astrocytomas are negative for telomerase expression (Carroll *et al*. 1999). This is an unexpected finding given the high degree of malignancy that characterises these tumours (Kleinschmidt-DeMasters *et al*. 1998a).

One of the earliest studies, reported by Langford *et al* (Langford *et al*. 1995), analysed telomerase activity in 90 gliomas. In this study, glioblastomas displayed telomerase activity in 38 of 51 cases (75%) overall, with 78% of primary tumours and 68% of recurrent tumours positive. Anaplastic astrocytomas, on the other hand, displayed very limited telomerase activity, with only 2 of 20 (10%) positive. Oligodendrogliomas in this study were uniformly found to display telomerase activity, with 19 of 19 tumours positive for the TRAP assay. Overall, the telomerase activity in this study correlated well with histological assessment of malignancy.

Nakatani *et al* reported slightly different results in their study of 41 brain tumours (Nakatani *et al*. 1997). In this study, 12 of 20 (60%) of glioblastomas were found to be positive for telomerase activity (57% of primary GBMs and 67% of recurrent GBMs). None of 3 anaplastic and 4 low

grade astrocytomas displayed telomerase activity, however, while 2 of 2 oligodendrogliomas were found to be positive for telomerase expression. In contrast to the study reported by Langford *et al*, however, there was no significant correlation between pathological diagnosis and telomerase activity, or between MIB-1 staining and telomerase activity. The relatively small number of samples of each tumour type, however, make interpretation of these results difficult.

Carroll *et al* examined telomerase activity in several glioblastomas as part of an investigation into the presence of dicentric chromosomes in brain tumours (Carroll *et al.* 1999). In the glioblastomas examined, telomerase activity was present in only 7 of 25 (28%), a figure substantially lower than earlier studies. None of the 4 low grade glial tumours demonstrated telomerase activity. The apparent disparity between the levels of telomerase activity detected in tumours of this study and previous studies may be explained by the different versions of the TRAP assay used in different studies. Earlier studies described telomerase activity in gliomas and meningiomas using the upstream primer TS and a downstream primer CX. These primers have been reported to result in primer-dimer formation and false positive generation, as reflected by Southern blots of TRAP assay products. However, as with several other reports, a relatively small number of tumours was examined, making comparison between tumour types difficult.

Santarius *et al* found that a small percentage of low grade astrocytomas, 71% of anaplastic astrocytomas and 89% of glioblastomas were positive for telomerase activity (Santarius *et al.* 1997). In contrast, Morii *et al* found no significant correlation between tumour malignancy (as assessed by WHO grade) and telomerase activity (Morii *et al.* 1997). In this study, 20 tumours were examined, including 1 pilocytic astrocytoma, 7 oligo-astrocytomas, 1 anaplastic astrocytoma and 11 glioblastomas, with 63% of grade I and II tumours and 67% of grade III and IV tumours displaying telomerase activity respectively. As with the study reported by Nakatani *et al* however, the small number of tumours examined hinders accurate assessment of telomerase activity with respect to proliferation.

A larger study that provided evidence for telomerase involvement in tumour progression was reported by Hiraga *et al* (Hiraga *et al.* 1998). This study examined telomerase activity and telomere length in 170 brain tumour tissues, and found telomerase expression in 20% (3 of 15) of grade II astrocytomas, 40% (6 of 15) anaplastic astrocytomas, and 72.3% (34 of 47) of glioblastomas. Overall, telomerase activity was present in 61.7% (66 of 107) of the neuroepithelial tumours examined. However, in grade II and anaplastic astrocytomas, telomerase activity was significantly correlated with early histological progression and reduced survival. Furthermore, a number of recurrent tumours which had been previously telomerase-negative were found to display telomerase activity following recurrence and progression to a higher grade. Telomerase activity was also detected in all primitive neuroectodermal tumours, anaplastic oligoastrocytomas, neuroblastomas and oligodendrogliomas. Telomere length was found to be significantly shorter in

tumours with telomerase activity present, including 10 glioblastomas which had progressed from lower grade tumours with normal telomere lengths, and which had acquired telomerase activity. Interestingly, this study also found that telomerase activity appeared to be a pre-requisite for the establishment of cell lines from tumour tissue, as cell lines were cultured from 5 of 29 (17.2%) telomerase-positive neuroepithelial tumours but not from telomerase-negative tumours.

Within the glioblastoma subgroup, Harada and colleagues provided evidence that the route to telomerase activation might vary between primary, or de novo tumours, and secondary tumours arising as a progression from more benign variants (Harada *et al.* 2000b). Primary tumours, which arise in older patients and are associated with EGFR and MDM2 gene mutations, and with LOH in chromosome 10, carry a worse prognosis than secondary tumours, which generally arise in patients under the age of 45 and which are associated with inactivation of p53. Harada and colleagues found that only 36% of primary tumours possessed telomerase activity, as assessed by TRAP assay, as compared with 90% of secondary tumours. In addition, hTERT expression was higher in secondary tumours with telomerase activity than in primary tumours with telomerase activity. Overall, 62% of all the glioblastomas examined had telomerase activity.

Kleinschmidt-DeMasters *et al* (Kleinschmidt-DeMasters *et al.* 1998a) examined gross total resection samples to determine whether sampling of multiple regions throughout tumours could also explain the conflicting findings of previous studies. In addition, telomerase expression was examined to determine whether there was any connection with the histological features of each type of tumour, along with proliferation (as characterised by MIB1 staining) and p53 staining. In this study, 3 of 10 oligodendrogliomas were found to express telomerase activity. Interestingly, of the 10 tumours, 9 were found to possess uniform activity with all regions sampled either uniformly positive or negative for telomerase expression. In contrast, high grade astrocytomas showed more variable telomerase activity. Of the 11 glioblastomas examined, all tumours showed at least one region positive for telomerase expression. However, only 3 tumours showed homogenously positive activity, the remainder exhibiting negative telomerase activity in some of the samples taken from each tumour. Negative telomerase activity was not explained by the presence of necrosis (as assessed from mirrored histological samples). Of the anaplastic astrocytomas examined, 2 of 3 showed heterogenous positive telomerase activity. Interestingly, although not statistically significant given the small number of these tumours examined, there was a tendency for regions positive for telomerase expression to display histological anaplasia. Interestingly, the study by Cabuy and de Ridder supported the finding that telomerase activity varies across different regions of glioblastomas (Cabuy & de Ridder 2001). Overall, this study found strong statistical association between the presence of telomerase activity and MIB1 staining, but not with any other histological feature or with p53 staining. The variation in telomerase activity within tumours may well provide an explanation for previous, conflicting results.

Another method that evaluates telomerase activity within selective regions of tumours rather than within the entire tumour, was described by Weil *et al* (Weil *et al.* 1999). In this study, the authors used microdissection to evaluate telomerase activity via the TRAP assay in specific areas of tumours, free of other components such as vascular proliferation, necrosis and inflammation. Using this method, 8 of 8 glial tumours (comprising 1 low grade oligodendroglioma, 1 anaplastic oligodendroglioma, 3 anaplastic astrocytomas and 3 glioblastomas) were positive for telomerase activity, in addition to an aggressive B cell lymphoma. In contrast, normal brain tissue and a meningioma were negative for telomerase activity. These findings, along with those of Kleinschmidt-DeMasters *et al* (Kleinschmidt-DeMasters *et al.* 1998a) suggest that a high proportion, if not all, malignant glial tumours express telomerase activity, but that detection may require analysis of selective areas within tumour tissue.

Chong *et al* focused specifically on non-astrocytic glial tumours in their study of 45 cases (Chong *et al.* 2000). Telomerase activity along with hTERT mRNA expression was examined in 27 oligodendrogliomas (11 oligodendrogliomas, 10 anaplastic oligodendrogliomas, 4 oligo-astrocytomas and 2 anaplastic oligo-astrocytomas) and 18 ependymal tumours (14 ependymomas, 1 anaplastic ependymoma, 1 myxopapillary ependymoma and 1 subependymoma). Telomerase activity appeared to correlate with malignancy, with 3 of 11 (27%) grade II and 7 of 12 (58%) grade III oligodendrogliomas positive for telomerase activity as assessed by the TRAP assay. Of the oligoastrocytomas, 1 of 4 (25%) grade II and 1 of 2 (50%) anaplastic tumours were telomerase-positive. Interestingly, no statistical difference was found between telomere lengths of telomerase positive and negative tumours, or between telomere length and degree of malignancy, although other authors have reported short telomeres in telomerase positive gliomas (Hiraga *et al.* 1998; Morii *et al.* 1997). This study also examined mRNA expression of hTERT and found that the expression of hTERT mRNA was significantly greater in telomerase positive (100%) vs telomerase-negative (50%) oligodendrogliomas. These results suggest that the expression of hTERT mRNA is an important factor for telomerase activation. However, the significance of the presence of hTERT mRNA in telomerase-negative tumours is unclear, as it is possible the PCR product detected in this study may have been derived from alternative, non-functional spliced transcripts of hTERT.

While the majority of reports describe detection of telomerase activity by the TRAP method, some researchers have utilised *in situ* hybridisation (ISH) to assess telomerase activity. Sallinen *et al* (Sallinen *et al.* 1997) examined telomerase activity in 46 astrocytic tumours, consisting of 2 grade I, 16 grade II, 4 grade III and 24 grade IV tumours (glioblastomas), using ISH directed against hTR. This study found that hTR expression was significantly higher in malignant (grade III and IV) than benign (grade I and II) tumours. In addition, hTR expression was significantly correlated with proliferation in the tumours studied, although the significance of this is unclear given the lack of correlation between hTR and telomerase activity reported by other authors, and since hTR is

commonly expressed in both cancerous and non-cancerous tissue (Gunther *et al.* 2000) (Chong *et al.* 2000).

3.2.1.4.2 Meningiomas

Meningiomas constitute the most common benign neoplasm affecting the central nervous system in both humans and dogs. In humans they are divided into three histopathological grades: benign, atypical and anaplastic (Kleihues & Cavenee 1999), which possess features consistent with an increased growth rate (mitotic figures and increased likelihood of recurrence following surgery). In concordance with their generally benign nature, telomerase activity in these tumours has been generally reported to be low or absent in the benign variant. In the study of 41 tumours reported by Nakatani *et al* (Nakatani *et al.* 1997), 9 were meningiomas, all of which were found to be negative for telomerase activity. Similarly, Carroll *et al* (Carroll *et al.* 1999) found that none of 25 meningiomas expressed telomerase activity, including 4 atypical variants. Another study found that 7/26 (27 %) of benign meningiomas assessed with TRAP and RT-PCR possessed telomerase activity, as compared with 6/6 (100 %) of atypical and anaplastic variants (Boldrini *et al.* 2003).

The study reported by Carroll *et al* also examined the presence of dicentric chromosomes and their relationship with telomerase activity. Dicentric chromosomes have been considered to represent telomere end associations associated with very short telomeres in cells approaching senescence, and have been described in up to 50% of meningiomas (Carroll *et al.* 1999). Consequently, dicentric chromosomes should be absent in cells which possess telomerase activity (Park *et al.* 1996). This study reported that of the telomerase negative meningiomas examined, 11 of 23 were found to have dicentric chromosomes present. The authors of this study remarked that earlier studies had utilised TRAP assays with primers that could form primer-dimer associations, resulting in false positive results (Kim & Wu 1997; Krupp *et al.* 1997), and that their study utilised a TRAP assay designed to prevent these associations. Consequently, this could explain the lower number of TRAP positive tumours detected.

Simon *et al* (Simon *et al.* 2000), in their study of 53 meningiomas, found that telomerase activity correlated strongly with malignancy and progression of meningiomas. In this study, 7 of 34 (21%) benign, 9 of 12 (75%) atypical, and 7 of 7 (100%) anaplastic meningiomas displayed telomerase activity as reported by TRAP assay. The involvement of telomerase activity in tumour progression was also supported by the finding that one possible radiation-induced tumour was found to be TRAP-positive, while tumour multiplicity was not associated with telomerase activity. Radiation induced tumours are known to follow a more aggressive course. Additionally, all recurrent tumours and tumours that recurred during the follow up period were found to be telomerase positive. In this study, expression of hTERT mRNA as assessed by Reverse Transcriptase PCR (RT-PCR) was also found to correlate with malignancy. However, of 30 tumours positive for

hTERT mRNA expression, only 23 were TRAP positive. The level of telomerase activity in this study was not found to correlate with malignancy, with some malignant tumours displaying low levels of TRAP activity.

3.2.1.4.3 Pituitary tumours

Tumours arising from the pituitary gland in humans are frequently benign, with the most common variant being the pituitary adenoma. Pituitary adenomas themselves are amongst the most benign of CNS neoplasms, and rarely metastasise. However, in rare instances, these tumours may transform. Harada *et al* reported on a pituitary adenoma in which both activation of telomerase activity and decreasing telomere length measured over successive resections were associated with malignant transformation and metastasis (Harada *et al.* 2000a).

3.2.1.4.4 Neuroblastomas

A number of studies have focused on neuroblastomas, the most common solid tumour in children younger than 5 years of age (Falchetti *et al.* 2002). As a tumour group, they are markedly heterogenous in terms of their biologic, morphologic and clinical characteristics (Poremba *et al.* 2000a). Additionally, approximately 80% of a particular subset of metastatic neuroblastomas (defined by localised primary tumour and distant metastasis including liver, skin and/or bone marrow) often regress spontaneously independently of anticancer therapies (Falchetti *et al.* 2002). Several characteristics have been identified that are associated with clinical outcome, including age, stage, histology, MYCN copy number, deletions within the short arm of chromosome 1, DNA content and TPK-A (high-affinity receptor protein for nerve growth factor) expression (Streutker *et al.* 2001). However, none of these factors alone has been sufficient to accurately predict tumour behaviour. Telomerase, however, has been shown to be a prognostic indicator by several groups (Isobe *et al.* 2004; Krams *et al.* 2003; Poremba *et al.* 2000a; Poremba *et al.* 2000b; Streutker *et al.* 2001). Hiyama and colleagues found that 94% of neuroblastomas overall express telomerase activity (Hiyama *et al.* 2003). Importantly, 75% of tumours with high telomerase activity in this study had a poor prognosis, as compared with 97% of tumours with low telomerase activity, which had a good prognosis. Indeed, 100% of tumours without detectable telomerase activity regressed. Telomerase activity also correlates strongly with expression of the full length hTERT transcript in these tumours (Krams *et al.* 2001; Krams *et al.* 2003). Other studies have corroborated these findings and have suggested that, apart from tumour stage, telomerase is the only independent predictor of survival (either disease-free or overall), and that telomerase activity increases with tumour progression (Poremba *et al.* 2000a; Streutker *et al.* 2001). Collectively, these studies strongly recommend that telomerase activity should be assessed in all patients with newly diagnosed neuroblastoma as an important prognostic indicator. Moreover, they provide two possible pathways for the development of neuroblastomas. Firstly, it may be that since

neuroblastomas arise in infancy or early childhood, telomerase activity is present due to the inappropriate retention of activity in underlying stem cells which form the precursor for the tumour. In this group, such tumours have lower levels of telomerase activity, similar to that found in normal adrenal glands. Alternatively, it may be that telomerase activity is switched off during normal development, but then reactivated as a consequence of a transforming event (eg expression of other oncogenes). Neuroblastomas in this second group then show much higher levels of telomerase activity.

3.2.1.4.5 Metastases

Metastatic tumours commonly affect the brain as it receives 15% of cardiac output (Johnson 1990). In the human field, the incidence of silent brain metastases suggests that most epidemiological studies underestimate their occurrence, reported to comprise between 30% and 50% of all CNS neoplasms. Up to 20% of people with malignant tumours have brain metastases at death, but many of these patients die of systemic disease before these metastases become symptomatic. Telomerase activity has been investigated rarely in metastatic CNS tumours, but has been detected with high frequency. Kleinschmidt-DeMasters *et al* found that 32 of 35 (91%) brain metastases were positive for telomerase activity using the TRAP assay (Kleinschmidt-DeMasters *et al.* 1998b). This study examined tumours that commonly metastasise to the brain, including melanoma, adenocarcinoma (with primary sites including breast, lung, prostate, gastrointestinal and colon), squamous cell carcinoma, hepatocellular carcinoma, mixed embryonal-yolk sac germ cell neoplasm, osteogenic sarcoma and secondary immunoblastic lymphoma. However, quantification of telomerase products showed high variability (fourfold logarithmic variation) and no correlation of telomerase levels with tumour subtype, or with survival times, even within one tumour type. It was noted that one tumour in this study showed a rise in telomerase activity with progression, such that the highest telomerase levels were found in the sample closest to patient demise, but the small number of tumours studied made interpretation of this finding difficult.

3.2.1.4.6 Telomerase activation and crisis

The occurrence of dicentric chromosomes has been proposed to be due to telomere associations and is considered to represent abnormal telomere functioning. Post crisis, telomere association formation stabilises or decreases, and cells acquire telomerase activity. In the study reported by Carroll *et al*, approximately 50% of meningiomas were found to show random dicentric chromosomes (Carroll *et al.* 1999). However, malignant glioblastomas did not demonstrate telomere associations despite telomerase activity being present in a considerable percentage of the tumours examined. If the M1/M2 hypothesis is applicable to tumours of the nervous system, meningiomas with no telomerase activity and a high percentage of dicentric chromosomes would be consistent with a pre-crisis state. In addition, those tumours that are followed over multiple

resections during progression frequently evolve telomerase activity (Harada *et al.* 2000a; Hiraga *et al.* 1998; Simon *et al.* 2000). These findings would suggest that immortalisation is not necessarily a prerequisite for tumour formation in the central nervous system, but that it occurs only in some tumours. Most commonly, this occurs in parallel with the development of a more malignant phenotype, although primary glioblastomas would appear to use a different pathway to malignancy, one that does not necessarily require immortalisation (Harada *et al.* 2000c).

3.2.1.4.7 Telomerase activity and proliferation

The correlation of both telomerase activity and proliferation with malignancy has led several researchers to examine whether these two features of cancer are linked. In several studies, telomerase activity does indeed seem to correlate with proliferation at least in meningiomas and gliomas (Cabuy *et al.* 2001; Kleinschmidt-DeMasters *et al.* 1998a; Macs *et al.* 2005; Santarius *et al.* 1997). Interestingly, in examining breast cancer cells, Ikeda *et al*, using an immunocytochemical restaining method, found that the same cells that expressed TERT simultaneously displayed immunopositivity for Ki67, suggesting that in breast cancer cells telomerase activity is more directly linked to cell proliferation than in meningiomas, at least (Ikeda *et al.* 2003). One important possible connection between telomerase activity and cellular proliferation could be the p21 protein. Telomerase-positive glioma cells, when transfected with wild type P21, demonstrate a decrease in cell proliferation rate with accumulation of cells at the G0/G1 phase of the cell cycle (Harada *et al.* 2000c). This cell cycle arrest is accompanied by a subsequent decrease in TERT mRNA levels and telomerase activity, suggesting that these two factors may be linked. Other researchers have also demonstrated an association between telomerase activity and the cell cycle (Holt *et al.* 1997; Sharma *et al.* 1995). Together these findings suggest a situation in which TERT expression and telomerase activity are closely tied to the mechanics of cell proliferation in ways which are as yet poorly understood.

3.2.1.4.8 Interpreting Telomerase Repeat Amplification Protocol (TRAP) Results

The majority of the existing data on telomerase activity in human brain tumours has been derived from use of the TRAP assay. However, the sensitivity of the TRAP assay together with the marked heterogeneity of certain tumour types create potential pitfalls in evaluating current data. One major complication is the possibility that homogenised tumour samples may contain small numbers of telomerase positive non-neoplastic cells, such as lymphocytes and stem cells, which may lead to falsely positive TRAP results (Hiyama & Hiyama 2002). In addition, recent findings have suggested that as well as telomerase activity possibly being present in bystander cells included with tumour biopsy unintentionally, in some brain tumours telomerase activity is actively switched on in vascular endothelial cells. Pallini and co-workers, using *in situ* hybridisation, found that telomerase activity within vascular endothelial cells directly correlated with malignancy in

astrocytic tumours (Pallini *et al.* 2001). In fact, 100% of glioblastomas in this study contained vascular endothelial cells expressing hTERT mRNA. In addition, glioblastoma cells were found to directly induce telomerase activity *in vitro* in vascular endothelial cells, either following co-culturing of the two cell types, or following addition of glioblastoma-conditioned medium to the endothelial cells (Falchetti *et al.* 2003).

A study that further illustrates this point was also reported by Falchetti *et al* (Falchetti *et al.* 2000). These authors used ISH against hTERT RNA and correlated hTERT RNA expression with TRAP analysis of telomerase activity, with RT-PCR analysis of hTERT and with cellular proliferation in 34 glioblastomas. This study found that while telomerase activity as assessed by the TRAP assay was present in only 23 (67.6%) tumours, ISH detected hTERT RNA expression in all tumours, and RT-PCR detected hTERT RNA in all but one tumour. As was described by Kleinschmidt-DeMasters *et al* (Kleinschmidt-DeMasters *et al.* 1998a), significant variation in ISH staining was found within glioblastomas, with 19 cases (55.9%) showing a diffuse pattern, but the remaining 15 (44.1%) having only focal areas of hybridisation with hTERT-positive cells scattered within neoplastic tissues.

The difference between hTERT RNA expression and TRAP assay results could potentially be explained by the presence of non-functional splice variants amplified along with full length hTERT. Indeed, Kotoula *et al* showed that the expression of different splice variants varies across astrocytoma subtypes, with non-functional variants Adel (deletion of the first 36 nucleotides of exon 6) and Bdel (deletion of 182 nucleotides from exons 7 and 8) present in all grades of tumour (Kotoula *et al.* 2004). However, the full length hTERT mRNA transcript was only present in high grade tumours and was the predominant variant found in those tumours.

Despite this finding, the difference between RT-PCR and ISH reported by Falchetti *et al* could not be explained by the presence of different splice variants, as RT-PCR was also performed using primers specific to the regions of splice variants. This did not detect any differences between the sizes of PCR fragments as would be expected with any splice variants present. These results could be explained in several ways. It is possible that samples used for the TRAP assay did not contain enough telomerase positive cells due to the considerable variability in telomerase activity between regions within glioblastomas, as has been noted by other authors (Kleinschmidt-DeMasters *et al.* 1998a; Weil *et al.* 1999). Other possible reasons include: 1) degradation of telomerase protein and/or RNA may have lowered the amount of detectable telomerase in some cases 2) post-translational modifications of hTERT might be necessary to convert it into the active enzymatic form 3) the presence of hTERT alone may not necessarily imply functioning due to incorrect interaction of telomerase-associated proteins. From these results, it would appear that telomerase activity exists in all glioblastomas, but that it may be undetectable by the TRAP assay in approximately one third of cases.

3.2.2 Canine Brain Tumours

Intracranial neoplasms represent a substantial proportion of all neoplasms found in dogs. In this species, the incidence of intracranial neoplasia would appear to be greater than in humans, with a reported incidence of 14.5 tumours per 100,000 dogs compared to 3-4 per 100,000 people (Braund 1994; Holt *et al.* 1997; Lingaas *et al.* 2003; Morrison 1998; Summers *et al.* 1995). Other figures suggest an incidence of CNS tumours found in 1% - 3% of all canine necropsies, including one report that found 2.83% of 6,175 dogs having intracranial tumours at post-mortem examination (McGrath 1960). One report examining tumours in young dogs indicated that the 3 most common sites for neoplasia were the haematopoietic system, the brain and the skin, in decreasing order (Keller & Madewell 1992).

The majority of intracranial tumours seen in dogs and cats are of primary CNS origin. The spectrum of primary brain tumours reported is broad and is similar to that described in humans (see Table 3.2). The most common types of primary tumours affecting the brains of dogs are meningiomas and glial tumours (Morrison 1998). Pituitary tumours, choroid plexus papillomas and ependymomas are also commonly described. Meningiomas are reported to comprise between 30 and 39% of all intracranial neoplasms (Braund & Ribas 1986; Heidner *et al.* 1991; Lingaas *et al.* 2003; Turrel *et al.* 1986). Of the neuroectodermal brain tumours, astrocytomas are probably the most common, representing 55% of one report of 215 cases of neuroglial tumours (Braund 1994). Of the remaining cases, oligodendrogliomas comprised 28%, glioblastoma multiforme approximately 12%, choroid papillomas approximately 12% and ependymomas 2%. Metastasis of primary brain tumours to non-neural tissue is rare, although it has been reported (Dugan *et al.* 1993; Helman *et al.* 1980; Lingaas *et al.* 2003; Schulman *et al.* 1992). However, a number of tumours may spread within the CNS along CSF pathways, notably medulloblastoma and choroid plexus papilloma (LeCouteur *et al.* 1981), although this is more properly referred to as implantation rather than metastasis (Johnson 1990).

Tumours of the nervous system are most commonly reported in mature and older dogs, with one study reporting a peak incidence at 9 year of age (Heidner *et al.* 1991; Lingaas *et al.* 2003), although there are sporadic reports of brain tumours in dogs less than 1 year of age (Keller & Madewell 1992). There would appear to be no gender predisposition, but there is an increased incidence of brain tumours in brachycephalic breeds such as boxers, English bulldogs and Boston terriers, especially of glial tumours (Braund 1994). Meningiomas are more commonly reported in mesocephalic and dolichocephalic breeds such as golden retrievers, Dobermans and collies (Gavin *et al.* 1995).

Table 3.2: Tumour types and predilection sites in dogs

Tissue of Origin	Tumour Type	Predilection Site
Neuron	Ganglioneuroma	Variable: cerebellum, cranial nerve roots, eye, cervical ganglion
	Ganglioneuroblastoma	
	Neuroblastoma	
Neuroepithelium	Ependymoma	Third and lateral ventricles
	Neuroepithelioma	Meninges, TL spinal cord
	Choroid plexus papilloma	Fourth ventricle
Neuroglia	Astrocytoma	Piriform area, convexity of cerebral hemispheres, thalamus, hypothalamus
	Anaplastic Astrocytoma	As for astrocytoma
	Glioblastoma multiforme	As for astrocytoma
	Oligodendroglioma	Cerebral hemispheres, periventricular white matter
	Anaplastic oligodendrogliomas	As for oligodendroglioma
	Spongioblastoma	Variable, ependymal surfaces: cerebellum, optic nerve tracts
	Medulloblastoma	Cerebellum
	Gliomas (unclassified)	Periventricular area, esp cerebral hemispheres
Peripheral nerves and nerve sheaths	Schwannoma	Peripheral nerves
	Neurofibroma	
	Neurinoma	
	Neurofibrosarcoma	
Meninges, vessels and other mesenchymal structures	Meningiomas	Convexities of cerebral hemispheres, floor of vault, cerebellopontine angle, falx cerebri
	Angioblastoma	Variable
	Sarcoma	Variable
Pineal gland, pituitary gland and craniopharyngeal duct	Pinealoma	Pineal body
	Pituitary adenoma	Pituitary gland
	Craniopharyngioma	Hypophyseal/infundibular areas
Heterotopic tissues (malformation tumours)	Epidermoid cyst	Fourth ventricle, cerebellopontine angle
	Dermoid cyst	Variable
	Teratoma	Variable
	Germ Cell Tumours	Base of brain above sella turcica
Metastatic tumours	Mammary gland adenocarcinoma, pulmonary carcinoma, prostatic carcinoma, chemodectoma, malignant melanoma, lymphosarcoma, salivary gland adenocarcinoma, haemangiosarcoma etc	Variable
Primary tumours from surrounding tissues	Osteosarcoma, lipoma, chondrosarcoma, fibrosarcoma, nasal adenocarcinoma, haemangiosarcoma, multiple myeloma, calcifying aponeurotic fibromatosis, epidermoid cyst	Variable

The incidence of metastatic brain neoplasms in animals is unknown, but it is likely that it is underestimated, as the cranial vault is uncommonly evaluated during routine post mortem examination in cats and dogs. In the human field, the incidence of silent brain metastases suggests that most epidemiological studies underestimate their occurrence, reported to comprise between 30% and 50% of all CNS neoplasms (Johnson 1990). Up to 20% of people with malignant tumours have brain metastases at death, but many of these patients die of systemic disease before these metastases become symptomatic. Metastases often become apparent after neoplastic involvement of the lung, as 15% of cardiac output returning from the lung goes to the brain.

Classification of brain tumours in animals has traditionally employed criteria used for human tumours, and most commonly follows the grading system established by the World Health Organisation for prognostic purposes (Koestner *et al.* 1999) (see Table 3.3). However, not all of these tumours have been reported in animals, and some authors question the value of such a grading system in animals given the comparatively infrequent attempts at antemortem biopsy and definitive therapy (Johnson 1990; Lingaas *et al.* 2003; Summers *et al.* 1995). Classification is primarily based on the histological and cytological determination of the cell of origin, the degree of differentiation, pathological behaviour, topographic pattern and secondary changes within and surrounding the tumour (Braund 1994; LeCouteur 1999). Classification may also be facilitated by the use of immunocytochemical studies, although substantial differences exist between staining characteristics of human and animal brain tumours, limiting their usefulness (Summers *et al.* 1995).

Research has also investigated the expression of oncogenes and genes which code for growth factors or their receptors and thus allow more effective competition with other cells for selective growth advantage. Within neoplastic glial cell tumours in humans, growth factors examined have included epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factors (IGF), all of which are coded for by separate genes. Progesterone and oestrogen are also thought to play a role in the growth of human meningiomas, and receptors for these sex steroids have been identified in some canine meningeal tumours (Adamo *et al.* 2003; Summers *et al.* 1995). The expression of Vascular Endothelial Growth Factor (VEGF) in canine meningiomas has also been examined (Platt *et al.* 2005). All of these studies are indicative of the increasing search for molecular targets present in canine tumours. This search has also been extended to the level of the genome, with some papers examining the profile of canine brain tumours using comparative genomic hybridisation (CGH) to examine chromosome-wide gains and losses of genetic material (Dunn *et al.* 2000). Most recently, the development of cDNA microarray chips has also allowed a survey of more than 2000 clones matching genes or expressed sequence tags (ESTs) and identified a number of genes expressed differentially in canine brain tumours (Thomson *et al.* 2005). Importantly, several of these, including ApoD, CREG and NDRG4 show similar patterns of expression in both human and canine brain tumours. In addition, the recent

publication of linkage maps of the canine genome has allowed some authors to investigate the possibility of LOH in the canine equivalent of human chromosomes 1p and 19q in canine oligodendrogliomas, and suggests that the same finding may be present in dogs although this research is still ongoing (Olby 2005). All of these factors suggest that the similarities between canine and human brain tumours extend beyond the histochemical level to the molecular level.

Treatments used to treat canine tumours have largely paralleled methods used in human tumours, and include surgical removal, radiotherapy and chemotherapy (LeCouteur 1999). Approaches have been described for surgical excision of masses in most part of the canine brain, including most regions of the cerebral cortex, cerebellum and brain stem. Removal of lesions from the ventral brain and brainstem, and from within the deep cortical grey matter still pose a significant challenge, however. Various protocols have been described for irradiation of intracranial tumours in dogs, including hypofractionated protocols and stereotactic radiosurgery (Brearley *et al.* 1999; Evans *et al.* 1993; Goossens *et al.* 1998; Lester *et al.* 2001). In addition, limited case reports have described the use of various chemotherapeutic agents including carmustine, lomustine and cytosine arabinoside (Dimski & Cook 1990; Fulton & Steinberg 1999). Analysis of survival times is complicated by the fact that many of the case series described to date have included relatively small group sizes for comparison. In addition, one of the major challenges in canine neurology has been the identification of outcomes for different tumour types, since many cases are euthanased without definitive histopathological diagnosis. At present, the consensus for the majority of tumours seen in dogs is that the longest survival times are seen in those cases treated with surgical excision followed by radiotherapy (Platt *et al.* 2004).

3.2.2.1 Telomerase activity in canine neoplasms

Given the similarities shared between human and canine brain tumours, investigating telomerase activity in canine brain tumours would appear to be an important starting point from which to develop telomerase-directed therapies to be used either in the treatment of canine tumours or as a preclinical testing ground for human tumour therapies.

The presence of telomerase in canine tumours in general is considerably less well-established than in man. In one of the earliest reports, Biller *et al* reported that 24 of 26 malignant tumours of various origin were telomerase-positive, as compared with 1 of 4 benign tumours and none of 3 normal tissues (Biller *et al.* 1998). In a survey of 27 mammary tumours, Yazawa *et al* found that 26 of 27 tumours were positive for telomerase activity (Yazawa *et al.* 2001). However, there appeared to be little correlation between the level of telomerase activity and tumour grade, and 4 of 12 normal specimens were also found to be positive for telomerase activity. In a separate study, Yazawa *et al* reported telomerase activity in a panel of 16 tumours and its correlation with RT-

PCR, although they did not provide the number of telomerase-positive tumours (Yazawa *et al.*, 2001). Nasir *et al* reported telomerase activity in a range of canine cell lines, and in both normal and neoplastic lymph nodes (Nasir *et al.* 2001). Overall these studies combined suggest that approximately 95% of the canine tumours so far examined are positive for telomerase activity (Argyle & Nasir 2003). However, none of these reports have yet investigated the presence of telomerase activity in canine brain tumours.

3.2.2.2 Proliferation in canine tumours

In validating canine brain tumours as a model for research into human tumours, the correlation between telomerase activity and proliferation must also be shown to exist in this species. Several reports have described the use of BrdU, Ki-67 and PCNA labelling in tumours in domestic animals (reviewed in (Madewell 2001)), all of which have been shown to correlate well with other prognostic factors in a variety of tumours (Fournel-Fleury *et al.* 1997; Griffey *et al.* 1999; Lohr *et al.* 1997; Platz *et al.* 1999; Sarli *et al.* 1994; Simoes *et al.* 1994; Zacchetti *et al.* 2003). Of these markers, only PCNA has been examined in brain tumours. Theon *et al* showed that PCNA correlated significantly with outcome in meningiomas treated with surgery and radiotherapy (Theon *et al.* 2000). In addition, Uchida *et al* reported the use of PCNA in the diagnosis of a ganglioglioma (Uchida *et al.* 2003). However, PCNA staining in this tumour was negative, not unexpectedly, since gangliogliomas are typically benign tumours. To date, no studies have reported the use of Ki67 in canine brain tumours.

3.2.3 Methods of Assessing Telomerase Activity

3.2.3.1 The Telomerase Repeat Amplification Protocol

Specific detection of telomerase activity in biological samples has been made possible through the use of the PCR based TRAP assay. This assay, first described by Kim and others at the Geron corporation in 1994 (Kim *et al.* 1994), allows detection of telomerase activity based on the presence of active enzyme in as few as 10 cells in any given sample (Hiyama & Hiyama 2002). Using this assay, Shay and Bacchetti went on to demonstrate that approximately 90% of all human cancers are TRAP-positive (Shay & Bacchetti 1997).

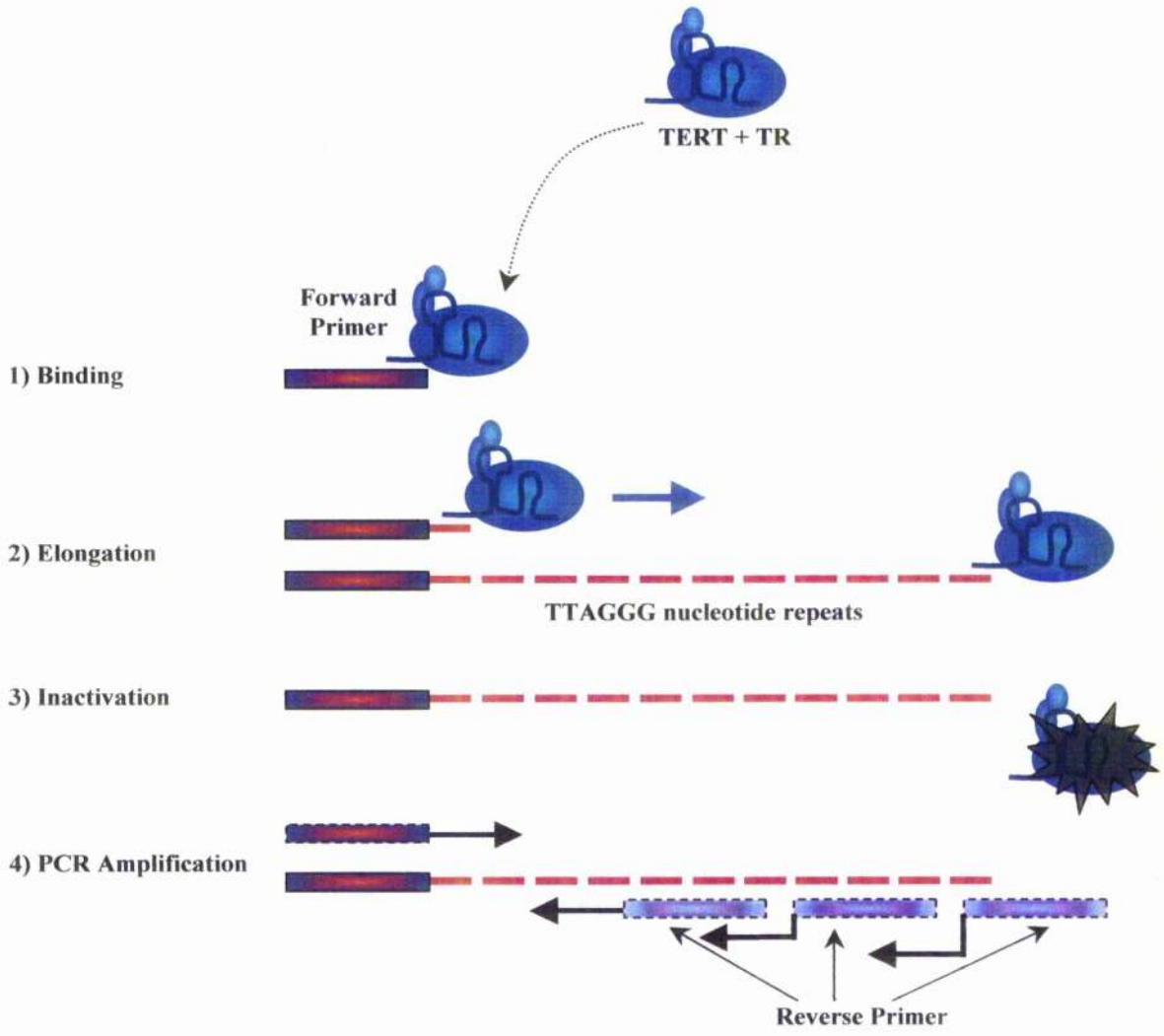
The TRAP assay relies on the presence of both the TERT and TR components of telomerase in the sample acting as a functional enzyme. In the first step, a synthetic primer acts as a substrate which is bound by telomerase, comprised minimally of the TERT and TR subunits. For this reason, the primer, referred to as TS, contains within its sequence a number of TTAGGG repeats. In addition, the TS primer may be labelled in a way that allows detection of the final products (eg by conjugation with biotin for detection by ELISA, or by radiolabelling with ^{32}P).

In the second step, the primer is then elongated by the addition of further TTAGGG repeats. Following this, telomerase is first deactivated by heating the sample. Then, the elongated products are subjected to PCR amplification using the labelled TS as a forward primer, and a reverse primer (CX) complementary to a run of four TTAGGG sequences. Since the reverse primer is able to bind in multiple places along the telomerase-elongated sequence (composed of TTAGGG repeats), the final product lengths differ from each other by multiples of 6 nucleotides (see Figure 3.1).

In the final step, the PCR products are detected in one of a number of ways. In the original assay, described by Kim *et al*, addition of radioactive bases to the PCR reaction mix (eg [^{32}P]-labelled dCTP or dGTP) resulted in a product that could be detected by autoradiography (Kim & Wu 1997). Products can then be subjected to polyacrylamide gel electrophoresis (PAGE) (Kim & Wu 1997) In this case, a characteristic 'TRAP ladder' is generated, with consecutive bands increasing in size by multiples of 6 nucleotides. Alternatively detection can be performed using an ELISA-based system, in which the TS primer, previously labelled with biotin, binds to a streptavidin-coated microplate and products are then detected with an antibody against digoxigenin that is conjugated to horseradish peroxidase (Anti-DIG-HRP) and the sensitive peroxidase substrate TMB.

Figure 3.1: The Telomerase Repeat Amplification Protocol

Principal features of the TRAP assay. In the first step, telomerase binds to the forward primer to allow elongation to occur (step 2). Then, telomerase is heat-inactivated, leaving the elongation products composed of tandem (TTAGGG)n repeats. Following this step, PCR amplification occurs, but due to the presence of repeats containing the same hexanucleotide sequence, the reverse primer is able to bind in multiple places, producing amplification products of different lengths, varying by 6 base pairs. In the final stage (not shown), the amplification products are then detected, most commonly by gel electrophoresis or ELISA techniques.



Szatmari and colleagues described an alternative method of detecting the PCR products, in which, following incorporation of [^{32}P]-labelled bases in the PCR reaction mix, samples were precipitated following amplification and the radioactivity of the sample measured (Szatmari *et al.* 2000). Particular features of the primers used and their annealing temperatures meant that reverse priming of the PCR products occurred primarily at the 3' end of amplified constructs, reducing the random internal reverse priming that occurs with conventional primers. In this way, the radioactive signal generated by PCR products increased in a linear fashion with the amount of telomerase present in the sample, rather than in an exponential or hyperbolic fashion. This technique also reduced the amount of post-PCR processing required before detection, shortening the time required.

Quantification of telomerase activity within samples required modification of the original procedure. In fact, the number of PCR products generated depends on two factors: the processivity of the enzyme within the sample, and the number of enzyme molecules present. The first of these factors reflects the number of telomeric repeats the enzyme adds to the substrate, and the second reflects the number of substrate molecules simultaneously extended by telomerase. Therefore, those methods which measure the strength of radioactive signal generated following incorporation of radioactive bases into the PCR reaction do not distinguish between enzyme processivity and the amount of enzyme present in each sample.

Kim and Wu described a refinement of the original TRAP protocol in which only the TS primer, rather than all bases, was radiolabelled (Kim & Wu 1997). In this way, strength of the signal generated by PCR products depends only on the number of substrate molecules elongated and not on the processivity of the enzyme. In order to quantify the test, a comparison was then made between the strength of the signal generated from the test sample following PCR amplification, with the strength of the signal generated from a sample of known telomerase activity. This positive control was obtained from an extract of 293 cells, which are known to be highly positive in telomerase activity (Wick *et al.* 1999). Using this positive control, Kim and Wu described a linear relationship between the quantity of telomerase present in a sample and the total strength of signal generated by amplified products (Kim & Wu 1997). Using this scale, the total product generated (TPG) was defined such that 1 unit of TPG is equivalent to 600 molecules of primer TS extended for at least 3 telomeric repeats by telomerase within the extract. One TPG is approximately the quantity of telomerase activity present in a single immortal cell. In current versions of the test, quantitiation of telomerase activity in any given sample is performed by expressing it relative to telomerase activity present in a positive control run alongside, often comprising an extract of 293 cells. In this way, telomerase activity can be compared between samples.

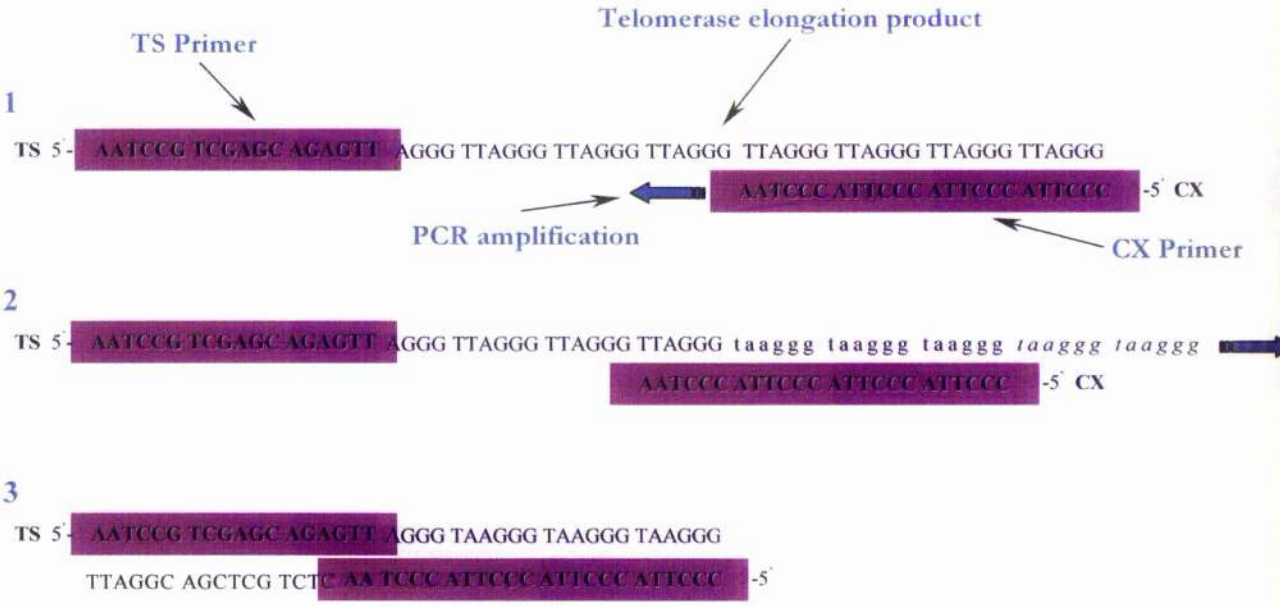
3.2.3.1.1 Technical Problems with the Telomerase Repeat Amplification Protocol

3.2.3.1.1.1 Primer-Dimer formation and other artefacts

When the first report of the TRAP assay was described, it became apparent that certain artefacts were commonly found (Kim *et al.* 1994). Following amplification of a synthetic oligonucleotide containing four TTAGGG repeats using the TS and CX primers (without prior telomerase elongation), PAGE revealed a product ladder with 6 base pair steps extending to the top of the gel, rather than a single product of original size (Krupp *et al.* 1997). This was found to be due to staggered annealing of the CX primer to the substrate. The CX primer was originally designed with a number of mismatches, so that efficient priming of the substrate occurred only when the entire primer binds. However, it was shown that binding of only the first repeat of the CX primer could occur, tolerating the single nucleotide mismatch present. In this case, subsequent amplification leads to extension of the original product by a further 3 repeats (18 nucleotides) with each round of amplification (Figure 3.2).

The second artefact that arose was due to primer-dimer formation. PCR amplification in the absence of any substrate could potentially result in a TRAP ladder being formed, resulting in a false positive result. In this situation, the last two nucleotides at the 3' end of TS and CX would bind in a complementary fashion, leading to a 40-bp product. This could be extended, following staggered annealing of the CX primer in successive rounds of amplification, to yield a complete TRAP ladder in the absence of telomerase activity (Kim *et al.* 1994) (Figure 3.2).

Figure 3.2: Basis of artefacts associated with the TRAP assay



1. Normal TRAP assay. The TS primer is used by telomerase present within the test sample to synthesise TTAGGG repeats (in capitals). The CX primer then binds to a run of four TTAGGG repeats during PCR amplification of the elongation products.
2. Continued extension of TTAGGG repeats during PCR amplification. Although the CX primer is designed to pair with a run of four TTAGGG repeats, in some cases it was found that CX could bind a single TTAGGG repeat, tolerating the single nucleotide mismatch. PCR amplification then results in extension of the elongation product by a further 3 TTAGGG repeats with every cycle. Lower case ttaggg shows extension after first cycle, italicised *ttaggg* shows extension after second cycle etc.
3. Primer-Dimer formation. In some cases, the last two nucleotides of the TS primer could bind to the first two primers of CX. This primer-dimer could be extended during the PCR amplification step, resulting in complete TRAP ladder in the absence of any telomerase in the sample.

Elimination of both of these artefacts was made possible through changes to the CX primer. Krupp and colleagues added three additional nucleotides to the 5' terminus of the CX primer which were non-complementary to telomeric sequence. In this way, amplification of primer-dimer formation or subsequent extension of already-elongated products was virtually eliminated (Krupp *et al.* 1997). Kim and Wu, who were amongst the group that reported the original TRAP assay in 1994, described a very similar modification using a 6bp 'anchor' at the 5' end and solved the problem in the same way (Kim & Wu 1997).

3.2.3.1.1.2 Taq-polymerase Inhibitors

Some tissues contain Taq-polymerase inhibitors that have been found to inhibit the PCR amplification of telomerase-elongated products (Wright *et al.* 1995). In these tissues, performing a TRAP assay with the standard amount of protein (6μg) leads to a negative result, whilst a reduced protein level would reveal a positive result. To control for the presence of either Taq-polymerase or other PCR inhibitors within the sample, a number of modifications of the TRAP assay include an internal standard. The internal standard is an oligonucleotide substrate of known size, the 5' end of which can be primed by TS, and the 3' end of which can be primed either by the same reverse primer as is used in the initial PCR reaction or a second, specific reverse primer. In both cases, the substrate is amplified in the initial PCR reaction along with the telomerase-elongated sequence. However, PCR amplification of the internal standard results in a product of known size.

In the an early report of the protocol first described by Kim *et al*, the internal standard was a product 36 nucleotides in length (Kim & Wu 1997). The presence of Taq-inhibitors in the sample results in a failure of amplification of the internal standard, enabling detection of false-negative results. Later modifications substituted an internal standard that was 216 bp in length, since it was found that the shorter internal standard could sometimes be amplified even in the presence of Taq inhibitors, leading to false negative results. An additional feature of the inclusion of the internal standard is that it aids quantification of telomerase activity in samples, since telomerase activity, once normalised to the internal standard, displays a linear relationship to the number of telomerase-positive cells in the sample (Wright *et al.* 1995).

3.2.3.2 Reverse Transcriptase-PCR of TERT and TR

RT-PCR allows the detection of messenger RNA (mRNA) in samples through PCR amplification. RT-PCR techniques involve isolating either total RNA or mRNA. This is then followed by reverse-transcribing the RNA using a reverse transcriptase enzyme, which synthesises DNA complementary to the RNA template (cDNA). This cDNA encodes a modified form of the gene of interest as all non-coding introns have been removed during synthesis of mRNA. cDNA can then be amplified using standard PCR techniques and in this way the expression of genes in cells can be

evaluated. PCR amplification of the gene of interest is usually performed in conjunction with amplification of a constitutively expressed gene which serves as a positive control for the reaction.

RT-PCR analysis of telomerase in humans has most frequently involved the amplification of the hTERT gene, as hTERT expression is the rate-limiting step in telomerase activation. In addition, some authors have investigated the expression of hTR (Dome *et al.* 1999; Gunther *et al.* 2000; Ohyashiki *et al.* 2005; Swiggers *et al.* 2004; Takakura *et al.* 1998; Yi *et al.* 2001). As well as using conventional PCR techniques, quantitative RT-PCR (qRT-PCR) techniques have also been used to analyse hTERT expression (Kotoula *et al.* 2004; Ohyashiki *et al.* 2005; Tchirkov *et al.* 2003). qRT-PCR allows the quantity of the product of interest to be measured by normalising amplification levels against those of a positive control gene which is amplified simultaneously. This requires initial optimisation of the PCR reaction so that the amplification plateau is calculated for both the product of interest and the positive control, with the same amount of each template included at the start of the reaction. Once this has been performed, using the same conditions, amplification of the product of interest can then be quantified using a fluorescent marker incorporated into the reaction mix such as a Taqman probe or SYBR Green I (Bieche *et al.* 2000; de Kok *et al.* 2000; Krams *et al.* 2003; Tchirkov *et al.* 2003; Wu *et al.* 2000).

There appears to be good correlation between hTERT mRNA expression and telomerase activity in some tumour types, including leukaemia, soft tissue sarcomas, thyroid tumours, gastrointestinal tumours, lung carcinomas and cervical cancer (Gunther *et al.* 2000; Kumaki *et al.* 2000; Ohyashiki *et al.* 2005; Saji *et al.* 1999; Takakura *et al.* 1998; Yan *et al.* 1999). Within the brain tumour subgroup, similar findings have been reported for glial tumours and neuroblastomas (Kotoula *et al.* 2004; Krams *et al.* 2003). Expression of hTR mRNA, however, does not seem to correlate well with telomerase activity, either *in vivo* or *in vitro* in the cell lines in which it has been examined (Ohyashiki *et al.* 2005; Swiggers *et al.* 2004; Takakura *et al.* 1998). In addition, there appears to be little correlation between the expression of hTERT mRNA and the expression of hTR mRNA (Gunther *et al.* 2000).

However, although expression of the hTERT gene generally correlates well with telomerase activity, this is not always the case. In colorectal cancer and Wilms' tumours in humans, for instance, the expression of hTERT mRNA correlates only weakly with the activity of telomerase (Dome *et al.* 1999; Palmqvist *et al.* 2005). In addition, Swiggers and colleagues reported that in primary human fibroblast clones with reconstituted telomerase activity, hTERT mRNA levels do not seem to correlate with telomerase activity or with telomere length (Swiggers *et al.* 2004). Similarly, Yi and colleagues found that the correlation between telomerase activity and hTERT mRNA expression in a number of immortalised and telomerase-positive cancer cell lines is surprisingly weak (Yi *et al.* 2001). Taken together, these reports suggest that post-transcriptional modification of hTERT is important for producing active telomerase, and therefore that analysis of

the presence or absence of gene expression alone does not provide sufficient information to establish this. One important mechanism involved in post-transciptional modification is the presence of splice variants, first described in humans by Kilian and colleagues (Kilian *et al.* 1997) (see 5.2.2.2: Post-Transcritptional Modification of hTERT). Whilst several splice variants have been discovered, only the full-length version of hTERT is functional in activating telomerase. Depending on the location of the primers used, therefore, some reports analysing hTERT mRNA expression may not have necessarily distinguished between full length, active hTERT, and non-functional splice variants. For instance, Kotoula and colleagues, in investigating the presence of hTERT splice variants in astrocytic gliomas, reported that in 5 grade I tumours examined, 3 displayed the presence of hTERT mRNA (Kotoula2004). However, in all 3 cases, the transcript was found to contain two of the common deletions (α-del and β-del), resulting in a non-functional mRNA. Conversely, all of the high grade tumours expressing hTERT mRNA showed the full length transcript. Expression of the full length hTERT mRNA transcript has also been found to be important for the activity of telomerase in neuroblastomas (Krams *et al.* 2001). On the face of it, these findings might suggest that telomerase-positive cells only express the full-length version of hTERT mRNA. However, Yi and colleagues showed that, of the total hTERT mRNA expressed within many of the telomerase-positive cell lines so far characterised, only a small fraction (approximately 5%) of the transcripts were the full length form (Yi *et al.* 2001). An extra complication was uncovered in a report by Ohyashiki and colleagues, who, in examining the relationship between hTERT expression and telomerase expression in patients with leukaemia, found that generally telomerase expression correlated very well with expression of the full length transcript (Ohyashiki *et al.* 2005). However, in those cases where discrepancy occurred, it was found that expression of hTR RNA was significantly lower, suggesting that although hTR expression alone does not correlate with telomerase activity, telomerase activity may require the synthesis of both gene transcripts in a coordinated fashion.

In summary, whilst investigation of hTERT and hTR mRNA through RT-PCR methods has been performed both in vivo and in vitro, there appears to be some disagreement about its correlation with telomerase activity. This may be due to post-transcriptional modification, and splice variants are likely to play a role in this.

3.2.3.3 In Situ Hybridisation (ISH)

In situ hybridisation techniques make use of the presence of single-stranded mRNA in tissue samples. Probes consisting of DNA sequence are designed complementary to the gene of interest, and labelled in one of a number of ways, including with dixogenin, or fluorescein (FISH). Addition of the probe to the sample without prior denaturation ensures that the probes bind to the only single-stranded sequence present – mRNA. Prior treatment with RNase together with the use

of probes designed to be identical to the sequence of interest (sense) rather than complementary (anti-sense) provide negative controls.

ISH techniques have been described by several authors (Athanassiadou *et al.* 2003; Baykal *et al.* 2004; Fukushima *et al.* 2004; Kolquist *et al.* 1998; Liu *et al.* 1999; Nieh *et al.* 2005; Norrback *et al.* 2001; Saretzki *et al.* 2002; Soria *et al.* 2001). Most commonly, probes synthesised from TERT have been used, and ISH has been used to examine TERT expression in cytological preparations, paraffin-embedded sections and in fresh or frozen tissues. In many of these reports, the use of ISH has provided important findings regarding the localisation of telomerase activity, such as the finding that germinal centres of lymphocytes contain telomerase activity only within activated B cells (centroblasts) but not in resting or active naïve B cells (Norrback *et al.* 2001). In addition, using ISH, Kolquist *et al* showed that telomerase activity was present in breast epithelial cells with long-term proliferative activity, and that it appeared in tumour cells early in breast tumorigenesis, suggesting that in these tumours telomerase activity may possibly precede other neoplastic changes (Kolquist *et al.* 1998).

Whilst ISH examination of telomerase activity in situ has been used extensively, the technique is somewhat hampered by the fact that it is time- and labour-intensive (Maes *et al.* 2005). In addition, the detection of mRNA in paraffin-embedded tissues is affected by the length and quality of sample fixation, due to the rapid degradation of RNA. Therefore, some samples with TERT mRNA expression may stain falsely negative if fixation is not optimal.

3.2.3.4 Immunohistochemical Detection of Telomerase Activity

The complications associated with assessing telomerase activity through either TRAP assay or RT-PCR techniques are best addressed by utilising techniques that examine telomerase activity *in situ*. In addition to microdissection techniques and *In Situ* Hybridisation (ISH) analysis of hTR and hTERT mRNA described, other techniques have been described for detecting telomerase activity in cancer cells such as immunomagnetic bead separation and sorting by flow cytometry (Waguri *et al.* 2003). However, these techniques result in limited recovery of molecular targets, and it may be difficult to precisely assess the degree of sample contamination by non-neoplastic cells because of the sensitivity of molecular amplification assays.

Another potential method for examining telomerase activity in individual cells is through the immunohistochemical detection of the TERT protein. The advantages of immunohistochemical techniques are that they provide an overall picture of telomerase activity while also allowing examination of individual cells and cell types (Shroyer 2002). Importantly, they may be used on paraffin-embedded archival material, allowing a larger survey of samples to build a more comprehensive picture of telomerase activity both within and across tumour subtypes. Fixed

material can potentially be used for ISH techniques and RT-PCR, depending on the quality of the material, but is technically difficult because of DNA and RNA degradation that occurs during the fixation. Thus, histochemical approaches remain the optimal method in many circumstances for defining the expression of specific proteins or RNAs at the cellular level (Shroyer 2002).

Several studies have examined the detection of hTERT in tumour samples, including in paraffin-embedded material, in frozen sections, and in cytological preparations (Cheung *et al.* 2004; Hiyama & Hiyama 2002; Ikeda *et al.* 2003; Poremba *et al.* 2000b). Those tumours examined include breast tumours, lung cancers, meningioma, neuroblastoma, ameloblastoma, mesothelioma, gynecological cancers and colorectal carcinomas (Hiyama *et al.* 2001; Hiyama & Hiyama 2002; Ikeda *et al.* 2003; Kumaki *et al.* 2000; Kumamoto *et al.* 2001; Kyo *et al.* 2003; Maes *et al.* 2005; Poremba *et al.* 2000b; Tahara *et al.* 1999). Several antibodies have been used, and the staining pattern varies slightly depending on the antibody used. The majority of reports suggest that immunopositivity is restricted to the nucleus, and in some cases specifically to the nucleolus (Maes *et al.* 2005), frequently with a granular or speckled pattern. However, in some tumours staining also appears to extend to the cytoplasm of neoplastic cells (Cheung *et al.* 2004; Kyo *et al.* 2003). Staining for hTERT has also been detected in a small number of non-neoplastic cells within normal colonic mucosa, located at the bottom of intestinal crypts, and in associated activated lymphocytes (Hiyama *et al.* 2001). hTERT staining has also been reported in telomerase positive BJ+hTERT cells (hTERT transfected fibroblasts) and HT1080 cells grown in serum, which possess high telomerase activity when actively growing, but not in SW13-ALT immortal cells or HT1080 cells deprived of serum, both of which lack telomerase activity (Hiyama *et al.* 2001). In the study reported by Hiyama *et al* (Hiyama *et al.* 2001), immunohistochemical staining of hTERT was not detected in the majority of normal tissues, including cardiac/skeletal muscle, liver, prostate, breast, pancreas, brain, lung and kidney. Low to high levels of telomerase activity were found in germ-line tissues, proliferative pre-menopausal endometrium and tissues containing activated lymphocytes. Staining for hTERT was also detected in a small number of cells within colonic mucosa, located at the bottom intestinal crypts, and in associated activated lymphocytes. hTERT staining was present in telomerase positive BJ+hTERT cells (hTERT transfected fibroblasts) and HT1080 cells grown in serum, which possess high telomerase activity when actively growing, but not in SW13-ALT immortal cells or HT1080 cells deprived of serum, both of which lack telomerase activity.

Correlation between TERT immunostaining and TRAP activity has not been extensively investigated. However, telomerase staining has been found to correlate with TRAP activity in some tumours, with those tumours possessing low TRAP activity displaying hTERT staining in a small number of cells, and those tumours with high TRAP activity showing more widespread hTERT staining. Poremba *et al* reported that in the neuroblastomas examined, hTERT staining correlated well both with the level of TRAP activity and with the presence of hTERT mRNA

(Poremba *et al.* 2000b). Similar results were reported in ameloblastomas and lung carcinomas (Kumamoto *et al.* 2001; Nakanishi *et al.* 2002). In addition, TERT staining appears to correlate with malignancy, with cellular proliferation and with outcome in the majority of tumours examined (Cheung *et al.* 2004).

These reports confirm that the immunohistochemical detection of TERT provides a useful technique for the assessment of telomerase activity in paraffin-embedded tissue, because of the ability to examine telomerase status of individual cells *in situ*, the correlation with other methods of assessing telomerase activity, and the ability to survey large groups of archival samples.

To date, few reports have described the use of hTERT immunohistochemistry in human brain tumours. In one report which examined telomerase expression in 133 neuroblastomas, 39 (29.3%) were found to be TRAP positive (Poremba *et al.* 2000b). The quantification of both TRAP activity and hTERT staining in this study showed that hTERT staining correlated well with TRAP activity. In five neuroblastomas with low telomerase activity, 1% to 10% of cells were found to stain positively for hTERT. Of four samples with intermediate telomerase activity, 10% to 50% of cells were found to stain positively for hTERT. Three neuroblastomas with high telomerase activity displayed hTERT staining in more than 50% of cells. In meningiomas Maes *et al* described hTERT immunoreactivity in a series of meningiomas that recurred following resection (Maes *et al.* 2005). In this study, of the 27 meningiomas that recurred, positive hTERT staining was found in 26 tumours, as compared with 6 of 28 non-recurrent meningiomas. To date, no reports have examined TERT immunohistochemistry in canine brain tissues.

3.3 Aims of the chapter

To date, no reports have evaluated the immunohistochemical detection of TERT in canine tumours. The first aim of this chapter was therefore to evaluate a number of commercially available hTERT antibodies in canine tissues by correlating TERT staining with TRAP assay, Western blotting and RT-PCR. In addition, no reports to date have described the assessment of cellular proliferation in canine brain tumours using Ki-67. Therefore, the second aim of this chapter was to evaluate Ki-67 staining in a range of canine brain tumours and to correlate the MIB-1 labelling index (LI) with conventional histological grading to determine its usefulness as a prognostic indicator. Once the MIB-1 LI was validated in these tumours, the third aim of this chapter was to correlate TERT staining with proliferation and with malignancy.

3.4 Materials and Methods

3.4.1 Sample details

3.4.1.1 Normal samples

Normal brain tissue samples were obtained from a dog that had been euthanased for a non-neurological cause and submitted for post-mortem at GUVS. All necropsies performed at this Institute are carried out with informed owner consent. Approximately $1cm^3$ tissue samples were taken under aseptic conditions from the olfactory lobe, subventricular zone at the level of the caudate nuclei, and cerebellum within 30 minutes of euthanasia. Samples were divided into two portions, one of which was flash frozen in liquid nitrogen (LNO_2) before storage at -70° C, and the other fixed in 10% Buffered Neutral Formalin (BNF). To serve as a positive control, archival material from 3 normal canine testes was retrieved from the pathology archives of the Institute of Comparative Medicine, University of Glasgow. These sample had been previously taken, fixed in 10% BNF and paraffin-embedded as part of routine post-mortem examination for other reasons.

3.4.1.2 Tumour samples

Tumour samples were retrieved from a total of 93 paraffin-embedded brain tumours retrieved from the pathology archives of the Institute for Comparative Medicine, University of Glasgow Veterinary School and collaborating Institutes, namely, Institute of Animal Neurology, University of Berne; College of Veterinary Medicine, North Carolina State University; Department of Pathology, Utrecht University Faculty of Veterinary Medicine; Centre for Small Animal Studies, Animal Health Trust, UK. Where available, Haematoxylin and Eosin (H&E)-stained slides were retrieved from the archives for review. Where slides were not available, fresh sections were cut from paraffin-embedded tissue blocks and restained. In some cases, special immunohistochemical stains, including glial fibrillary acidic protein (GFAP), neurofilament (NF), vimentin, synaptophysin and other stains as required were used to confirm the original diagnosis.

All sections used in the study were re-evaluated with Dr S Robinson, NHS Consultant Neuropathologist, Department of Neuropathology, Southern General Hospital, Glasgow, and the diagnosis of intracranial neoplasia was confirmed. Each tumour sample was classified according to the World Health Organisation classification system of tumours (Koestner *et al.* 1999). Tumours were then divided into two grades, 1 and 2, based on their classification, as described in Table 3.3.

Table 3.3: WHO Classification of Canine Brain Tumours and Grading Scheme Used

WHO Classification	Grade
Meningioma	
Low grade meningioma	1
Anaplastic meningioma	2
Oligodendroglioma	
Oligodendroglioma	1
Anaplastic oligodendroglioma	2
Astrocytoma	
Low grade astrocytoma	1
Anaplastic astrocytoma	2
Glioblastoma multiforme	2
Other glial tumours	
Gliosarcoma	2
Oligoastrocytoma	1
Anaplastic oligoastrocytoma	2
Choroid plexus tumours	
Choroid plexus papilloma	1
Choroid plexus carcinoma	2
Pituitary tumours	
Pituitary adenoma	1
Pituitary adenocarcinoma	2
Primitive Neuroectodermal Tumours	
Medulloblastoma	2
Ependymal tumours	
Ependymoma	1
Anaplastic ependymoma	2
Metastatic tumours	2

3.4.1.3 Cell Lines

Cells used in this chapter include the D17, CMT7, U87-MG and K9SF cell lines. The D17 and CMT7 cell lines are canine osteosarcoma and mammary tumour cell lines, and the K9SF cell line is a canine skin fibroblast primary culture. The U87-MG cell line is a human glioblastoma cell line. All cell lines are detailed in Chapter 2. Cells were grown in T75 flasks and passaged routinely upon reaching confluence.

For immunohistochemical evaluation, artificial clots were created using cells from the CMT7, D17, U87 and K9SF cell lines. Following trypsinisation, cells were counted and an aliquot of 5 x 10^5 cells was transferred to a 1.5ml eppendorf tube and pelleted at 3000 rpm for 5 minutes. The growth medium was discarded and the pellets washed twice in sterile PBS at 4° C. Following the second wash, cells were centrifuged at 3000 rpm for 5 minutes at 4° C and supernatant was carefully decanted. Fifty µl of bovine plasma was added to the resultant cell pellet, followed by 50 µl bovine thrombin, and the cell pellet agitated at 37° C until a clot had formed. Clots were then fixed in 10% BNF and processed as described below.

For Western blotting, cell pellets were created using cells from the CMT7, D17, U87 and K9SF cell lines. Following trypsinisation, cells were counted and an aliquot of 2 x 10^6 cells was transferred to a 1.5ml eppendorf tube and pelleted at 3000 rpm for 5 minutes. The growth medium was discarded and the pellets washed twice in sterile PBS at 4° C. Following the second wash, cells were centrifuged at 3000 rpm for 5 minutes at 4° C and supernatant was carefully decanted. Aliquots were then flash-frozen in LNO_2 for storage prior to Western blotting.

3.4.2 Telomerase Activity

3.4.2.1 Telomerase repeat amplification protocol (TRAP assay)

Telomerase activity was measured using the TRAP assay as described in 2.2.10.1: Telomerase repeat amplification protocol.

3.4.2.2 Reverse transcriptase-PCR

3.4.2.2.1 Primers

Primers were designed as detailed in 2.2.6.1: Primer Design, and supplied by Sigma-Genosys.

3.4.2.2.2 Sample Preparation

To assess TERT gene expression in D17, CMT7, K9SF and U87 cells, reverse-transcriptase PCR was performed. Following trypsinisation and counting, aliquots of 2 x 10^6 cells were transferred to a DEPC-treated 1.5ml eppendorf tube and pelleted at 3000 rpm for 5 minutes. The growth medium was discarded and the pellets washed twice in sterile PBS at 4° C. Following the second wash, cells were centrifuged at 3000 rpm for 5 minutes at 4° C and supernatant was carefully decanted. Cell pellets were then flash-frozen in LNO_2 and then stored at –70° C prior to RNA extraction.

3.4.2.2.3 RNA Extraction

RNA was extracted from cell pellets using RNA-Wiz™ (Ambion, UK) as described in 2.2.4.1: Extraction and Purification of RNA.

3.4.2.2.4 Assessing RNA Quality

RNA quality was assessed in 2 ways. Firstly, the RNA was visualised using agarose gel electrophoresis on a 1% gel. This allowed visualisation of the 28S and 18S ribosomal subunits. In addition, 2µl of each sample was diluted 1:50 in autoclaved distilled water and analysed using spectrophotometric analysis to assess the concentration and quality of RNA in the sample.

3.4.2.2.5 DNA Removal

To ensure RNA samples were not contaminated with DNA, DNA-free™ (Ambion, UK) was used as described in 2.2.4.1.2: DNase Treatment of RNA.

3.4.2.2.6 cDNA First Strand Synthesis

Following RNA extraction, cDNA was synthesised using Superscript III™ reverse transcriptase (Promega, UK) as described in 2.2.5: First strand cDNA synthesis.

3.4.2.2.7 PCR amplification

Following synthesis of cDNA, PCR was performed using the primers EX2F and EX2R for canine TERT, and DNHT001F and DNHT001R for human TERT. As a control, amplification of a fragment of the canine cyclophilin gene was simultaneously performed using the primers CYCLOF and CYCLOR, and a fragment of the human ß-actin gene, both of which are constitutively expressed housekeeping genes. These primers are detailed in Table 3.4.

Table 3.4: Primers Used in RT-PCR

Primer Identification	Oligonucleotide primer sequence (5'-3')	Tm (°C) & GC (%) content
DNHT001F	ACTGTTCAGCGTGCTCAACTA	62.6° C : 47.6 %
DNHT001R	TCATTCAGGGAGGAGCTCTGCT	68.5° C : 54.5 %
CycloF	CGTCCTCTGAGTACTGGAGAGAAGGGA	80.2 C : 55.6 %
CycloR	CCACTCAGTCTTGGCGGTGCAGATGAA	75.0° C : 60.0 %
EX2F	CAGGAGCTGCTTGGGAACCA	69.8 C : 60.0%
EX2R	CTGGGTTCCCGTGCAGCCAG	74.4 C : 70.0%
Actin F	CAAGAGATGGCCACGCCTGCT	58.0° C : 61.9%
Actin R	TCCTTCTGCATCCTGTCGGCA	56.0° C : 57.1%

Platinum High-Fidelity Taq polymerase (Invitrogen, UK) was used for second strand PCR. For each sample, 1-5 µl of cDNA was mixed in a 0.2µl thin-walled PCR tube with 5µl of High Fidelity PCR buffer, 1µl dNTP mixture, 2µl $MgSO_4$, 1µl of each primer (forward and reverse) used and 0.2µl Platinum Taq High Fidelity. The reaction mix was made up to a total volume of 50µl with autoclaved, distilled water. Samples were kept on ice during pipetting and prior to PCR. As a negative control, 5 µl of total RNA was also included in each reaction to ensure resultant products were not amplified from genomic DNA. Cycling conditions involved denaturation of the template and activation of the Taq polymerase at 94° C for 2 minutes followed by 30 cycles of the following: denaturation at 94° C for 30 seconds, annealing at 55° C for 30 seconds, extension at 68° C for 1 minute. Finally, the mix was incubated at 72° C for 10 minutes to add a run of adenosine residues to the 3' end of each template, before being maintained at 4° C.

3.4.2.2.8 Assessment of RT-PCR results

Following PCR, visualisation of reaction products was performed using 1% agarose gel electrophoresis using 5 µl of the PCR product.

3.4.3 Immunohistochemistry

Immunohistochemistry (IHC) is a technique that allows identification of cellular or tissue antigens by means of interaction between a specific antigen-antibody combination. Since the early direct labelling experiments performed using a primary antibody conjugated directly to a fluorochrome

(Coons *et al.* 1941). Commonly, a modification involving an indirect immunoperoxidase labelling approach is used (Sternberger *et al.* 1970), and this is the method used for the experiments described here. This method involves a dual antibody system in which an unlabelled primary antibody is bound to a secondary biotinylated 'bridging' antibody, derived from a species immunised against immunoglobulins of the species from which the first antibody is derived. A complex formed from streptavidin and horseradish peroxidase (HRP) is then conjugated to this secondary antibody to provide signal at the site of the primary antibody. The substrate for the HRP is 3,3 diaminobenzidine (DAB; Sigma, USA) which produces a brown end-product at the antigen site that is highly insoluble in alcohol and other inorganic solvents. Sections are then stained with a counterstain, typically haematoxylin, to visualise negatively-stained structures.

3.4.3.1 Staining Technique

3.4.3.1.1 Staining Procedure

For this project, staining was performed by Mr C Nixon, University of Glasgow Vet School. Paraffin sections were dewaxed in histo-clear (National Diagnostics, USA), rehydrated in alcohol and incubated in 0.5% H_2O_2 methanol solution for 20 minutes. Sections were then subjected to antigen retrieval with 0.01M sodium citrate (pH6) in a pre-warmed microwave pressure cooker for 6 minutes. All sections were blocked in 1% normal unlabelled serum (Scottish Antibody Production Unit, UK) in 0.01M Tris buffered saline for 30 minutes at room temperature and incubated for 2 hours at room temperature with one of a panel of antibodies against hTERT (at a range of concentrations) or with the MIB-1 monoclonal antibody (at a concentration of 1/100). A standard 1/200 dilution of the appropriate secondary antibody (polyclonal anti-goat/mouse/rabbit, biotinylate; Dako, UK) was then applied and sections were incubated for 45 minutes at room temperature. Sections were then washed three times for five minutes in wash buffer before signal amplification was achieved by incubation with HRP-conjugated streptavidin-biotin complex (DakoCytomation, UK) for 45 minutes. The chromagen DAB (Sigma, UK) was reconstituted in 5 ml tap water as per manufacturers instructions as ions contained in the tap water enhance the reaction and improve the intensity of final staining. The chromagen was then applied for 5 minutes to produce the stable brown insoluble product visible when viewed by light microscopy. Counterstaining was performed using Gills' haematoxylin followed by washing of the stained cells in water, dehydration via a series of graded alcohols and clearing in Histo-Clear (AGTC Bioproducts). Cover slips were then permanently mounted using DPX mounting medium (BDH, UK) before being examined by light microscopy. Negative controls were run in parallel with omission of the primary antibody step.

3.4.3.1.2 Staining Optimisation

Optimisation of the staining procedure was performed with a panel of antibodies against hTERT to identify the concentration of primary antibody that provided the maximum amount of specific staining with the least amount of background. A range of dilutions (1/50, 1/100, 1/200, 1/500, 1/1000, 1/1200, 1/2000, 1/10,000, 1/20,000, 1/40,000) was used to stain the positive control tissue (canine testis). Evaluation of staining by light microscopy then identified the most favourable dilution for each antibody which was then used on all subsequent sections.

3.4.3.2 TERT evaluation

For each section the distribution of staining within cells and throughout each section was noted. In addition, the nature of TERT staining within cells was noted. To evaluate TERT staining in tumour sections, sections were examined at high power for areas of TERT positivity and an assessment of the number of nuclei staining positive was made. In fields with TERT-positive cells, a staining percentage of greater than 10% positive-staining nuclei in each high power field was regarded as positive, based on the findings by Poremba *et al* that >10% of cells from tumours with intermediate telomerase activity stain positively for TERT (Poremba *et al.* 2000b).

3.4.3.3 Ki67 evaluation

In order to evaluate proliferation in tumour sections, Ki-67 staining was evaluated and a MIB-1 Labelling Index (LI) calculated. A minimum of 5 fields were selected for each slide from areas with the highest number of positive-staining nuclei. Within these fields, a minimum of 500 nuclei per case were counted, and the number of positive cells expressed as a percentage of all nuclei counted.

3.4.3.4 Statistical Analysis

For comparison of MIB-1 LI, histological grading, and TERT positivity, the Mann-Whitney U Test was used, with the level of significance set at $p<0.05$. For comparison of MIB-1 LI and histological grade, the mean MIB-1 LI of grade 1 tumours in each tumour subgroup was compared with the mean MIB-1 LI of grade 2 tumours. For comparison of TERT staining and histological grade, the number of TERT-positive grade 1 tumours in each subgroup was compared with the number of TERT-positive grade 2 tumours. For comparison of MIB-1 LI and TERT staining, the mean MIB-1 LI of TERT-negative tumours in each subgroup was compared with the mean MIB-1 LI of TERT-positive tumours in each subgroup.

3.4.4 Antibody Evaluation

3.4.4.1 Western Blotting

In order to assess the specificity of the antibody used, Western blotting was performed using the NuPage® Bis-Tris Electrophoresis System (Invitrogen, UK). The NuPage system uses a neutral pH, discontinuous Sodium Dodecyl Sulphate (SDS)-PAGE pre-cast mini-gel. The pre-cast gel is formulated with a varying gradient of polyacrylamide (4%-12%) to allow separation of a range of proteins over a range from 1-200 kDa. Samples are reduced using a reducing agent containing 500 mM dithiothreitol (DTT) at a 10x concentration. This prevents oxidation of the samples and allows sharper resolution of bands during electrophoresis. Following addition of the reducing agent, ectrophoresis is then performed with protein either in a denatured (through the addition of an antioxidant to the running buffer) or non-denatured (native) state. Protein is then transferred to a nylon membrane and stained with the primary antibody of interest followed by detection with a secondary antibody which is directed against the immunoglobulin molecule of the species in which the primary antibody was generated. This secondary antibody is conjugated to a molecule (horseradish peroxidase) which produces a colour change when a chromagen is applied that can be viewed. This system therefore produces amplification of the original signal sufficient to allow visualisation by light microscopy.

3.4.4.1.1 Sample Preparation

In order to assess the specificity of staining, several samples were used of known telomerase activity (see 3.4.1: Sample Details):

Normal canine brain
D17 Cells
CMT7 Cells
K9SF Cells
K9SF Cells transfected with the human DNhTERT construct (see 4.4.1.3: Generation of stable transfectants)
U-87 Cells

Tissue samples approximately 0.2 mm x 0.2 mm x 0.2 mm in size were used to extract protein. For all cell lines, 2 x 10^6 aliquots previously frozen in LNO_2 were used. To each sample, 200 µl of lysis buffer was added. Lysis buffer was composed of 0.5% NP40, 150mM NaCl, 50 mM TrisHCl (pH 8.0) and shortly before use 1 Complete Mini Protease Inhibitor Cocktail Tablet (Roche, UK) was added per 10 ml. Samples were then centrifuged at 13,000 rpm for 30 minutes at room temperature. The supernatant was then removed and placed in a fresh 1.5 ml eppendorf tube. The

protein concentration of each sample was then assayed using the BCA assay (see 2.2.9: Protein analysis).

For Western blotting, initial experiments were performed using 10-30 µg of protein. Subsequently, a total of 10 µg of protein from each sample was used. To the volume of sample was added 5 µl of LDS Sample Buffer containing marker dye (Invitrogen, UK) and 2 µl of Reducing Agent (Invitrogen, UK). The total volume was then made up to 20 µl using deionised water. Samples were then heated at 70° C for 10 minutes for denaturation prior to electrophoresis. Following heating, samples were flash-spun to collect contents in the bottom of each tube.

3.4.4.1.2 Polyacrylamide Gel Electrophoresis (PAGE)

To perform PAGE, the precast gel was taken out of its wrapping, the comb and tape removed, rinsed with deionised water, rinsed twice with running buffer and then loaded into the Xcell *SureLock*™ Mini-Cell (Invitrogen, UK). Gels were run singly, using the buffer dam instead of the opposing gel. SDS Running Buffer was then made up to a volume of 1000 ml by adding 50 ml of NuPAGE® 20x MES Running Buffer Stock Solution (Invitrogen, UK) to 950 ml of deionised water and mixed thoroughly. To the outer (lower) buffer chamber of the Xcell *SureLock*™ Mini-Cell 800 ml of running buffer was then added. To the remaining 200 ml of running buffer, 500 µl of NuPAGE® Antioxidant (Invitrogen, UK) was added and mixed thoroughly, before being added to the inner (upper) chamber. The antioxidant is added to perform electrophoresis under reducing conditions and migrates with the proteins during electrophoresis in order to prevent reoxidation and maintain the proteins in a reduced state. The antioxidant also protects sensitive amino acids such as methionine and tryptophan from oxidising.

Once the gel was loaded into the Mini-Cell, 20 µl of each sample was then loaded into each well. Twenty microlitres of a protein mass ladder (SeeBlue™ Plus2 pre-stained standard; Invitrogen, UK) was added to the end well of the gel. Electrophoresis was then performed with a constant voltage of 200 V for 50 minutes.

3.4.4.1.3 Membrane Transfer

Following electrophoresis, proteins were transferred to a nitrocellulose membrane, supplied pre-cut with filter paper (Novex® membrane/filter paper sandwiches; Invitrogen, UK). NuPage® transfer buffer (Invitrogen, UK) was then made up by adding 50 ml transfer buffer (20x) to 100 ml of methanol. The total volume was then made up to 1000 ml using deionised water. The membrane and filter papers were briefly soaked in transfer buffer prior to transfer.

The gel sandwich was then oriented so that the notched (well side) plastic plate was on top and the slotted plate underneath the gel. The plastic plates were separated using the gel knife and the upper plate discarded. The wells of the gel were then cut off using the gel knife. One piece of pre-soaked filter paper was then carefully placed on the gel so no air bubbles were trapped between the gel and the filter paper. The lower plate, gel and filter paper were then inverted and the gel pushed off the plate by pressing the foot of the gel out of the slot in the plate, and the lower plate discarded. The nitrocellulose membrane was then placed on top of the gel, again making sure that no air bubbles were trapped underneath, and the second piece of filter paper placed on top.

Two blotting pads were soaked in transfer buffer before being placed in the cathode (-) core of the Xcell II™ Blot Module. The gel/membrane/filter paper sandwich was then placed on these pads, making sure the gel was closest to the cathode core. Another two blotting pads (pre-soaked) were placed on top and the anode (+) core of the blotting module placed on top. The entire assembly was then placed into the buffer chamber using the guide rails and locked in place using the gel tension wedge. To the 1x transfer buffer, 1ml of antioxidant was added and mixed well before adding to the centre of the blot module. The buffer chamber was then filled with deionised water outside the blot module to dissipate heat, the chamber lid was put in place, and transfer performed using 30V constant for 90 minutes.

3.4.4.1.4 Protein staining

Following membrane transfer, the gel and membrane sandwich was removed and the orientation of the membrane marked so that the lane containing the protein ladder could be identified later. The membrane was then briefly stained with Ponceau S solution to confirm protein transfer. The membrane was placed in a shallow tray and approximately 5 ml of Ponceau S was added. Colour was allowed to develop for approximately 30 seconds before the membrane was washed with deionised water. Successful transfer was noted by visualising multiple red bands in each lane.

3.4.4.1.5 Blocking

Prior to staining, the membrane was blocked overnight in dried skimmed milk (5%) dissolved in 0.1% PBS – Tween at 4° C. Alternatively, the membrane was blocked at room temperature on a shaker for 1 hour.

3.4.4.1.6 Staining – primary antibody

After blocking, the blocking solution was discarded and the membrane was washed briefly in 0.1% PBS-Tween. In order to titrate the concentration of antibody required, for the initial experiment the

membrane was cut into strips, each strip containing a single lane. Each strip was then stained with antibody at one of the following dilutions: 1:100, 1:500, 1:1000, 1:2000. For all subsequent experiments, a 1:500 dilution of antibody was used with the whole membrane. The appropriate dilution of antibody was made up in 5% dried skimmed milk dissolved in 0.1% PBS-Tween. Five ml was then added to the membrane which was agitated either on a plate shaker or on a roller for 90 minutes at room temperature.

3.4.4.1.7 Staining – secondary antibody

Following initial staining, the membrane was then washed four times in 0.1% PBS-Tween for five minutes per wash. The secondary antibody, consisting of goat anti-mouse IgG conjugated to horseradish peroxidase (Amersham, UK) was then made up to a 1:5000 dilution using 5% dried skimmed milk dissolved in 0.1% PBS-Tween. Ten ml was added to the membrane and incubated for 60 minutes on a plate shaker at room temperature.

3.4.4.1.8 Visualisation

After staining with the secondary antibody, the membrane was washed four times with 0.1% PBS-Tween for five minues per wash. Excess liquid was drained from the membrane which was then placed on clingfilm. ECL-Plus (Amersham, UK) was made up by adding 125 µl to 5 ml of reagent which was then evenly distributed over the membrane and incubated for five minutes at room temperature. The membrane was then sealed within clingfilm and placed inside an autorad x-ray film cassette.

In a darkroom, x-ray film was placed inside the cassette together with the membrane and developed for 1 minute before being removed and processed using an automatic x-ray film processor. This was repeated with exposure times of 2 minutes, 5 minutes and 10 minutes. From these exposures, the most appropriately exposed film was then selected for analysis.

3.5 Results

3.5.1 Telomerase Activity

3.5.1.1 Telomerase Repeat Amplification Protocol analysis of positive and negative control cell lines

In order to confirm the telomerase status of the cell lines used as positive and negative controls, TRAP assays were performed on D17, CMT7, U87 and K9SF cell lines. TRAP results showed that D17, CMT7 and U87 cell lines were all positive for telomerase activity, whilst the K9SF cell line was found to be negative (Table 3.5).

3.5.1.2 Telomerase Repeat Amplification Protocol analysis of telomerase activity in normal canine brain

Tissue extracts obtained from the cerebellum and olfactory lobe of the canine brain were negative for telomerase activity. In contrast, tissue extracts prepared from the caudate nucleus were positive for telomerase activity (Table 3.6).

Table 3.5: TRAP Analysis of telomerase activity in cell lines used as positive and negative controls.

A_S is the absorbance of the sample, A_{S0} is the absorbance of the heat inactivated version of each sample, ΔA_S is the corrected absorbance of each sample (calculated by subtracting the mean of all heat inactivated samples ($\bar{A}_{S0}$)from A_S), $\Delta A_S/\bar{A}_{S0}$ is the ratio of sample to background activity and RTA is the telomerase activity relative to the positive control. Samples were considered telomerase-positive if the corrected absorbance (ΔA_S) was greater than twice the background activity, and if $\Delta A_S/\bar{A}_{S0}$ was greater than 2.0. Using these criteria, samples were designated positive (POS) or negative (NEG). The results shown are the mean of at least two experiments, and the assay gave a mean background absorbance of 0.049 and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 2.353 after 10 minutes of colour development using the high activity positive control.

Cell Line	A_S	A_{S0}	ΔA_S	$\Delta A_S/\bar{A}_{S0}$	RTA	Result
CMT7	0.39	0.07	0.32	4.0	1.7	POS
D17	0.24	0.07	0.17	2.1	0.9	POS
U87	2.61	0.10	2.51	24.0	10.2	POS
K9SF	0.18	0.15	0.03	0.0	0	NEG

Table 3.6: TRAP Analysis of samples taken from normal canine brain.

A_S is the absorbance of the sample, A_{S0} is the absorbance of the heat inactivated version of each sample, ΔA_S is the corrected absorbance of each sample (calculated by subtracting the mean of all heat inactivated samples ($\bar{A}_{S0}$)from A_S), $\Delta A_S/\bar{A}_{S0}$ is the ratio of sample to background activity and RTA is the telomerase activity relative to the positive control. Samples were considered telomerase-positive if the corrected absorbance (ΔA_S) was greater than twice the background activity, and if $\Delta A_S/\bar{A}_{S0}$ was greater than 2.0. Using these criteria, samples were designated positive (POS) or negative (NEG). The results shown are the mean of two samples, and the assay gave a background absorbance of 0.10 and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 2.597 after 10 minutes of colour development using the high activity positive control.

	A_S	A_{S0}	ΔA_S	$\Delta A_S/\bar{A}_{S0}$	RTA	Result
Olfactory Lobe	0.07	0.05	0.02	1.4	0.5	NEG
Cerebellum	0.08	0.04	0.03	1.6	0.6	NEG
Subventricular Zone	0.21	0.05	0.16	4.3	1.7	POS

3.5.2 Reverse transcriptase-PCR

RT-PCR analysis of U87, D17, CMT7 and K9SF cell lines was performed. The primer pair Ex2F/Ex2R was used to amplify a 200 bp fragment from exon 2 of the canine TERT gene in D17, CMT7 and K9SF cells. The primer pair DNHT001F/DNHT001R was used to amplify a 450 bp fragment of human TERT in U87 human cells. As a positive control, the CycloF/CycloR primer pair was used to amplify a 200 bp fragment of the canine cyclophilin gene in canine cell lines and the ActinF/ActinR primer pair was used to amplify a 200 bp fragment of the human beta actin gene in U87 cells. Agarose gel electrophoresis of PCR products revealed a 200 bp product in D17 and CMT7 cells, whilst K9SF cells had no such product. Similarly, a 200 bp product was amplified from U87 cells, confirming the existence of the hTERT transcript.

3.5.3 Immunohistochemistry

3.5.3.1 Antibody Evaluation

3.5.3.1.1 Antibodies

Three commercially available anti-hTERT antibodies were tested. Staining was performed with hTERT antibodies supplied by Oncogene (Anti-TERT, which recognises an internal 21 amino-acid sequence), Novocastra (NCL-hTERT, an antibody recognising a 147 amino-acid sequence near the N terminal region of hTERT), and Alpha Diagnostics (EST-22A, an antibody directed against a 21 amino-acid sequence found in the mid-region of hTERT) in formalin-fixed and paraffin-embedded human tissues by immunohistochemistry (IHC). Staining patterns observed using the Oncogene and Alpha Diagnostics antibodies were non-specific and revealed uniform cytoplasmic staining of all cells in positive and negative controls. No nuclear staining was visible and all cells were stained equally.

3.5.3.1.2 Staining Patterns

Only one antibody (NCL-hTERT; Novacastra) showed sufficient specificity in canine tissues and this antibody was used in all subsequent experiments. To determine whether the anti-hTERT antibody could recognise canine TERT protein, TERT immunoreactivity was first examined using the NCL-hTERT antibody in positive control D17, CMT7 and U87 cells and in canine testis tissue sections. K9SF cells were used as a negative control. In all telomerase-positive cell lines, TERT staining was restricted mainly to the nucleus (Figure 3.4A-B), although faint cytoplasmic staining was also observed. Staining was particularly prominent in nucleoli, and in some cells nucleolar staining was the only detectable pattern. In other cells, staining was distributed throughout the

nucleus, either in a punctuate/granular pattern or diffusely. Similarly, in normal canine testis tissues staining was confined to the germinal cells of the sperminiferous tubules (Figure 3.4D). In contrast, no immunoreactivity was detected in the negative controls (Figure 3.4C).

3.5.3.1.3 Antibody Titration

Staining using the NCL-hTERT antibody was performed with the following dilutions: 1/50, 1/100, 1/200, 1,500, 1/1000, 1/10,000, 1/20,000, 1/40,000 in sections of canine testis. At dilutions of 1/200 and below, strong nuclear and nucleolar staining was seen but some background staining of cells was also observed. At dilutions above 1/500, staining became very faint. At 1/500 dilution, staining was restricted to the nucleus, with occasional nucleolar staining. For this reason, staining of all subsequent sections was performed with a 1/500 dilution of the antibody.

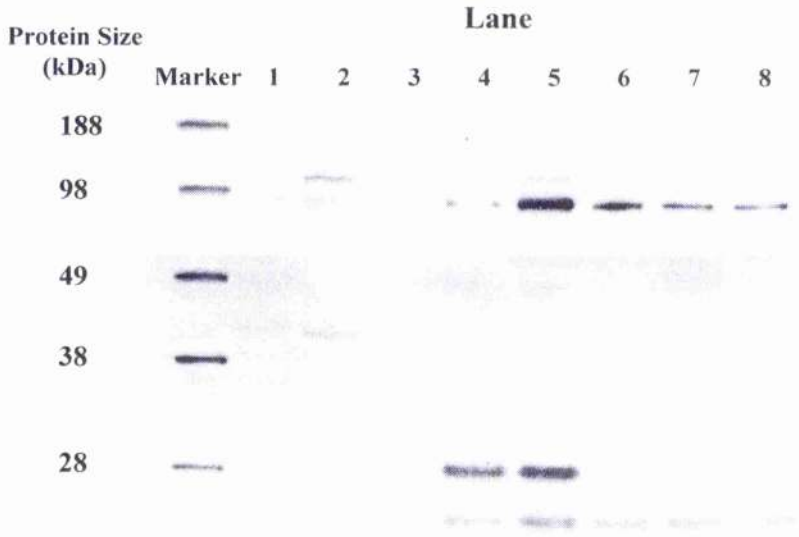
3.5.3.1.4 Western Blotting

Western blotting was performed on samples obtained from the olfactory lobe, subventricular zone (in the region of the caudate nuclei) and the cerebellum of a normal dog. In addition, Western blotting was performed on the TRAP-positive cell lines D17, CMT7 and U87, as well as the TRAP-negative K9SF cell line.

Staining of blots with the NCL-hTERT antibody revealed a number of bands in all samples (see Figure 3.3). The largest band was present in all samples, approximately 100 kDA in size. However, no bands of 127 kDa were present.

Figure 3.3: Western Blots

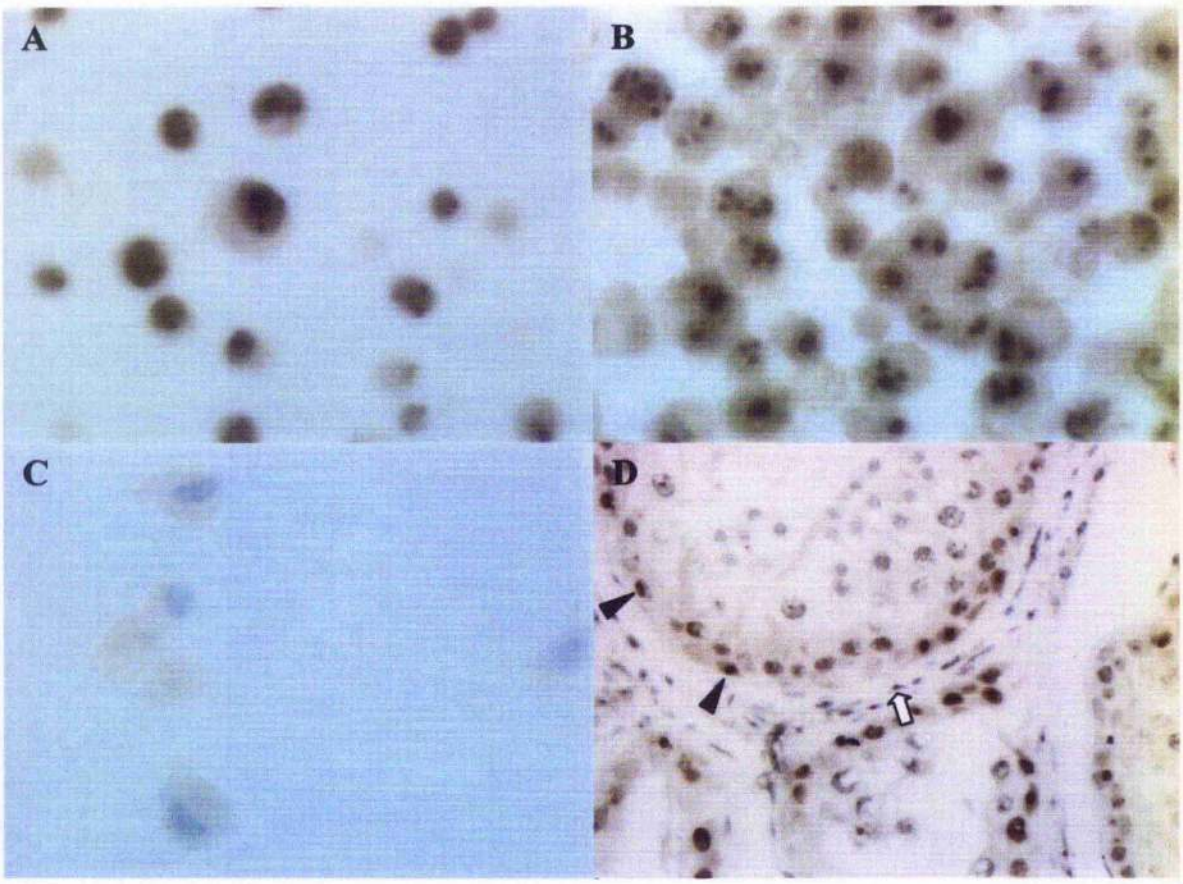
No clear band was identified approximately 127 kDa in size (the expected size of the canine TERT protein) in any lane. Strong bands approximately 100 kDa in size were detected in the CMT7, D17, U87 and K9SF cell lines but not in normal brain. A slightly larger, weak band was detected in samples derived from the subventricular zone of the normal brain and in CMT7 and D17 samples, but not in U87 samples.



Sample Details:

Lane	Sample
1	Normal brain: Olfactory lobe
2	Normal brain: Subventricular Zone
3	Normal brain: Cerebellum
4	D17 Cell Line
5	CMT7 Cell Line
6	U87 Cell Line
7	K9SF Cell Line
8	K9SF Cell Line

Figure 3.4: Immunohistochemical staining of TERT in positive and negative controls



A: D17 Cells, TERT staining. Note intense nucleolar staining, diffuse strong nuclear staining and weak cytoplasmic staining. X 400 Magnification.

B: CMT7 Cells, TERT staining. Note similar staining pattern to D17 cells but with weaker nuclear staining. X 400 Magnification.

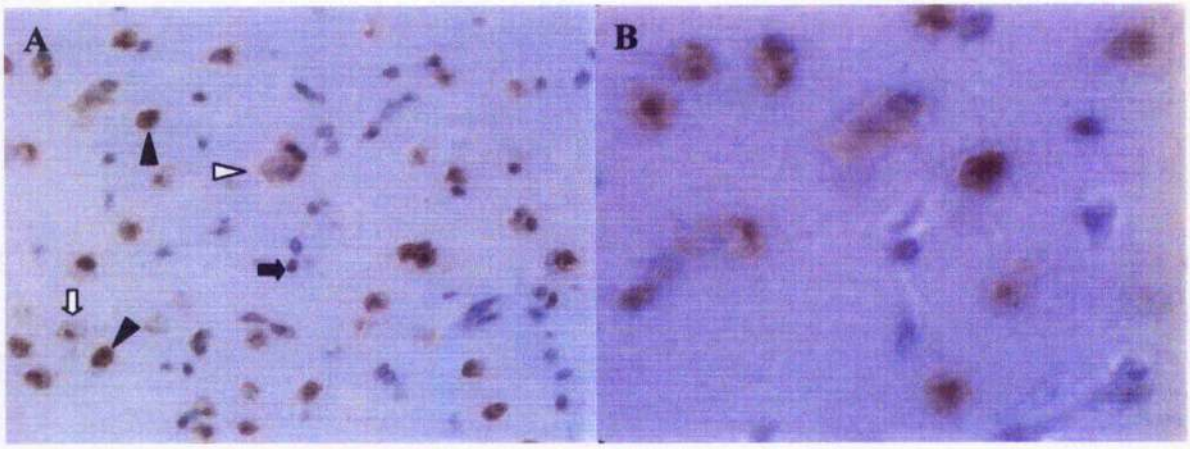
C: K9SF Cells, TERT staining. Note negative nuclear staining. X 400 Magnification.

D: Normal canine testis, TERT staining. Note germinal layer with diffuse nuclear staining (arrowheads) but progressive loss of staining of cells with further differentiation. In addition, some cells in interstitial areas (white arrow) were found to have isolated nucleolar staining. X 100 Magnification.

3.5.3.2 Immunohistochemical detection of TERT in the normal canine brain

To determine whether TERT protein is expressed in normal canine brain, immunohistochemistry was performed on brain sections of the olfactory lobe, subventricular zone at the level of the caudate nuclei, and the cerebellum. Some cells were found to stain positively in all sections of the brain, largely restricted to neuronal cells (Figure 3.5A-D). In the cerebellum, Purkinje cells appeared to be the only cell type stained positively (Figure 3.5E). In general staining appeared to be confined to nuclei of cells. In Purkinje neurons, nucleoli were not found to stain positively. The region of the brain that showed greatest staining was the subventricular zone, in the region of the caudate nucleus, with large numbers of neurons and glial cells staining positively (Figure 3.5A-B).

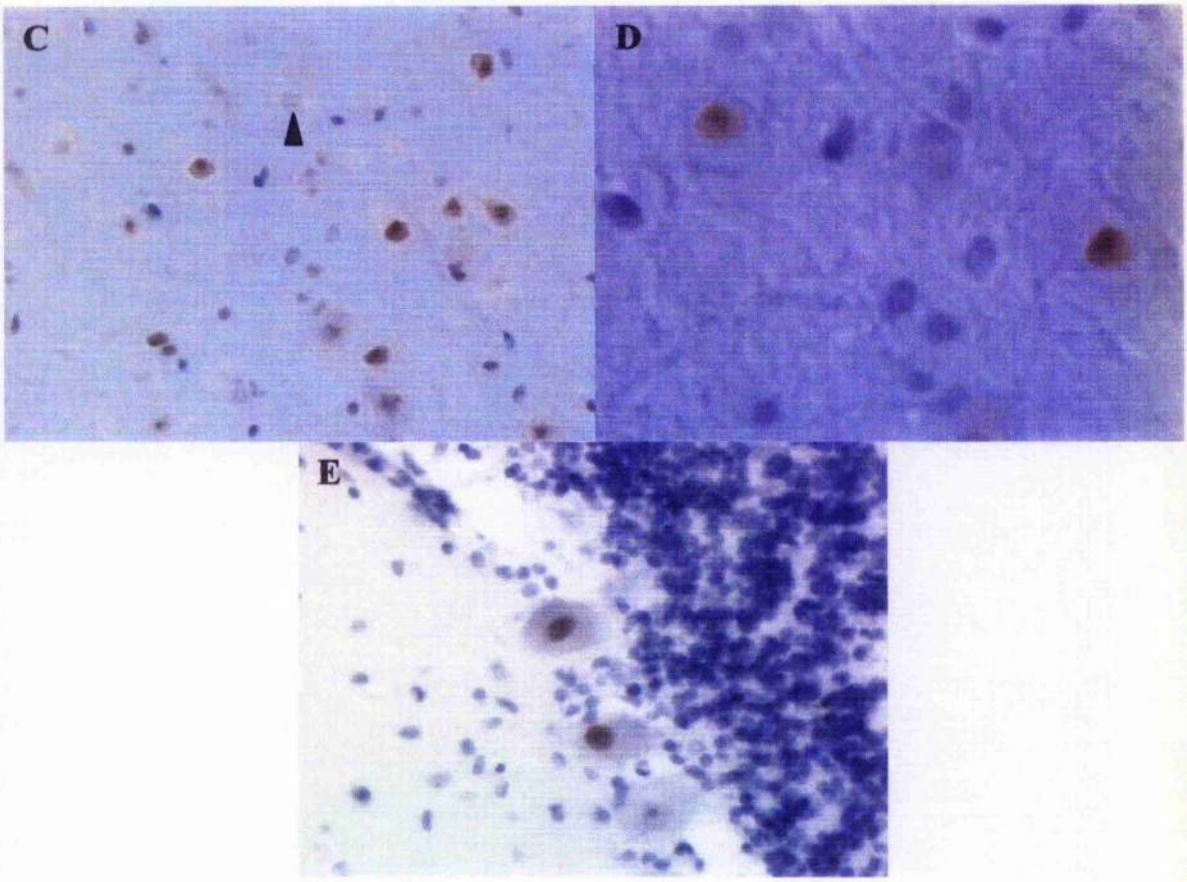
Figure 3.5: TERT staining of normal canine brain



A: Normal canine brain, TERT staining – subventricular zone. Note large number of positively stained cells. The majority of these cells are neurons, displaying a mixture of diffuse nuclear and nucleolar staining (black arrowheads), nucleolar staining only (white arrow) and cytoplasmic staining (white arrowhead). However, some smaller cells, comprising oligodendroglia and astrocytes, also display positive staining (black arrow). x 200 Magnification

B: Normal canine brain, TERT staining – subventricular zone. Higher power view of A showing neuronal staining. x 400 magnification.

Figure 3.5 (cont)



C: Normal canine brain, TERT staining – olfactory lobe. Note lower number of TERT-positive cells, all of which are neurons, and some negatively staining neurons (arrowhead).
x 200 magnification.

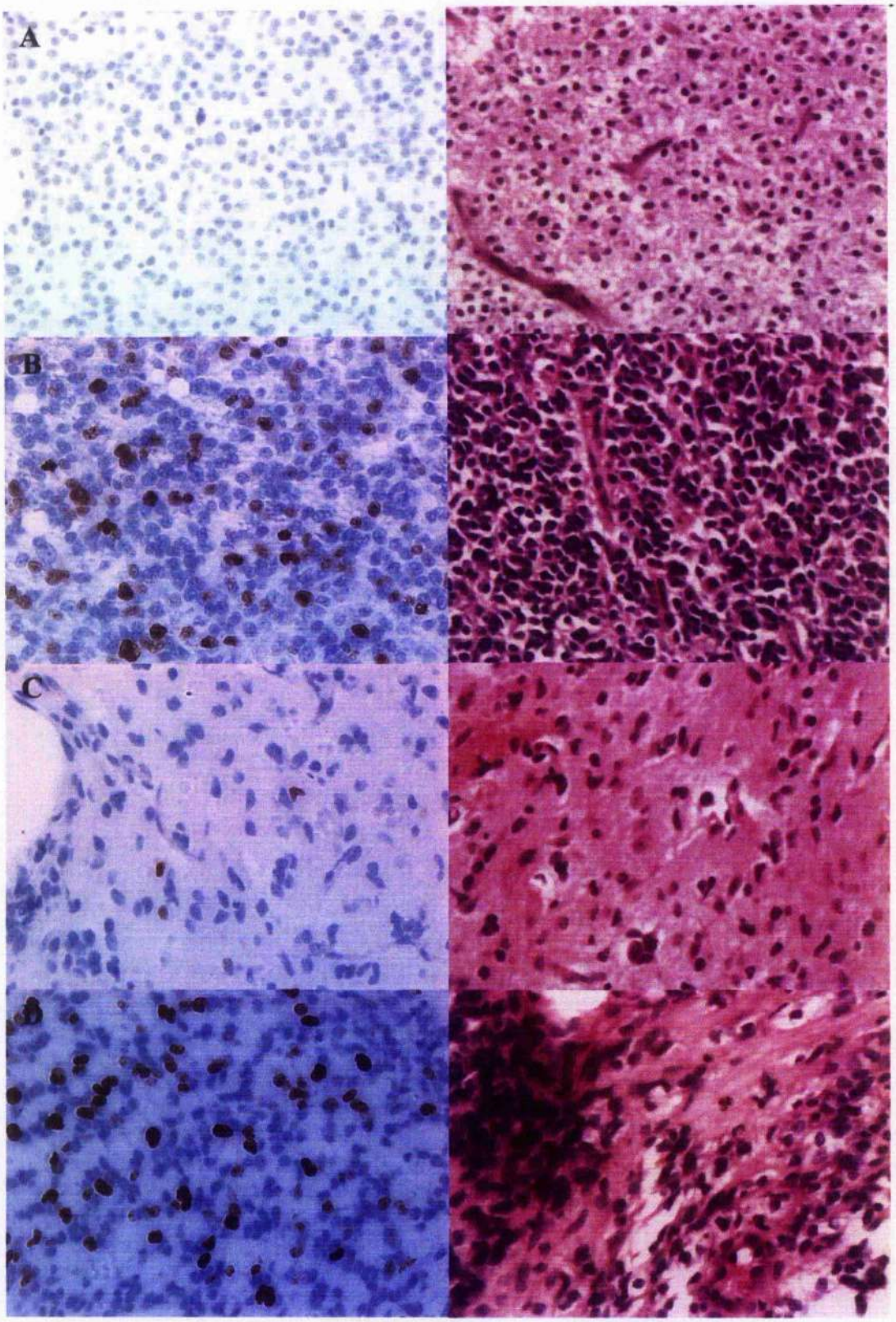
D: Normal canine brain, TERT staining – olfactory lobe. Higher power view of C showing neuronal staining. x 400 magnification.

E: Normal canine brain, TERT staining – cerebellum. Note positive staining of Purkinje neurons only, with adjacent granule cells staining negatively. x 400 magnification.

3.5.3.3 Validation of Ki-67 Staining

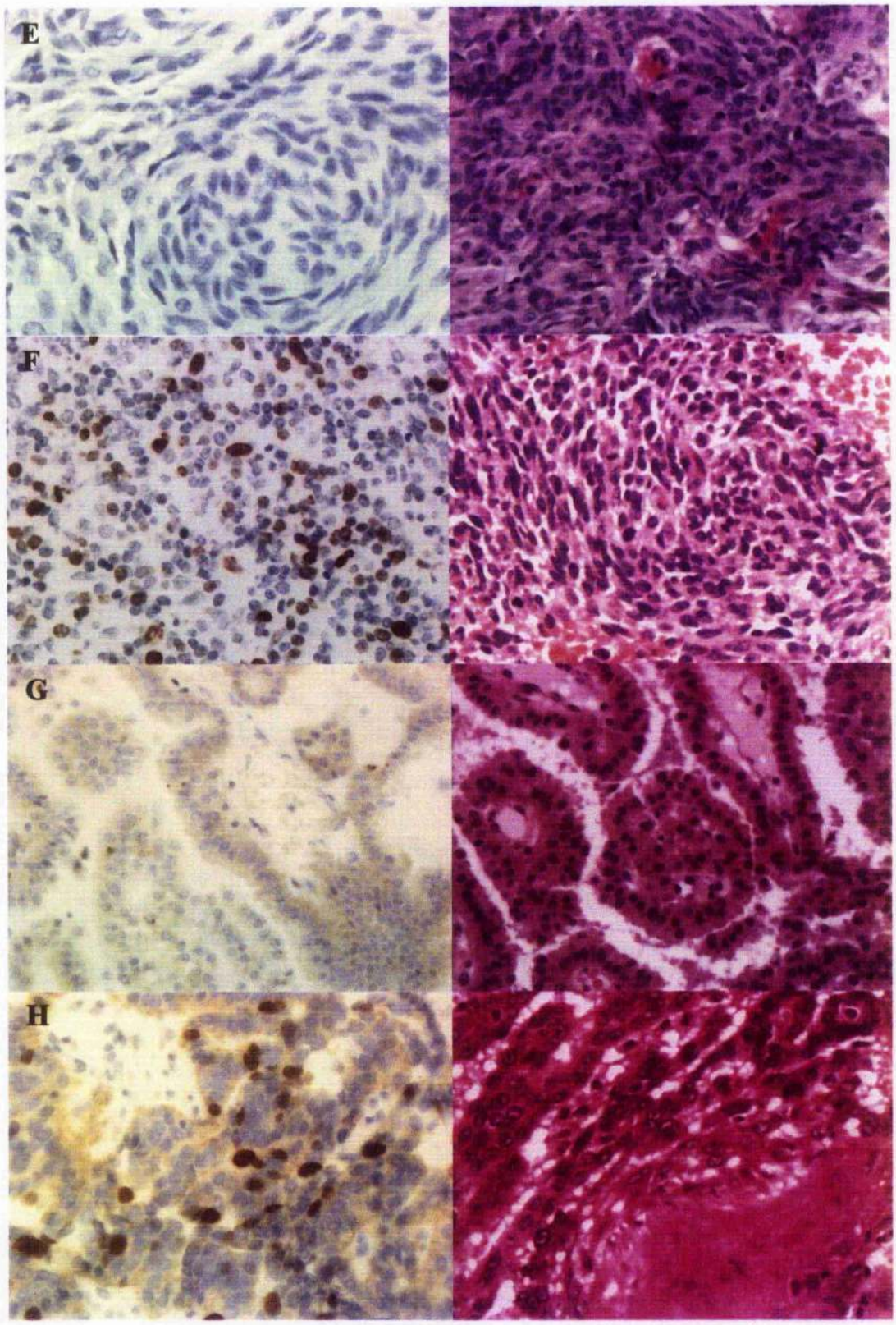
Immunoreactivity for the Ki67 antigen was exclusively confined to the nucleus with no evidence of cytoplasmic staining. Staining was strong and diffuse throughout the nucleus. Whilst normal cells showed no positivity, proliferating cells stained strongly positive for Ki-67 in all tumour types examined. Examples of staining are shown in Figure 3.6. In those tumours stained for Ki-67, MIB-1 LI correlated significantly with tumour grade, with a mean MIB-1 LI of 1.5% for grade 1 tumours, as compared with a mean MIB-1 LI of 21.7% for grade 2 tumours ($p<<0.0001$). The highest MIB-1 LI for all grade 1 tumours was found to be 8.9%, which was also the lowest MIB-1 LI for grade 2 tumours (Figure 3.7, Table 3.8).

Figure 3.6: Examples of MIB-1 staining in canine brain tumours. Right: Corresponding H&E sections



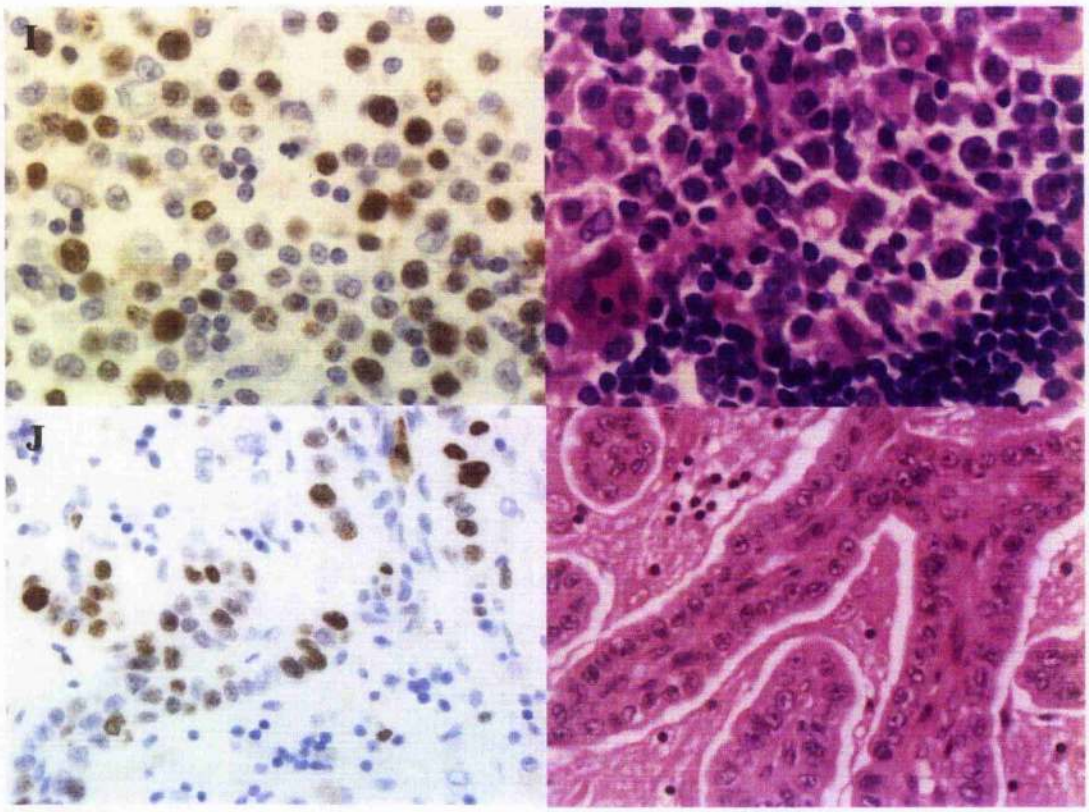
A: Oligodendroglioma. MIB-1 LI 0.0% x 200 magnification
B: Anaplastic oligodendroglioma. MIB-1 LI 25.0% x 200 magnification
C: Astrocytoma. MIB-1 LI 3.2% x 200 magnification
D: Glioblastoma multiforme. MIB-1 LI 26.1% x 200 magnification

Figure 3.6 (cont).



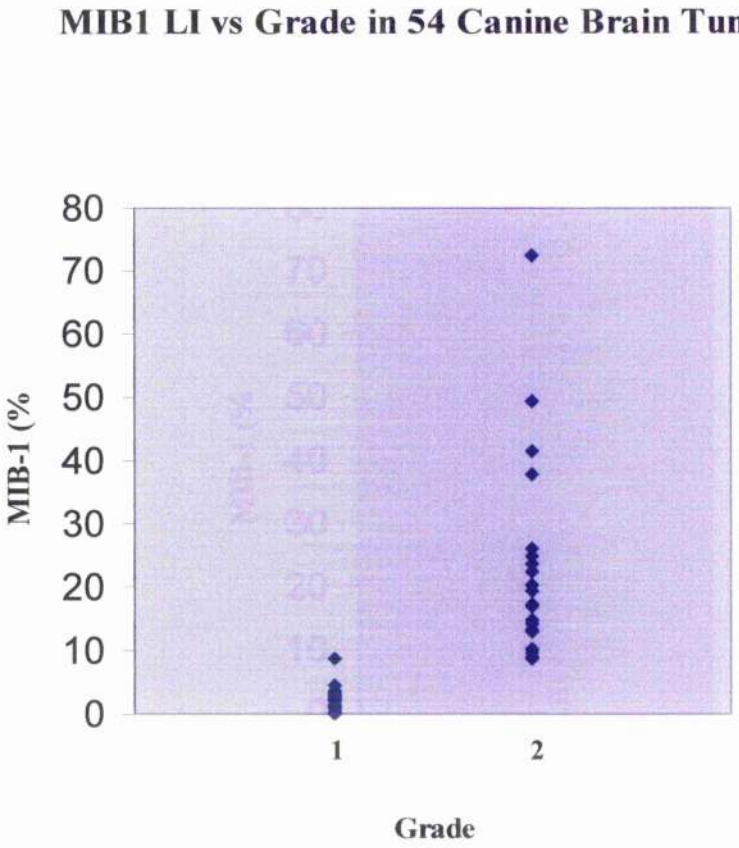
E: Meningioma. MIB-1 LI 0.0% x 200 magnification
F: Anaplastic meningioma. MIB-1 LI 14.8% x 200 magnification
G: Choroid plexus papilloma. MIB-1 LI 3.0% x 200 magnification
H:Choroid plexus carcinoma. MIB-1 LI 9.2% x 200 magnification

Figure 3.6 (cont).



I: Metastatic Lymphoma. MIB-1 LI 49.3% x 400 magnification
J: Metastatic carcinoma. MIB-1 LI 10.4% x 200 magnification

Figure 3.7: Graph showing MIB-1 LI vs grade in 51 canine brain tumours

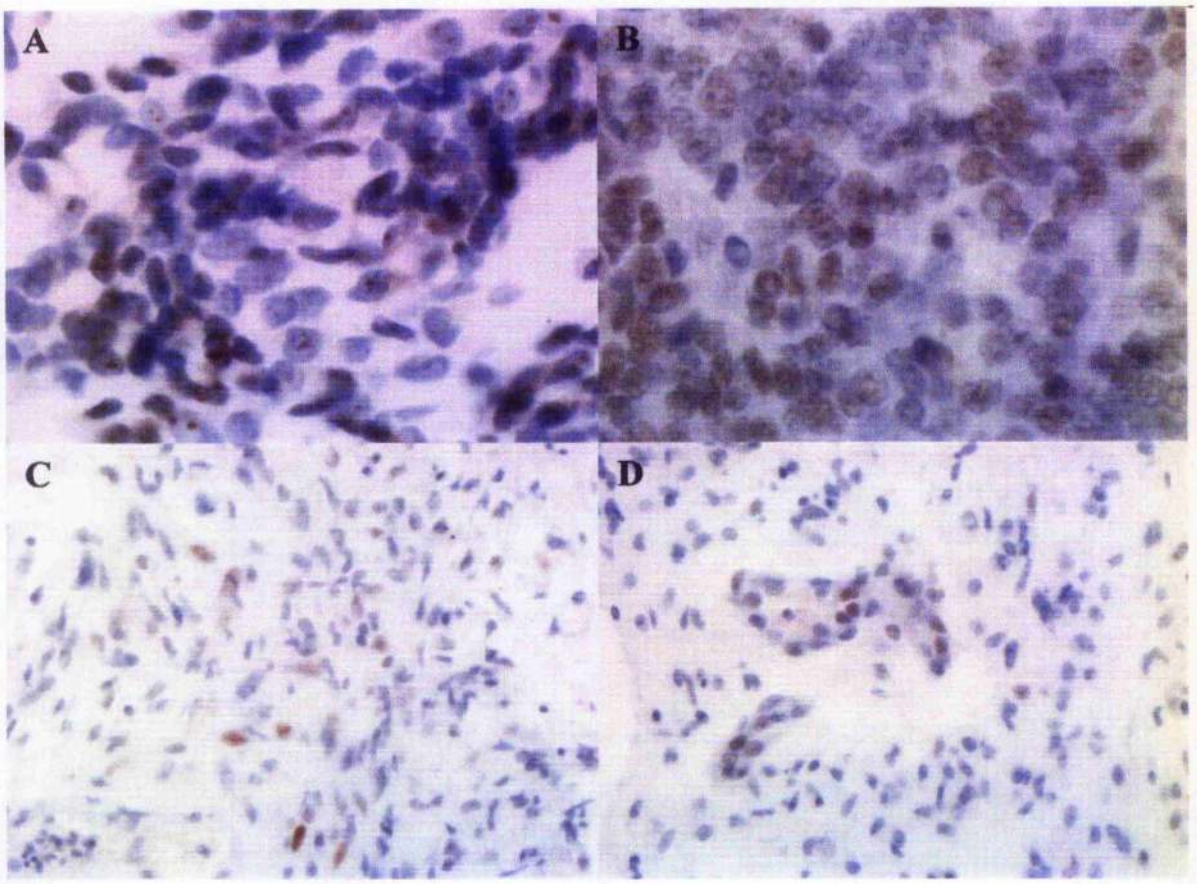


3.5.3.4 TERT staining in canine brain tumours

In general TERT staining in canine brain tumours appeared to be remarkably similar to that described in human tumours. The staining pattern seen varied between tumours, with discrete nucleolar staining in some tumours, and more diffuse nuclear staining in others. The strength of this staining also appeared to vary, with some tumours showing only faint nuclear staining and other others very strong staining. Some examples of this staining are shown in Figure 3.8.

There appeared to be no consistent staining pattern associated with particular tumour types, although some observations can be made. Overall, oligodendrogliomas and meningiomas tended to show diffuse staining patterns, with almost the entire tumour either staining positively or negatively (Figure 3.8B). Astrocytomas, on the other hand, tended to show more patchy staining patterns, with some areas focally positive, and with individual cells showing positive staining (Figure 3.8C). In addition, in some glioblastomas, TERT staining was not restricted to tumour cells but was also found to be present in areas of vascular endothelial hyperplasia (Figure 3.8D). In these areas, endothelial cells stained positively, either with nucleolar staining or with a more diffuse nuclear localisation. In addition, there appeared to be some background cytoplasmic staining in these cells. Cytoplasmic staining was also found in choroid plexus papillomas and carcinomas, both of which tumour types contain cells of endothelial origin.

Figure 3.8: TERT staining in canine brain tumours



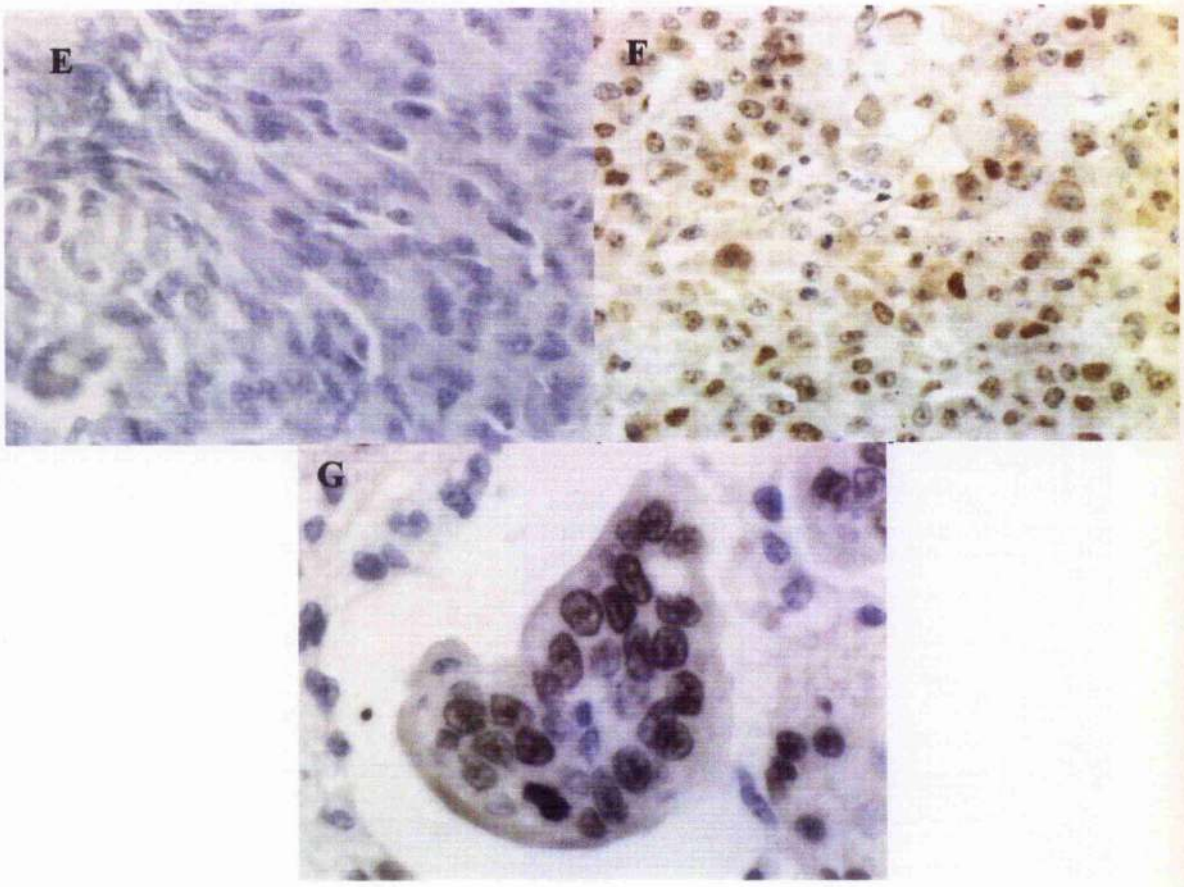
A: Anaplastic astrocytoma, TERT staining. Note discrete nucleolar staining with little nuclear staining. X 400 magnification.

B: Anaplastic oligodendroglioma, TERT staining. Note that the vast majority of cells in this section exhibit diffuse nuclear staining. X 200 magnification.

C: Glioblastoma multiforme, TERT staining. Note the focus of positive-staining cells within a predominantly negative-staining field. X 200 magnification.

D: Glioblastoma multiforme, TERT staining. Note the central vascular endothelial cells with positive-staining cells. X 200 magnification.

Figure 3.8 (cont.)



E: Meningioma, TERT staining. Note absence of immunoreactivity X 200 magnification.

F: Anaplastic meningioma TERT staining. Note mixture of cytoplasmic and nuclear staining. X 200 magnification.

G: Metastatic adenocarcinoma, TERT staining. X 400 magnification.

3.5.3.5 Correlation between TERT and Ki67 staining in canine brain tumours

A total of 93 canine brain tumour sections were evaluated for TERT staining, of which 50 were classified as Grade 1 and 43 as Grade 2 (Table 3.7). Positive immunostaining for TERT was evident in 16 of 50 Grade I tumours (32%) compared to 29 of 43 Grade 2 tumours (67%), showing a significant association with histological grade (p=0.00012) (Figure 3.9, Table 3.9). Similarly TERT staining was found to correlate with malignancy in meningioma and oligodendroglioma tumour subtypes (p=0.01 and p=0.004 respectively), but no significant association was evident for the remaining subtypes (Figure 3.10).

Of these tumours, 51 were also available for MIB-1 staining, of which 26 were classified as Grade 1 and 25 as Grade 2 (Table 3.8). When TERT staining was compared with MIB-1 LI, a significant association was also found between the mean MIB-1 LI value and TERT expression in brain tumours overall ($p << 0.0001$) and in the meningioma and oligodendroglioma subtypes ($p = 0.002$ and $p = 0.007$ respectively) (Table 3.9, Figure 3.10). A trend for association between TERT staining and MIB-1 LI was obvious in other tumour subtypes, although this was not significant (Figure 3.10, Table 3.9).

Table 3.7: Diagnosis, grading and TERT staining in 93 brain tumours

Tumour Type	Grade	No.	No. TERT +ve	P Value
Astrocytomas				
Astrocytoma	1	5	0	
Astrocytoma anaplastic	2	5	3	
Astrocytoma glioblastoma	2	4	2	
		14	5 (36%)	0.10
Meningiomas				
Meningioma	1	21	8	
Meningioma anaplastic	2	6	4	
		27	12 (44%)	0.008
Oligodendrogliomas				
Oligodendroglioma	1	17	7	
Oligodendroglioma anaplastic	2	10	8	
		27	15 (56%)	0.004
Choroid plexus tumours				
Choroid plexus papilloma	1	6	0	
Choroid plexus papilloma: anaplastic	2	1	1	
Choroid plexus papilloma: carcinoma	2	1	0	
		8	1 (13%)	0.19
Other				
Ependymoma	1	1	1	
Gliosarcoma	2	1	1	
Medulloblastoma	2	1	1	
Pituitary adenocarcinoma	2	1	1	
		4	4	N/A[a]
Metastases				
Adenocarcinoma	2	6	4	
Lymphoma	2	7	4	
		13	8 (61%)	N/A[a]
All tumours				
Grade 1		50	16	
Grade 2		43	29	
		93	45	0.00012

a Not applicable due to insufficient numbers in group

Table 3.8: Diagnosis, grade, MIB-1 LI and TERT staining in 51 canine brain tumours

Tumour Type	Grade	No.	Mean MIB-1 LI (%)	MIB-1 Range (%)	TERT +ve	P value
Astrocytomas						
Astrocytoma	1	1	3.2	3.2	0	
Astrocytoma anaplastic	2	2	11.6	8.9-14.2	1	
Astrocytoma glioblastoma	2	2	22.9	19.6-26.1	1	
		5			2	0.44
Meningiomas						
Meningioma	1	10	0.0	0-2.0	0	
Meningioma anaplastic	2	5	19.3	13.0-41.4	4	
		15			4	0.0015
Oligodendrogliomas						
Oligodendroglioma	1	10	2.5	0.-8.9	2	
Oligodendroglioma anaplastic	2	9	22.3	9.2-37.8	8	
		19			10	0.007
Choroid plexus tumours						
Choroid plexus papilloma	1	5	0.8	0-0.3	0	
Choroid plexus papilloma: carcinoma	2	1	9.2	N/A	0	
		6			0	0.19
Other						
Gliosarcoma	2	1	26.1	N/A[a]	1	
Pituitary adenocarcinoma	2	1	16.9	N/A[a]	1	
		2			2	N/A[a]
Metastases						
Lymphoma	2	3	47.1	19.4-72.5	2	
Adenocarcinoma	2	1	19.4	N/A[a]	0	
		4			2	N/A[a]
All tumours						
Grade 1		26	1.5	0-8.9	2	
Grade 2		25	21.7	8.9-72.5	18	
Total		51			20	<<0.001

a Not applicable due to insufficient numbers in group

Figure 3.9: Graph showing TERT staining vs Malignancy in 93 canine brain tumours

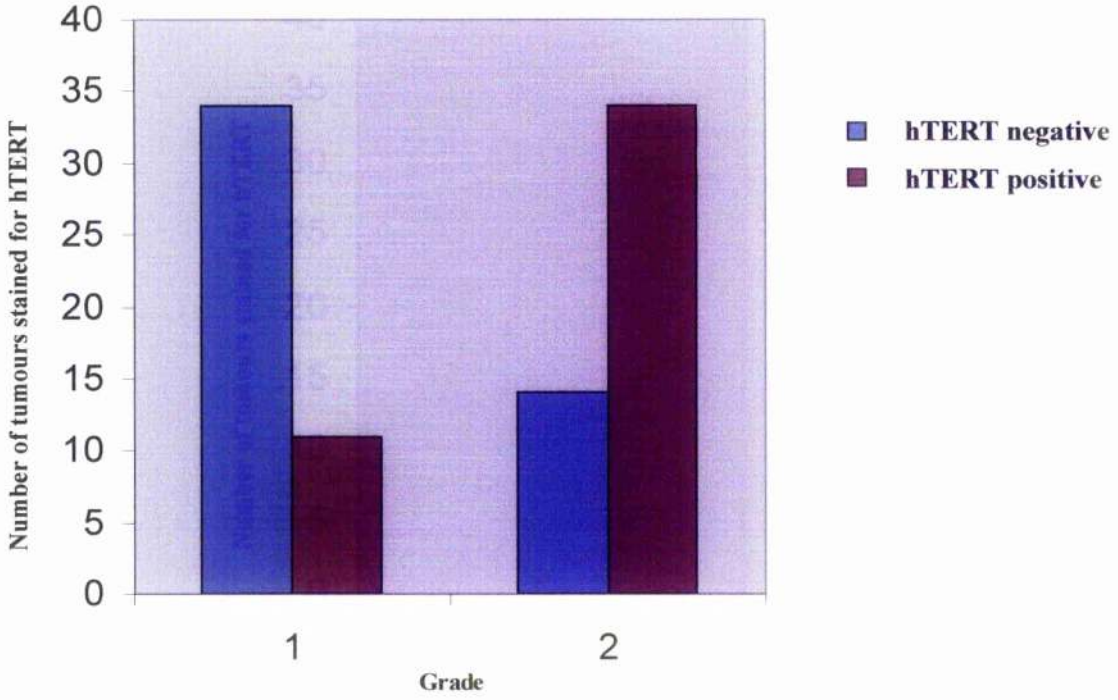
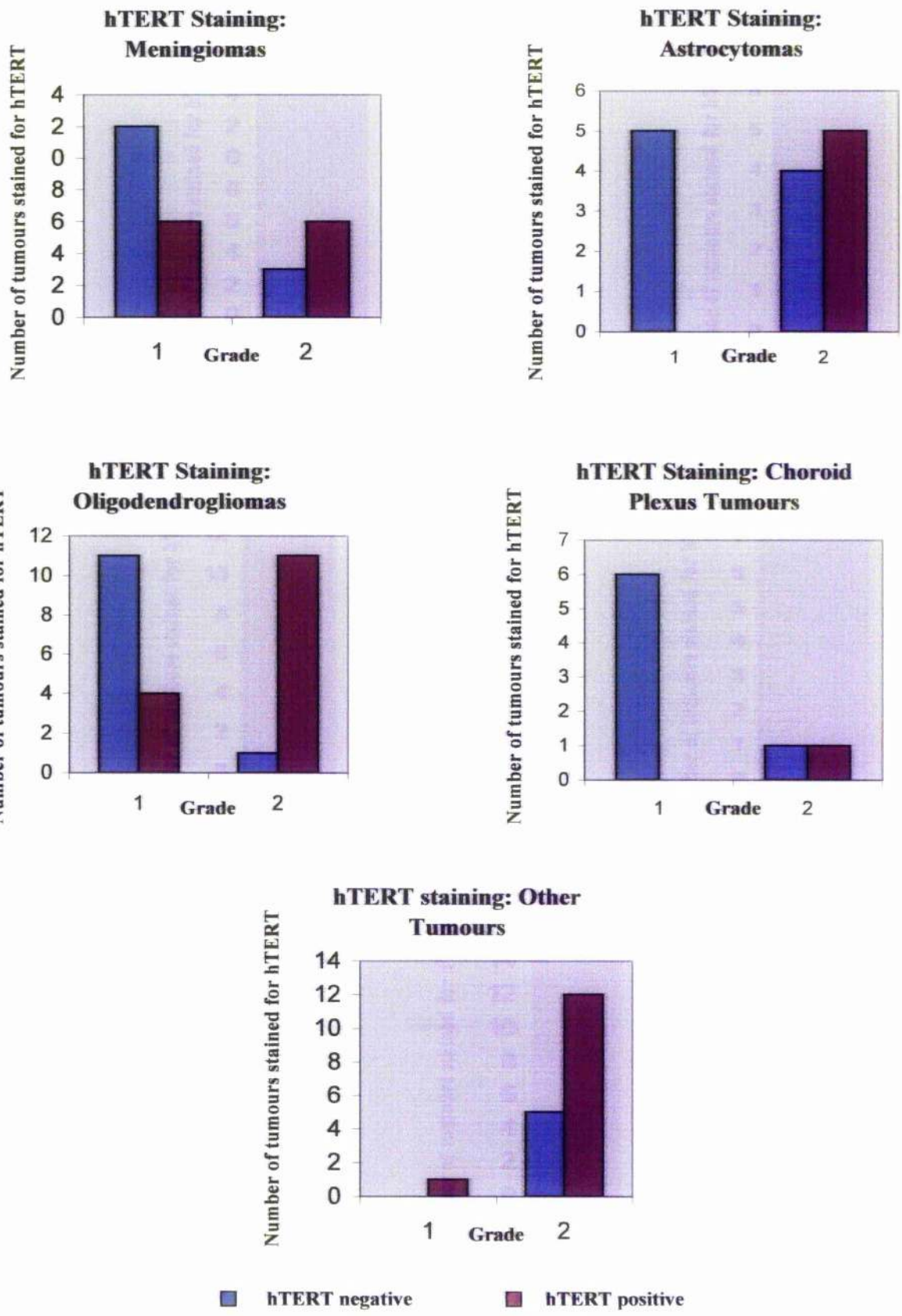


Table 3.9: Mann-Whitney U Test Calculations – TERT staining vs Malignancy. P values are highlighted in bold

	Tumour type					
	Meningioma	Astrocytomas	Oligodendrogliomas	Choroid plexus tumours	Other tumours	All tumours
TERT negative	14	9	15	7	12	48
TERT positive	6	5	12	1	5	45
n1	14	9	15	7	12	48
n2	6	5	12	1	5	45
U	78	35	148.5	6.5	32.5	1581
normal: z=	2.55	1.67	2.85	1.30	0.26	3.85
P (2 tail)	0.013	0.11	0.00	0.25	0.80	0.00
	0.01	**0.10**	**0.00**	**0.19**	**0.79**	**0.00**
P (1 tail)	0.00	0.06	0.00	0.13	0.40	0.00
	0.01	0.05	0.00	0.10	0.40	0.00

Figure 3.10: Immunohistochemical hTERT staining vs Malignancy in 93 brain tumours by subtype



3.6 Discussion

The primary aims of this chapter were to examine and validate the use of commercially-available hTERT antibodies in canine paraffin-embedded tissues. In addition, the secondary aim of the chapter was to validate the Ki-67 proliferation marker in canine brain tumours, and then to correlate proliferation with telomerase activity in canine brain tumours. Analysis of three commercially available antibodies in telomerase-positive and negative cell lines and in canine testis showed that the novocastra antibody NCL-hTERT specifically stained telomerase-positive cells and tissues at a dilution of 1/200.

3.6.1 Antibody Validation

In general, staining with the hTERT antibody in canine tissues correlated well with TRAP and RT-PCR results, with all TRAP-positive cell lines revealing strong TERT staining, and TRAP-negative cell lines showing no staining. However, Western blot analysis failed to demonstrate the presence of specific binding of NCL-hTERT in telomerase-positive samples. Instead, the NCL-hTERT antibody detected a 100 kDa band in all samples, irrespective of telomerase activity (Figure 3.3). Since performing this work, the NCL-hTERT antibody has also been found to recognise a 100 kDa band in canine cells exogenously expressing wild-type hTERT (I. Nicholson, unpublished data). A slightly larger, faint band was detected in samples obtained from the subventricular zone, D17 and CMT17 cells, but not in U87 cells. In addition, a number of smaller bands were present in some samples, in particular a strong 28 kDa fragment in D17 and CMT7 cells, and a smaller fragment was present in all samples with the exception of those obtained from the normal brain. A single faint band approximately 40 kDa in size was also detected in samples obtained from the subventricular zone that was not present in any other samples.

Several reasons could explain the lack of a single 127 kDa band on Western analysis. Firstly, technical issues with the process of Western blotting could be involved. The use of Sodium Dodecylsulphate (SDS) linearises the proteins in the sample and coats them with a negative charge. In addition, the presence of reducing agents (DTT) reduces the disulphide bonds between peptides. This denaturation alters the three-dimensional structure and in some cases may also alter the antigenicity of the protein. Some antibodies will only recognise the native, unreduced antibody, and this may be one of the reasons the hTERT antibody did not recognise TERT in our samples. However, as non-denatured proteins often migrate anomalously through the SDS-PAGE gel used, it may be difficult to estimate the size of any bands detected by the antibody in a non-denatured state. Another possibility that may explain the presence of bands smaller in size than expected is proteolytic degradation of the sample prior to electrophoresis. The presence of proteases in samples can lead to proteolysis of proteins, and this can be avoided through the use of protease

inhibitors included during sample preparation. Although protease inhibitors were included in our sample preparation, it is possible that some proteolysis still occurred.

An alternative reason for multiple bands in the samples examined is the presence of alternately-spliced variants of the TERT transcript. The NCL-hTERT is raised against a 147 peptide sequence located at the amino terminal end of hTERT. This region does not contain any reported splice sites, and so the antibody would be expected to cross-react with all of the splice variants known. Thus, the NCL-hTERT antibody does not distinguish between catalytically and catalytically inactive hTERT. However, the presence of the same 100 kDa band in U87 cells as well as all canine cell lines would not be explained by alternate splicing, as splice sites would be expected to be different in dogs as compared to humans.

Another reason for the lack of a 127 kDa-sized band in our samples is the possibility that insufficient TERT protein was present in our samples for antibody detection. It is well known that only a small number of copies of the hTERT mRNA transcript (3-4) are present in any given telomerase-positive human cell (Yi *et al.* 2001). For this reason it may be that the TERT protein is equally scarce within telomerase-positive cells. In order to investigate this possibility, protein-concentrating or TERT-concentrating techniques such as immunoprecipitation techniques may help to clarify this issue by increasing the number of copies of TERT molecules present prior to Western blotting.

Another unexpected finding when Western blots were examined was the presence of smaller bands in all samples. It is possible that the NCL-hTERT antibody was detecting alternative antigens in addition to hTERT itself, suggesting that it was not entirely specific for hTERT. However, given the conformational changes undergone by samples when reducing SDS-PAGE is performed, it is unknown whether the peptides would also be detected by NCL-hTERT in a natural state. Alternatively, the fact that proteolytic digestion may have occurred prior to electrophoresis raises the possibility that these fragments are partially-digested forms of hTERT.

3.6.2 TERT staining patterns

NCL-hTERT staining of positive controls produced results that are similar to those reported by other authors reporting hTERT staining. In particular, the strong nucleolar localisation of hTERT has been reported by other studies investigating the NCL-hTERT antibody (Maes *et al.* 2005; Smith *et al.* 2004). This would make sense given that it has been shown that hTERT can be concentrated in the nucleolus, and that functional assembly of telomerase occurs there (Smith *et al.* 2004). However, after the hTERT mRNA is transcribed, it is transported to the cytoplasm where it is translated into protein before being phosphorylated and re-entering the nucleus and locating to the nucleolar region. For this reason, cells that are producing TERT in large quantities could be

expected to show in the nucleus and possibly the cytoplasm, in addition to the nucleolus. It is also likely that cells with lower levels of TERT expression may only show nucleolar staining, as was found in some cells in sections of the canine testis (Figure 3.4). Additionally, some authors have suggested that the speckling pattern seen within the nucleus could be the presence of the active telomerase complex at the chromosome ends (Maes *et al.* 2005).

3.6.3 TERT staining of the normal canine brain

An unexpected finding was the significant staining of cells in the canine brain. The number of nuclei stained positively in the subventricular zone was considerably greater than in other areas of the brain. In this area, predominantly astrocytes and neurons stained positively, whilst in other areas staining was restricted to neurons. In the cerebellum, the only cell type observed to stain strongly was the Purkinje neuron, whilst the adjacent granule cells did not stain with NCL-hTERT. Interestingly, the nucleoli of Purkinje neurons were found not to take up stain, whilst the rest of the nucleus stained diffusely (with no obvious speckling).

The staining pattern in the subventricular zone correlated with TRAP results, with this part of the brain showing weak telomerase activity. In itself this is an interesting finding, as the brain contains predominantly populations of terminally differentiated cells that in general are not able to undergo further proliferation. However, there is increasing evidence to suggest that even in the brain populations of stem cells and precursor cells exist, which may be stimulated to divide. In fact, the subventricular zone is now known to be one area in which these stem cells reside, and in mice this part of the brain does possess telomerase activity, which may account for this finding (Caporaso *et al.* 2003). However, this does not explain the staining seen in cells from other parts of the brain. Perhaps the simplest explanation for this is that the NCL-hTERT antibody cross-reacts with a similar epitope within neurons in the canine brain, and it would be very hard to disprove this possibility. Alternatively, as may be the case with Western analysis, it may be that the hTERT antibody is recognising a splice variant of TERT that is non-functional. At this point, it is not known whether splice variants of TERT exist within canine neurons, but an important further study would be to determine whether this is the case. Thirdly, it is possible that telomerase activity can be upregulated in neurons, under some circumstances. For instance, telomerase can be found in neurons in mice following hypoxia induced by middle cerebral artery occlusion, and overexpression of TERT protects these neurons from hypoxic damage (Kang *et al.* 2004). Telomerase has also been shown to promote survival of neurons in the brain of mice under other circumstances (Fu *et al.* 2000; Lu *et al.* 2001). For this reason, it is possible that telomerase expression is inducible in the brain under situations of stress, and that this may occur in canine brains. However, given the lack of telomerase activity in parts of the brain other than the subventricular zone as determined by TRAP assay, this seems an unlikely explanation for the staining pattern seen in this case. Finally, an important caveat to the findings so far described is

that at this stage only a single canine brain has been examined for telomerase activity and TERT staining, and an important first study required would be to determine by TRAP assay whether these results are also found in these and other areas of normal brains of other dogs. If TRAP assay results can be shown to correlate with TERT staining in the brain, it would be interesting to examine TERT staining in other pathological states such as hypoxia and cerebral oedema.

3.6.4 Validation of Ki-67

Our results correlating Ki-67 staining in canine brain tumours suggest that it provides a useful marker of proliferation. Our results are consistent with results described in human brain tumours. In our study, a MIB-1 LI of approximately 9% appeared to be the cutoff between grade 1 and grade 2 tumours (Figure 3.7). In general, the majority of grade 1 tumours had a low MIB-1 LI, usually less than 4%, as is seen in the majority of benign human brain tumours. One interesting finding in our study was that two of the grade 2 meningiomas had a MIB-1 LI of >20%. However, three had a substantially lower MIB-1 of between 9 and 14%. In humans, the WHO classification includes three grades of meningioma: the benign, atypical and anaplastic variants (Kleihues & Cavenee 2000). Of these, the atypical variant has been reported to have a MIB-1 LI of less than 20% whilst the anaplastic variant has a MIB-1 LI of greater than 20% (Kleihues & Cavenee 1999). It is possible that in dogs a third, atypical variant may be present based on a proliferative index of less than 20%, as described in people, although current WHO grading systems only describe two variants. Further work correlating survival with grading would be needed to determine this, however. Overall, it would appear that the MIB-1 LI for most canine brain tumours is similar to that seen in human brain tumours.

3.6.5 TERT staining in canine brain tumours

In general, the staining pattern seen in canine brain tumours appeared to be similar to that described in humans. However, some discrepancies were apparent in the proportion of some tumour types positive for TERT. A higher proportion of canine meningiomas appeared to be immunoreactive for TERT as compared with human meningiomas, with 8 of 21 canine meningiomas (38%) versus 0-27% of human meningiomas being TERT-positive. This may be related to the more aggressive behaviour of canine meningiomas, which show a tendency to invade into the Virchow-Robin spaces and surrounding brain tissue, and which recur as a matter of course following surgical resection (Koestner *et al.*, 1999). On the other hand, a lower proportion of canine astrocytomas appeared to be positive for TERT than has been reported in human tumours. Only 5 of 9 (54%) grade 2 canine tumours were positive for TERT. This includes 2 of 4 glioblastomas. Although the number of astrocytomas overall was small, some authors have suggested that in humans, 100% of glioblastomas are positive for TERT expression, although this expression varies throughout the tumour mass and may be missed if careful sampling is not used (Falchetti *et al.* 2000). In dogs, it

would appear that less than 100% of glioblastomas are positive for TERT expression, although a larger number would need to be examined to ascertain the true incidence. It may be that this reflects a fundamental underlying difference in biological behaviour between the canine and human glioblastoma. To date, little work has examined these tumours in dogs to confirm whether they possess similar genetic characteristics to those seen in humans, and minimal data (especially regarding survival) are available by which to judge their clinical behaviour. It must also be noted, however, that studies examining telomerase activity in human astrocytic tumours overall have produced wide variations in the stated proportion of tumours positive for telomerase activity.

Although the numbers within each tumour type positive for TERT appeared to differ slightly, staining patterns appeared to be similar for both species. In particular, the more homogenous nature of oligodendroglial tumours appeared to be reflected in the TERT staining pattern seen, although this was not always the case. The trend towards more focal, patchy localisation of TERT activity in astrocytomas also appears to parallel the human situation (Kleinschmidt-DeMasters 1998a). This may reflect the evolution of these different tumour types. The fact that glioblastoma multiforme may arise through one of several different pathways, either as a de novo tumour or as malignant transformation of more benign astrocytoma subtypes, demonstrates the genetic variation underlying this phenotype. Indeed, Harada *et al* found that a significantly greater proportion of secondary glioblastomas possessed telomerase activity as compared with de novo tumours (Harada *et al.* 2000b). Some authors have suggested that telomerase activation occurs early in glioblastomas, and that with increasing tumour growth and the development of necrosis, telomerase activity subsequently disappears in some areas (Falchetti *et al.* 2000). However, other authors have suggested telomerase activation may occur in association with areas of greater anaplasia, a finding which could argue against this as anaplasia is more likely to occur later in tumour development (Kleinschmidt-DeMasters *et al.* 1998a). Whether this pattern of telomerase loss with glioblastoma development occurs in dogs as well as in people remains to be seen. The finding that TERT was present in endothelial cells undergoing vascular endothelial hyperplasia has also been reported in humans (Falchetti *et al.* 2003; Pallini *et al.* 2001). The involvement of telomerase in tumour angiogenesis is likely to be the result of similar factors responsible for tumour development. For instance, the SP-1 transcription factor which is known to be important for the activation of TERT transcription, also acts on the promoter region of the Vascular Endothelial Growth Factor (VEGF) gene, a major player in the initiation of angiogenesis in tumours as well as in other situations (Falchetti *et al.* 2003). There appeared to be no association between TERT localisation and other features of malignancy, however, such as necrosis or mitotic activity.

At this stage the significance of cytoplasmic staining in samples is unclear. The staining of endothelial cells may reflect a non-specific interaction with the antibody, as several other antibodies stain endothelial cell cytoplasm in a non-specific manner. Indeed, the MIB-1 antibody

showed frequent cytoplasmic staining of endothelial cells in some sections of choroid plexus papillomas (Figure 3.6).

3.6.6 Correlation between TERT staining and proliferation in canine brain tumours

In our study, TERT staining generally appeared to correlate with proliferation in all tumours overall, and in the meningioma and oligodendroglioma subtypes. The association between proliferation and telomerase activity in human brain tumours is not entirely clear. Whilst many studies have found that telomerase activity is associated with proliferation (Cabuy & de Ridder 2001; Falchetti *et al.* 2000; Fukushima *et al.* 2002; Sallinen *et al.* 1997; Simon *et al.* 2000), some studies have not (Nakatani *et al.* 1997). Given that increased proliferation is the most useful prognostic indicator for many brain tumour types, and that telomerase activity is commonly associated with malignancy, it would be expected that these two characteristic features of tumours should overlap to some degree. Evidence for a link between cell proliferation and telomerase activity has also emerged from somatic cells (Masutomi *et al.* 2003). However, it is not entirely clear whether the two factors are directly linked or whether they stem from a common determinant of tumour behaviour. One interesting study in breast cancer cells showed by immunocytochemical restaining that many of the cells undergoing proliferation were also expressing telomerase, suggesting that at least in some tumour types the pathways initiating these two factors are shared by the same cells (Ikeda *et al.* 2003). However, it is possible that telomerase activation may occur either before or after the signal to proliferate. For instance, Maes *et al* found that TERT expression could be found in a subset of benign meningiomas, and that this predicted recurrence (Maes *et al.* 2005). Proliferation, however, was not significantly different between recurrent and non-recurrent tumours, suggesting that at least in these tumours TERT expression occurs before significant proliferation. Although in canine brain tumours overall we found a direct correlation between TERT expression and cellular proliferation, significant variation was found between the different tumour subtypes. For instance, the most proliferative tumour, a metastatic lymphoma, was found not to express TERT. In humans, the level of telomerase expression in metastatic brain tumours does not seem to correlate with either the tumour type or patient survival (Kleinschmidt-DeMasters *et al.* 1998b). However, telomerase activity is a very common finding in metastatic tumours, which makes our case surprising. It may be that in canine metastatic tumour cells TERT expression may be regulated in a different manner to human tumours.

3.6.7 Potential usefulness of TERT staining in canine tumours

Ultimately, the potential usefulness of TERT staining and telomerase activity in canine cancer cells depends on the correlation between telomerase activity and patient survival. In dogs, evaluating

this is less straightforward than in humans, because of the variability inherent in measuring patient survival. This is influenced by the fact that the endpoint of cancer in dogs is determined by owner issues more often than by the death of the patient. The decision to euthanase is based on a number of factors, some emotional, some financial, with the result that the point at which owners elect to euthanase varies enormously. This is accentuated in patients with brain disease, where the clinical signs of a tumour may be less dependent on the size of the tumour than its location and its growth rate – a small tumour within the brainstem growing rapidly may lead to severe clinical signs more quickly than a larger tumour growing slowly in the cerebrum. In addition, tumours in the cerebrum may lead to clinical signs such as seizures and blindness which some owners find unacceptable but others are able to accommodate to prolong life for as long as possible. As a result, the assessment of survival in dogs with brain tumours is complicated by the variability inherent in the decision to euthanase.

Given this limitation in assessing patient survival, it is important to try to correlate the TERT stain described here with clinical outcome. The advantage of the immunohistochemical stain we have described is that it can be applied retrospectively to archival material. Therefore, acquiring a sufficient number of cases to assess survival following definitive treatment can be simplified by including retrospective as well as prospective case material.

There is considerable evidence already from the human literature that TERT expression will indeed be a useful prognostic indicator. For example, in meningiomas, up to 20% of the most benign subtype will recur despite surgical resection. Whilst proliferative markers do not predict those tumours which go on to recur, Maes *et al* found that TERT expression does, since recurrent tumours were found to have significantly more cells TERT-positive than non-recurrent tumours (Maes *et al.* 2005). Simon *et al* reported similar findings, finding that all recurrent meningiomas in their study had acquired telomerase expression (Simon *et al.* 2000). Similarly, Harada *et al* found a similar situation in tracking a recurrent pituitary tumour, with successive resections showing an increasing level of telomerase activity (Harada *et al.* 2000a). Studies examining telomerase activity within individual tumours as they progress are likely to provide useful information on tumour progression which is not necessarily available from surveying larger groups of tumours. Given the expense and morbidity of these repeat resections in dogs, it is unlikely in the near future that we will be able to perform similar evaluation in dogs as in people. However, given the nature of the stain we have described here, should such a situation arise, it will be possible to examine the telomerase activity at any point, which would not be possible without prior preparation with other methods of assessing telomerase activity.

CHAPTER IV

TELOMERASE INHIBITION IN CANINE CANCER CELLS

4.1 Abstract

Given the importance of telomerase activation as a means for attaining an immortalised phenotype, considerable research has focused on inhibiting telomerase. Several approaches have been investigated: using strategies interfering with the function of either TR or TERT, strategies targeting telomerase-associated proteins, and strategies interfering with the interaction between telomerase and the telomere. A large number of naturally-occurring and synthesised molecules are now in existence that fall into one of these categories. The aim of this chapter was to investigate two of these in canine telomerase-positive cell lines: the dominant negative mutant of human TERT, DN-hTERT, and the G-quadruplex inhibitor telomestatin, isolated from *Streptomyces anulatus*. We tested the DN-hTERT dominant negative mutant by establishing cell lines stably transfected with this construct carried by the selection vector pCINeo, and found that over 50 population doublings, the DN-hTERT mutant failed to inhibit telomerase activity in telomerase-positive D17 and CMT7 cells. Over this period, growth rates and telomere lengths of transfected cells were similar to those of negative control mock-transfected cells. Surprisingly, the same construct reactivated telomerase activity in telomerase-negative K9SF fibroblasts. Moreover, K9SF cells transfected with DN-hTERT appeared to undergo morphological changes consistent with a cancerous phenotype and exhibited accelerated growth as compared to negative controls. Investigation of telomestatin treatment of telomerase-positive cell lines yielded more encouraging results. Treatment of D17 and CMT7 cells with telomestatin at varying concentrations showed that a concentration of 2 μM caused a decrease in telomerase activity by approximately 80% as compared with negative controls. Over a longer treatment period, telomestatin was also shown to cause slower growth and telomere shortening in these cell lines, together with an increase in the proportion of cells undergoing apoptosis. Treatment of K9SF cells, however, did not cause any change in growth rate or apoptosis. These findings suggest that telomestatin treatment specifically abrogates telomerase activity in canine telomerase-positive cell lines and warrants further investigation of this promising telomerase inhibitor in canine cells.

4.2 Introduction

Since telomerase represents a major pathway to the acquisition of an immortal phenotype in cancer cells, considerable research has been performed investigating ways in which to inhibit its activity. This work has been carried out for 2 reasons: firstly, to further elucidate the effects of telomerase activity within cells, and secondly to explore new forms of cancer therapy. Telomerase represents an ideal target for cancer therapy since expression is largely restricted to tumour cells and since it plays a critical role in promoting tumour cell survival and extended lifespan. This chapter aims to explore telomerase inhibition as a means of treating cancer in dogs. There are several potential methods by which this inhibition can be achieved. Broadly speaking, these methods can be divided into three groups: 1) strategies interfering with the activity of either TR or TERT; 2) strategies targeting the telomerase-associated proteins (eg protein inhibitors); and 3) strategies interfering with the binding of telomerase to the telomere. The agents which have shown most promise within each of these groups will be reviewed briefly here.

4.2.1 Strategies interfering with the activity of TR or TERT

4.2.1.1 Reverse transcriptase inhibitors

Since the TERT subunit of telomerase functions as a reverse transcriptase, early searches for telomerase inhibitors focused on drugs known to inhibit other reverse transcriptases. These included the drugs Azidothymidine Triphosphate (AZT), dideoxyguanosine, arabinofuranyl-guanosine, dideoxyinosine, dideoxyadenosine, didehydrothymidine and phosphonoformic acid (Kelland 2005). Although these drugs have been shown to inhibit retroviral reverse transcriptase, their ability to specifically inhibit the activity of hTERT have not been promising. Long-term treatment of telomerase-positive B-cell and T-cell lines with these agents in one study resulted in telomerase inhibition only with AZT and dideoxyguanosine (Strahl & Blackburn 1996) When these were then tested in other cultures, only dideoxyguanosine also induced telomere shortening, but had no effect on population doubling or cell survivability, despite exposure of cells to the drug for almost 12 months. The effects of dideoxyguanosine were shown to be due to binding and competitive inhibition at the nucleoside triphosphate binding site. The addition of other agents to dideoxyguanosine did not improve the efficacy of action (Strahl & Blackburn 1996). Importantly, a recent study by Argyle *et al* has demonstrated that AZT alone is not an effective inhibitor of telomerase activity in the canine telomerase-positive cell lines MDCK and CMT7 (Argyle *et al.* 2004).

4.2.1.2 Small molecule inhibitors of TERT

Small molecule inhibitors are molecules that target the active site of TERT. The most extensively studied non-nucleoside molecule is BIBR1532. BIBR1532 inhibits telomerase *in vitro* with a half-maximal inhibitory concentration (IC_{50}) of 93 nM (Damm *et al.* 2001) and is a mixed-type non-competitive inhibitor of telomerase with a proposed binding site distinct from the sites for deoxyribonucleotides or the DNA primer (Pascolo *et al.* 2002). However, studies *in vivo* have shown that a lag phase exists between enzyme inhibition and subsequent cell growth arrest, apoptosis and tumour growth slowing, similar to that encountered with dominant negative mutants. Thus, NCI-H460 non-small cell lung cancer cells required a treatment time of 120 days *in vitro* before a decrease in cell proliferation became apparent (Damm *et al.* 2001), and *in vivo* effects were only noticeable when tumour cells used to xenograft nude mice were first pre-exposed *in vitro* for over 100 days prior to implantation. For this reason, the *in vivo* antitumour efficacy of BIBR1532 appears to be marginal.

A similar mixed-type non-competitive inhibitor of hTERT is 2,3,7-trichloro-5-nitroquinoxaline (TNQX), which has an IC_{50} of 1.4 μM (Pascolo *et al.* 2002). However, as with BIBR1532, *in vitro* effects such as growth inhibition and induction of senescence in telomerase-positive cells have required extensive periods of treatment.

4.2.1.3 Dominant-negative TERT mutants

The characterisation of splice variants of human TERT (see 5.2.2.2: Post-transcriptional modification of hTERT) revealed that one of the splice variants, the α deletion variant, results in the loss of 12 amino acids in the RT domain A, as the result of a 36 bp deletion (Kilian *et al.* 1997). This splice variant, in addition to being non-functional, was found to inhibit the activity of wild-type TERT in telomerase-positive cells and thereby to inhibit telomerase activity by competing for binding with other components of the telomerase holoenzyme (Colgin *et al.* 2000). This finding led other groups to examine the impact of other mutations in the reverse transcriptase A domain (Zhang *et al.* 1999), and Hahn *et al* subsequently showed that a substitution of only 2 amino acids in the third (A) RT motif of hTERT was sufficient to create a dominant-negative mutant of hTERT, DN-hTERT (Figure 4.4) (Hahn *et al.* 1999a). Mutant hTERT subunits have been effective tools in studying the characteristics of telomerase and several studies have shown that DN-hTERT abrogates the expression of wild-type (WT) hTERT in several telomerase-positive cell lines, including ovarian, breast, colon and hepatic cancer cells (Hahn *et al.* 1999a; Zhang *et al.* 1999). Interestingly, Sachsinger *et al* demonstrated that telomerase activity was reactivated in murine RenCa cells expressing DN-mTERT, suggesting there are fundamental differences in regulation

between mouse cells and human cells (Sachsinger *et al.* 2001).

The use of the DN-hTERT mutant as an inhibitor of telomerase has led to some interesting findings that suggest telomerase inhibition may lead to clinical effects distinct from those induced by telomeric shortening. Typically, inhibition of telomerase in telomerase-positive cell lines leads to cell death through crisis over a time-course dependent on the initial length of telomeres in those cells. Cell lines with longer telomeres must therefore go through more population doublings before undergoing crisis as compared to cell lines with shorter telomeres. However, DN-hTERT mediated inhibition of telomerase activity has also been shown to reduce the growth rate of some cell lines *in vitro* and to reduce the ability of these cells to form tumours xenografted mouse models *in vivo*, confirming the involvement of telomerase in other features of malignancy (Hahn *et al.* 1999a; Zhang *et al.* 2002).

4.2.1.4 Hammerhead ribozymes

Ribozymes are catalytic antisense RNAs that cleave RNA substrates in a sequence-specific manner, first discovered in satellite RNA of tobacco ringspot virus (Komata *et al.* 2002a). Ribozymes have been developed that target both hTR and hTERT. Although ribozymal cleavage of hTR has been found to reduce telomerase activity in tumour cells in some studies, no change in proliferation occurred in treated cells, suggesting that it is of limited use in the clinical setting (Kanazawa *et al.* 1996; Yokoyama *et al.* 1998). However, other studies have shown that ribozymal cleavage of hTERT leads to shortened telomeres and apotosis as well as senstitising tumour cells to inhibitors of topoisomerase, suggesting that ribozymes targeting TERT may be worth considering for further development (Ludwig *et al.* 2001; Yokoyama *et al.* 1998).

4.2.1.5 Oligonucleotides

A number of strategies have been developed using synthesised oligonucleotides designed to bind in a complementary fashion to either hTERT or, more commonly, the hTR component of telomerase. Typical antisense oligonucleotides contain DNA bases that form DNA-RNA hybrids upon binding to mRNA. These hybrids are recognised by RNase H which cleaves them and, in thus destroying the target mRNA, reduces the level of expression of the desired protein. Problems with oligonucleotide therapy include their rapid destruction by DNase, the large doses necessary to achieve a therapeutic response and the difficulty associated with directing oligonucleotides to target cells. However, improvements in their design and synthesis suggest that they may present an attractive option for clinical use for several reasons. They can now be administered systemically by intravenous injection, methods have been developed for their large scale synthesis, their pharmacokinetics are well characterised and toxicity is low (Corey 2002). There are also some indications that adequate bioavailability can be achieved following oral administration (Khatsenko

et al. 2000). Telomerase is an atypical antisense target, because binding of the oligonucleotide to its target site is all that is required to interfere with telomerase activity, with cleavage by RNase H being of secondary importance. As a result, the inclusion of DNA bases in the structure of anti-telomerase oligonucleotides is unnecessary, and they can be composed entirely of chemically modified bases that bind complementary sequences with elevated affinity. Some of the modifications that have been examined include peptide nucleic acids (PNA), 2'-O-alkyl RNA and locked nucleic acid (LNA) (Braasch & Corey 2001; Elayadi *et al.* 2001; Herbert *et al.* 1999). In addition, modification to the linkages between bases within the oligonucleotide can alter the potency of effects (Corey 2002). The number of synthesised anti-telomerase oligonucleotides so far examined is considerable, and only a few will be discussed here.

4.2.1.5.1 2-5A-linked Antisense Oligonucleotides

Kondo *et al* showed that expression of a conventional antisense oligonucleotide targeting hTR eliminates telomerase activity from malignant glioma cells and results in apoptosis after a lag phase of approximately 30 population doublings (Kondo *et al.* 1998a). Interestingly, in some subclones of cells treated, apoptosis does not occur but cells enter a more differentiated state, as shown by increased expression of glial fibrillary acidic protein (GFAP) and decreased motility. This would suggest that telomerase activity is linked in other, as yet undetermined, ways to the dedifferentiated phenotype of these cells.

A more rapid onset of apoptosis can be readily induced in these cells by linking the antisense oligonucleotide to 2'-5' phosphorylated oligoadenylate (2-5A). 2-5A is an activator of the endoribonuclease Rnase L, a single-strand specific endoribonuclease that functions in the antiviral mechanisms of interferons (Kondo *et al.* 1998b). Although Rnase L is a ubiquitous protein, it has no detectable ribonuclease activity until it comes into contact with 2-5A. Linking 2-5A to an oligonucleotide therefore creates an RNA-degrading molecule that specifically targets a single RNA species. The use of 2-5A linked to the hTR antisense oligonucleotide resulted in rapid onset of apoptosis in malignant glioma cells, both *in vitro* and *in vivo* in a nude mouse model (Kondo *et al.* 1998b; Mukai *et al.* 2000). The onset of apoptosis in these cells occurred within 5-7 days, too rapidly to be accounted for by telomeric attrition, although the mechanisms underlying this are not well understood. In addition, the 2-5A-hTR antisense oligonucleotide shows an additive effect when used in conjunction with adenoviral vectors expressing wild type p53, but only in those cell lines with a mutated form of p53 (Komata *et al.* 2000).

4.2.1.5.2 Oligonucleotide N3'-P5' Phosphoramidates

N3'-P5' phosphoramidate (NP) oligonucleotides are compounds complementary to hTR sequence that have a high *in vitro* potency for telomerase inhibition. NP oligonucleotides contain a 3'-amino

group substituted for the 3' oxygen in the 2'-deoxyribose rings. These compounds form very stable duplexes with single-stranded RNA, are resistant to nuclease degradation and display high specificity for RNA and DNA targets. Oligonucleotides directed at both the template region and another region 100 nucleotides further downstream have been used, and both lead to abrogation of telomerase activity and consequent apoptosis in telomerase-positive cells after a lag phase that correlates with initial telomere length (Pruzan *et al.* 2002). However, the use of these agents in cells requires a lipid-based cellular uptake enhancer for efficient entry into cells. Substitution of a single sulphur atom for an oxygen atom, to create an NP *thio*phosphoramidate, results in a molecule with similar specificity for target RNA but which is able to enter cells more efficiently without the presence of cellular uptake enhancers.

The most advanced of the phosphoramidates so far examined is GRN163, which is able to inhibit telomerase activity at nanomolar concentrations (Herbert *et al.* 2002). However, as with BBR1532, antitumour effects are largely dependent on telomere length, with little effect in U266 multiple myeloma cells (with long telomeres of 9kb) after 56 days of cell culture, but more rapid growth inhibition in 28 days in MM.1S cells (with short telomeres of 2.5 kb) (Akiyama *et al.* 2003). Similar effects have been demonstrated *in vivo* using xenografted nude mice bearing tumours with short telomeres from prostate cancer, myeloma and glioblastoma cancer cell lines (Asai *et al.* 2003; Wang *et al.* 2004). GRN163L, containing a lipid covalently conjugated to an aminoglycerol thiophosphate linker, is currently undergoing late-stage preclinical development in preparation for Phase I clinical evaluation.

4.2.1.5.3 2'-O-alkyl- and 2'-methoxyethyl-RNA

The synthesis of 2'-O-alkyl RNA oligonucleotides has provided another potentially useful class of telomerase inhibitor. Both 2'-O-alkyl RNA and 2'-methoxyethyl (ME) RNA oligonucleotides comprising 13-base oligonucleotides complementary to hTR have been shown to inhibit telomerase activity with an IC_{50} value in cell lysate of approximately 10 nM (Elayadi *et al.* 2001; Herbert *et al.* 1999; Pitts & Corey 1998). Introducing phosphorothioate (PS) linkages into these molecules improves their pharmacokinetic profile by enhancing resistance to nuclease digestion and increasing the serum half-life, with the result that the IC_{50} can be lowered to 3-10 nM while retaining selective inhibition. These agents have been trialled in cell lines of varying telomere lengths, and show growth rate slowing after a time lag related to the initial telomere length of each cell line. Importantly, in all cell lines examined, telomere lengths returned to pre-treatment size after the agent was removed, and in the cell line with longest (12 kb) telomeres, no impact on growth rate was seen at all (Herbert *et al.* 1999). These results suggest that despite their pharmacokinetic advantages, the clinical usefulness of these agents is likely to be marginal.

4.2.1.6 RNA Interference

Most recently, considerable interest has focused on the growing field of RNA interference as a means of interfering with gene expression. dsRNA, a hybrid of a sense and an antisense strand of an endogenous RNA species, can initiate a cellular response that results in the sequence-specific degradation of homologous single-stranded RNA (Kosciolek *et al.* 2003). This occurs in a wide variety of eukaryotic organisms and is known as RNA interference, or RNAi. In this process, RNA synthesis is not itself affected, but rather the RNA transcript is specifically degraded so that the corresponding gene becomes silenced. This apparently represents an old and evolutionarily conserved defence mechanism against parasitic RNAs, and is reviewed in (Hannon 2002), (Hutvagner & Zamore 2002) and (Sharp 2001). The key trigger of this response is dsRNA, which is processed by double-strand-specific RNase to shorter RNA fragments of both polarities. The enzyme responsible, first identified in Drosophila, is known as Dicer, and is an RNase III-type enzyme (Bernstein *et al.* 2001). The product generated following Dicer's processing is a dsRNA of approximately 21-22 bp, also know as short RNAs. Rather than being composed of a simple double strand, this short RNA has characteristic termini consisting of two unpaired 3'-terminal nucleotides on either side (Elbashir *et al.* 2001a), and is an important intermediate of the RNAi reaction which then becomes incorporated into a multicomponent protein complex called RISC (Hammond *et al.* 2001). RISC acts as an RNase, the sequence-specificity of which is conferred by the short dsRNA.

The importance of the short RNAs in this process led several researchers to investigate the possibility of artificially-generated short dsRNAs to initiate the RNAi response and led to the important discovery that chemically-synthesised RNAs that could form a double strand with the characteristic two unpaired nucleotides at the 3' termini could initiate RNAi in mammalian cells (Elbashir *et al.* 2001b), and the term 'siRNA' was introduced for these short duplexes. As an effective means of silencing genes, siRNA has been utilised in a range of situations, and most recently has been used to silence telomerase expression both through targeting of hTR and hTERT (Kosciolek *et al.* 2003). The use of siRNA to inhibit telomerase activity has been successfully reported both *in vitro* and *in vivo* (Del Bufalo *et al.* 2005; Guo *et al.* 2005; Li *et al.* 2005; Nakamura *et al.* 2005; Sciamanna *et al.* 2005; Zou *et al.* 2005), suggesting that it may be a promising approach for clinical anticancer therapies. However, perhaps more importantly, results of some of these experiments have uncovered fundamental links between telomerase activity and cancer cell biology that were not previously known. Li *et al* showed, using RNAi directed at hTR, that telomerase inactivation led to rapid slowing of cell growth independent of telomere shortening, uncapping or the activation of DNA damage-response pathways such as p53 and p21 (Li *et al.* 2005). In addition, telomerase activity in telomerase-positive cancer cell lines seems to be inextricably linked to other characteristics of tumour cell behaviour, since inactivation of telomerase activity led to downregulation of a number of genes, including integrin αV, known to be related to cancer cell proliferation, angiogenesis and survival (Cruet-Hennequart *et al.* 2003;

Elicciri & Cheresh 2001; Hutvagner & Zamore 2002; Li *et al.* 2005). These findings have been supported by *in vivo* experiments in which inhibition of hTERT expression through RNAi resulted in reduced colony-forming ability and tumorigenicity in mice, and also increased sensitivity to ionizing radiation and chemotherapeutic agents (Nakamura *et al.* 2005).

A number of useful and important points are worth noting in relation to these studies. Firstly, it appears that many of the effects of telomerase inhibition such as growth inhibition occur irrespective of whether the target of RNAi is hTR or hTERT. Secondly, these effects occur rapidly, without the time lag attributable to telomere-shortening seen with the majority of other methods of inhibiting telomerase, such as antisense oligonucleotide treatment or DN-hTERT treatment, and also occur without interfering with telomere length or capping. Finally, RNAi techniques appear to work by reducing the overall level of telomerase ribonucleoprotein complex, rather than by interfering with the quantity of enzyme activity. For all these reasons, RNAi techniques are worth investigating as methods of telomerase inhibition.

4.2.2 Strategies targeting telomerase-associated proteins

Heat shock protein 90 (HSP90) is a molecular chaperone that plays a role in protein refolding in cells exposed to environmental stress. It is required for the conformational maturation, stability and activity of several proteins involved in signal transduction pathways, but acts on a more narrow range of targets than other chaperones. Target proteins include receptor and non-receptor kinases (eg HER-2, epidermal growth factor receptor and Src family kinases), serine/threonine kinases (eg c-Raf-1 and Cdk4), steroid hormone receptors (androgen and oestrogen) and cell cycle and apoptosis regulators (eg mutated p53) (Hartson & Matts 1994; Sepp-Lorenzino *et al.* 1995; Stancato *et al.* 1993; Stepanova *et al.* 1996; Veldscholte *et al.* 1992; Villa *et al.* 2003). HSP90 regulates the half-lives of these target proteins by forming conformation-dependent higher order chaperone complexes. Since all of these proteins are involved with the growth and or survival of cancer cells, HSP90 may be a potential anti-tumour target. Studies have also shown that HSP90 and the co-chaperone p23 are required for efficient telomerase assembly, in a proposed model whereby both proteins bind hTERT and influence its assembly with hTR in the formation of an active enzyme (Holt *et al.* 1999).

The benzoquinone ansamycin antibiotic geldanamycin (GA) and its derivative, 17-allylamino, 17-demethoxygeldanamycin (17-AAG) are both inhibitors of HSP90 (Neckers *et al.* 1999; Schulte & Neckers 1998). Treatment of JR8 melanoma cells resulted in inhibition of telomerase activity, and apoptosis, although no telomere shortening was seen (Villa *et al.* 2003). These effects were dependent on the level of telomerase activity within the particular clone of cells used. 17-AAG is currently undergoing Phase I clinical trials for its other anti-tumour effects, and it is possible that

the level of efficacy of the drug may be partly related to the levels of telomerase activity in the tumour being treated.

4.2.3 Strategies interfering with telomere-telomerase interactions

A potential strategy to target telomerase activity involves interfering with its ability to bind to the telomere. One important way of doing this would be to physically change the structure of the telomere in such a way that it is no longer accessible to the telomerase holoenzyme. The highly repetitive, G-rich nature of telomeric sequence allows it to potentially fold into a 4-stranded (quadruplex) intramolecular structure, and approaches exploiting this ability have been pursued since the 1990s (Kelland 2005). G-quadruplex ligands affect telomere structure by covalently binding to the quadruplex and preventing its dissociation. Since the first demonstration of telomerase inhibition by a G-quadruplex-interacting compound (a 2,6-diamidoanthraquinone) in 1997 (Sun *et al.* 1997), an enormous array of G-quadruplex ligands has been described, including cationic porphyrins, trisubstituted acridines, dibenzophenanthrolines, ethidium derivative, triazines, pentacyclic acridines, fluoroquinophenoxazines and 2,6-pyridine-dicarboxamide derivatives (Duan *et al.* 2001; Gowan *et al.* 2001; Koeppel *et al.* 2001; Mergny *et al.* 2001; Pennarun *et al.* 2005; Read *et al.* 2001; Riou *et al.* 2002; Shin-Ya *et al.* 2001; Wheelhouse *et al.* 1998). Given the vast array of agents, only two of the most promising will be discussed here – the trisubstituted acridine BRACO19 and naturally-occurring telomestatin.

4.2.3.1 BRACO19

Many of the first generation G-quadruplex ligands suffered from a relative lack of selectivity for 4-stranded over 2-stranded DNA, resulting in non-specific acute cytotoxicity at similar concentrations to those used to inhibit telomerase activity (Harrison *et al.* 1999). However, the resolved crystal structure of parallel quadruplexes from human telomeric DNA allowed the development of improved molecules, including BRACO19 (Parkinson *et al.* 2002). BRACO19 was shown to induce extensive end-to-end chromosomal fusions in DU145 prostate cancer cells and senescence from 7 days following exposure at non-acute cytotoxic concentrations (Incles *et al.* 2004). Senescence was associated with an increase in the CDK inhibitor p21, consistent with senescence being triggered by telomere shortening or loss of integrity (Herbig *et al.* 2004). *In vivo* work has also been promising, with significant growth inhibition (96% compared with controls) in a uterus carcinoma xenograft model and against DU-145 prostate cancer xenografts (Burger *et al.* 2005).

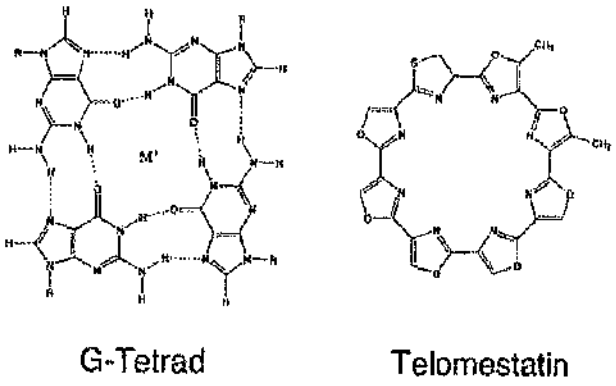
4.2.3.2 Telomestatin

Telomestatin is a natural product isolated from *Streptomyces anulatus* 3533-SV4 which has been

shown to be a very potent inhibitor of telomerase, with an IC_{50} of approximately 2 μM (Kim *et al.* 2002). It possesses structural similarity to the G-tetrad (Figure 4.1) and it is thought that its effects are due to its ability to either facilitate the formation of or stabilise pre-formed G-quadruplex structures, thereby sequestering the primer needed for elongation by telomerase. Telomestatin also shows preferential interaction with intramolecular G-quadruplex structures rather than intermolecular structures and is 70 times more selective in binding quadruplex structures than duplex DNA (Kim *et al.* 2003a). This affinity for intramolecular G-quadruplexes appears to be important in avoiding detrimental side effects such as the formation of anaphase bridges in telomerase-negative cells (Kim *et al.* 2003a). In addition to interfering with the function of telomerase, stabilising the formation of G-quadruplexes may also have other useful properties, such as the ability to down-regulate c-myc expression (Lemarteleur *et al.* 2004; Seenisamy *et al.* 2005).

The use of telomestatin in telomerase-positive cell lines results in delayed growth arrest and/or apoptosis (Shammas *et al.* 2004; Tauchi *et al.* 2003). In addition, telomere shortening is also observed in these cells, although it occurs earlier than expected for a single mechanism involving telomerase inhibition and is limited to only a few kilobases (Gomez *et al.* 2003). Its use in the leukaemic cell lines OM9; 22 and K562 in one study resulted in telomere shortening, and telomerase inhibition (Tauchi *et al.* 2003). Over a period of 10 days, treatment also resulted in significant cell death, through activation of p21, p27 and the ATM-dependent DNA damage response pathway, consistent with telomere uncapping. Similar results in multiple myeloma cells have also been reported (Akiyama *et al.* 2003). These results suggest that telomestatin holds considerable promise as a telomerase inhibitor.

Figure 4.1: Structures of G-quadruplex tetrad in relation to telomestatin



(Reproduced with permission from (Kim *et al.* 2002))

4.3 Aims of the Chapter

The aims of this chapter were to investigate two potential methods of inhibiting telomerase activity. The first aim was to investigate inhibition of canine TERT activity using a dominant negative mutant of canine TERT. However, at the time this work was performed, although the dogTERT cDNA sequence had been established, a vector containing the entire canine TERT cDNA was not available for mutational analysis of dominant negative forms of dogTERT. Given the close homology between the canine and human TERT molecules in the regions of the T and reverse transcriptase motifs, these studies were therefore performed using the human dominant negative mutant DN-hTERT.

Given the success of the G-quadruplex ligands in inhibiting human telomerase, the second aim of this chapter was to investigate inhibition of canine telomerase activity through the use of one of these - telomestatin, and to examine the effects of consequent telomerase inhibition in telomerase-positive canine cancer cell lines.

4.4 Materials and Methods

4.4.1 Inhibition of canine telomerase with the dominant-negative mutant DN-hTERT

4.4.1.1 Cell Lines

D17 (canine osteosarcoma) and CMT7 (canine mammary tumour) are canine cell lines which have previously been shown to be telomerase positive (Nasir *et al.* 2001). Canine primary skin fibroblasts (K9SF) are cells which have similarly been shown to be telomerase negative. (Nasir *et al.* 2001) The D17 cell line was cultured in DMEM (Gibco BRL, Grand Island, New York) supplemented with 10% fetal calf serum. The CMT7 cell line was cultured in RPMI (Gibco BRL, New York) supplemented with 10% fetal calf serum and 2mM L-glutamine. Canine primary skin fibroblasts (K9SF) were cultured in MEM-α (Gibco BRL, New York) supplemented with 10% fetal calf serum, 2 mM L-glutamine and epithelial growth factor (EGF).

4.4.1.2 Generation of pCINeo-DNhTERT Plasmid vector

DNhTERT was kindly donated by the laboratory of RA Weinberg, Whitehead Institute, within the plasmid vector pBabepuro. Following restriction digestion with *EcoR1* and *Sal1*, DNhTERT was cloned into the plasmid vector pCINeo, which conveys resistance to G418, a eukaryotic analogue of the antibacterial agent neomycin.

4.4.1.2.1 Restriction enzyme digestion of vectors

The pBabePuro vector containing the DN-hTERT dominant negative mutant was digested with *EcoR* I and *Sal* I restriction enzymes. The pCINeo vector was also digested with *EcoR* I and *Sal* I restriction enzymes. More specifically, the pBabePuro containing DNhTERT (5μg) was digested for one hour with *EcoR* I (5 units) in buffer D (10X) in 10μl at 37 C, before adding *Sal* I (5 units) to a final volume of 20μl and continuing with incubation for a further 3 hours at 37 C. The recipient pCINeo vector (5μg) was digested with *EcoR* I (5 units) and *Sal* I (5 units) using the same protocol. Samples (4μl) were analysed on a TAE agarose gel (1%) by comparing the bands created to a 100bp DNA ladder to confirm digestion before purification and ligation.

4.4.1.2.2 Purification, ligation and transformation

Digested DNA samples (linearised pCINeo and released DN-hTERT) were purified using the QIAquick®Gel Extraction Kit (Qiagen, UK) and eluted in 30μl of sterile water. The DNA was

quantified (2µl) by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a Low DNA Mass™ Ladder (2µl).

The quantity of vector (pCINeo) and insert (DN-hTERT) for each ligation reaction was calculated according to the equation shown in 2.2.3.7: Ligation of restriction digested DNA fragments. For each calculation the vector mass (X) was 100 ng, and the insert size (Y) was approximately 3.6 Kb. Ligation was performed using molar ratios of 3:1 and 1:1 insert to vector. The cut ends of the inserts were ligated to complementary ends of the cut pCINeo vector using T4 DNA ligase (Promega, UK) overnight at 16° C. The ligations (20ng) were transformed into 50µl of One Shot™ TOP10 competent cells which were heat shocked for 45 seconds at 42° C before adding 250µl SOC medium, shaking horizontally at 225 rpm at 37 C and then grown overnight at 37° C on LB/ampicillin agarose plates. Six to ten colonies were selected from each plate and transferred to LB broth for overnight culture at 37° C.

4.4.1.2.3 Isolation and screening of recombinant plasmids

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation using the QIAprep®PCR Spin Miniprep Kit (Qiagen, UK) and eluted in 50µl of sterile water. The DNA samples were then assessed by spectrophotometry for quantification. pCINeo promoter constructs were screened with *EcoR* I and *Sal* I restriction enzyme as described in 2.2.3.11: Screening of transformants for desired recombinant plasmids. Since all the promoter inserts were directionally cloned it was not necessary to screen for correct orientation.

4.4.1.2.4 Sequence evaluation

Recombinant plasmids were sequenced and analysed as described in section 2.2.3.11: Screening of transformants. Briefly, plasmid samples were prepared for sequencing with PCR reactions using plasmid DNA samples (400 ng) and the Big Dye™ Terminator Cycle Sequencing Ready Reaction (ABI Prism) with 0.5µM of both sense (DNHT001F) and anti-sense (DNHT001R) primers (Table 4.1). These primers were designed to amplify a 450 bp fragment containing the mutation site responsible for the dominant negative function of DNhTERT. Samples were amplified, and the DNA purified by precipitation methods before samples were loaded into the ABI 310 genetic analyser for generation of automated sequence data. Sequence files were downloaded from the chromas file, saved as Word documents using FASTA format, and then lined up with the relevant correct sequence for the hTERT sequence using VectorNTI (Invitrogen Bioinformatics).

4.4.1.3 Generation of Stable Transfectants

Liposomes are one of a growing number of chemical reagents used to deliver nucleic acids to eukaryotic cells. Transfections were performed using the TransFast™ liposome-based transfection reagent (Promega, UK). Liposomes are lipid bilayers that form colloidal particles in an aqueous medium. Liposome reagents specifically designed for transfection applications incorporate synthetic cationic lipids, often formulated together with the neutral lipid L-dioleoyl phosphatidylethanolamine (DOPE), which enhances the gene transfer ability of certain synthetic cationic lipids. The TransFast™ transfection reagent is comprised of the synthetic cationic lipid (+)-N,N [bis(2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide and the neutral lipid DOPE. The reagent is supplied as a dried lipid film that forms multilamellar vesicles upon hydration with water.

Incubation of cationic lipid-containing liposomes and nucleic acids results in quick association and compaction of the nucleic acid and the positively charged head group of the synthetic lipid. The liposome-nucleic acid complex is then presented to the cells that are to be transfected. The liposome complex neutralises the negative charge of the nucleic acids, allowing closer association of the complex with the negatively charged cell membrane. The liposome complex then gains entry into the cell either through endocytosis or by fusion with the plasma membrane via the lipid moieties of the liposome. Once inside the cell, the complexes often become trapped in endosomes and lysosomes. Endosomal disruption is facilitated by DOPE, which allows the complexes to escape into the cytoplasm. The manner in which the transfected DNA or liposome-DNA complex gains entry to the nucleus is not precisely known.

Transfections were performed following the supplier's recommended protocol using a ratio of TransFast™ Reagent:DNA of 2:1. The day before transfection, 16 x 10^5 cells were seeded into a 25 cm^2 flask to achieve a confluence of approximately 50% on the day of transfection. On the day of transfection, 3 µg of plasmid DNA (pCINeo-DN-hTERT and pCINeo negative control) was added to serum-free medium (2 ml) and vortexed. Then, 18 µl of TransFast™ reagent (6 µl/µg DNA) was added and the mixture vortexed again. The mixture was then incubated for 10-15 minutes at room temperature. While incubation was occurring, the growth medium was carefully aspirated from adherent cells. The TransFast™/DNA mixture was then added to the cells and flasks were returned to the 37° C incubator for 1 hour. At the end of this incubation, cells were overlaid with 4 ml of complete growth medium.

Forty-eight hours after transfection, cells were trypsinised, counted and plated in 25 cm^2 flasks at 4 different concentrations: 1:5, 1:10, 1:20, and 1:100. In addition, G418 was added to the medium at a concentration of 400 mg/ml. For the remainder of the experiment, medium was replaced every 3-4 days, and fresh G418 added. For the 14 days following addition of G418, cells were monitored for cell death. The flask containing surviving colonies was retained and the remainder discarded. When this flask reached confluence, cells were trypsinised and transferred to 75 cm^2 flasks. In

addition, following initial selection at 400 mg/ml, the concentration of G418 was increased to 600 mg/ml over approximately 2 weeks. Cells were trypsinised when they reached confluence (approximately every 7-10 days) and reseeded at a cell density of 1 X 10^5 cells per 75 cm^2 flask.

4.4.1.4 Confirmation of transfection with RT-PCR

4.4.1.4.1 RNA Extraction

RNA was also extracted from cell pellets containing 2 x 10^6 cells stored at –80°C using RNAwiz™ (Ambion, UK) as described in 2.2.4.1: Extraction and Purification of RNA.

4.4.1.4.2 cDNA Synthesis

Superscript III™ reverse transcriptase was used for first strand cDNA synthesis, which was performed as described in 2.2.5: First strand cDNA synthesis.

4.4.1.4.3 PCR amplification and sequencing

Following synthesis of cDNA, PCR was performed using the DNHT001F and DNHT001R primers. As a positive control, amplification of a fragment of the canine cyclophilin gene was simultaneously performed using the primers CYCLOF and CYCLOR. These primers are detailed in Table 4.1.

Platinum High-Fidelity Taq polymerase (Invitrogen, UK) was used for second strand PCR. For each sample, 1-5 µl of cDNA was mixed in a 0.2µl thin-walled PCR tube with 5µl of High Fidelity PCR buffer, 1µl dNTP mixture, 2µl $MgSO_4$, 1µl of each primer (forward and reverse) used and 0.2µl Platinum Taq High Fidelity. The reaction mix was made up to a total volume of 50µl with autoclaved, distilled water. Samples were kept on ice during pipetting and prior to PCR. As a negative control, 5 µl of total RNA was also included in each reaction to ensure resultant products were not amplified from contaminating genomic DNA.

Cycling conditions involved denaturation of the template and activation of the Taq polymerase at 94°C for 2 minutes followed by 30 cycles of the following: denaturation at 94° C for 30 seconds, annealing at 55° C for 30 seconds, extension at 68°C for 1 minute. Finally, the mix was incubated at 72° C for 10 minutes to add a run of adenosine residues to the 3' end of each product, before being maintained at 4° C. PCR products were analysed by agarose gel electophoresis (1.5%) using a 1 Kb molecular weight marker.

PCR products were purified using the QIAquick® Gel Extraction Kit (Qiagen, UK) and eluted in

30µl of sterile water 4µl of which was assessed by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a 1 kb molecular weight standard. Sequencing was performed directly from PCR products as described in 2.2.7: DNA sequence analysis. Briefly, plasmid samples were prepared for sequencing with PCR reactions using plasmid DNA samples (400 ng) and the Big Dye™ Terminator Cycle Sequencing Ready Reaction (ABI Prism, UK) with 0.5µM of both sense (DNHT001F) and anti-sense (DNHT001R) primers. Samples were amplified, and the DNA purified by precipitation methods before samples were loaded into the ABI 310 genetic analyser for generation of automated sequence data. Sequence files were downloaded from the chromas file, saved as Word documents using FASTA format, and then lined up with the relevant correct sequence for the hTERT sequence using VectorNTI (Invitrogen Bioinformatics).

Table 4.1: Primers used in RT-PCR

Primer Identification	Oligonucleotide primer sequence (5'-3')	Tm (°C) & GC (%) content
DNHT001F	ACTGTTCAGCGTGCTCAACTA	62.6°C : 47.6 %
DNHT001R	TCATTCAGGGAGGAGCTCTGCT	68.5°C : 54.5 %
CycloF	CGTGCTCTGAGTACTGGAGAGAAGGGA	80.2 C : 55.6 %
CycloR	CCACTCAGTCTTGGCGGTGCAGATGAA	75.0°C : 60.0 %
EX2F	CAGGAGCTGCTTGGGAACCA	69.8 C : 60.0%
EX2R	CTGGGTTCCCGTGCAGCCAG	74.4 C : 70.0%

4.4.1.5 Growth Rates

Cell lines were grown as an adherent monolayer in culture, in either 25 or 75 cm^2 tissue culture flasks stored in an incubator at 37°C with 5% CO_2. Cells were routinely split when they reached confluence, counted, and once stable transfectants had been generated, re-seeded at a cell density of 1 X 10^5 cells per flask. Population doublings were calculated according to the following formula:

$$PD = \frac{\text{Log}_{10}\,(\text{Cell count})_n - \text{Log}_{10}\,(\text{Cell count})_{n-1}}{0.301}$$

Where n=current passage and n-1= previous passage

4.4.1.6 Telomerase repeat amplification protocol

Telomerase activity was measured using the TeloTAGGG Telomerase PCR ELISA^{PLUS} assay (Roche, UK), following the manufacturers recommended protocol as described in 2.2.10.1: Telomerase repeat amplification protocol.

4.4.1.6.1 Sample Preparation

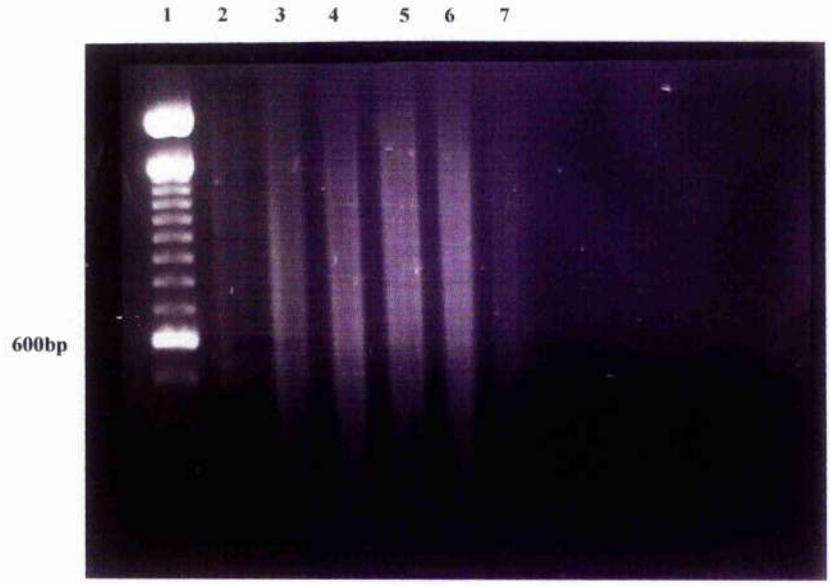
Samples used for telomerase assay analysis included cell pellets from the D17, CMT7 and K9SF and U87 cell lines. Cells were harvested and counted as described in 2.2.1: Growth and manipulation of mammalian cells. Aliquots of 2 x 10^5 cells were harvested for each assay. Cells were pelleted at 3000g for 5 minutes at 4° C, the supernatant withdrawn, and following a brief resuspension in 100 µl of PBS the cells were again pelleted and the supernatant carefully removed. Cell pellets were then resuspended in 200 µl of ice-cold lysis reagent (Roche, UK) and incubated on ice for 30 minutes. The lysate was then centrifuged at 16000g for 20 minutes at 4°C and the 175 µl supernatant was carefully removed with a micropipette, taking care not to disturb the pellet of cellular debris. Cell extracts were then either used immediately or snap frozen liquid nitrogen and stored at -80°C.

4.4.1.7 Telomere Length Analysis

4.4.1.7.1 Digestion of DNA with HinfI/RsaI

The selection of the restriction endonucleases *HinfI* and *RsaI* was based on the fact that these enzymes do not contain recognition sequences that cut within mammalian telomeric sequence. Although these enzymes are not alone in this respect, they are the most commonly used (Lauzon *et al.* 2000; Nakamura *et al.* 1999). The use of these enzymes allows complete digestion of non-telomeric DNA, up to the first recognition site for the enzymes. The remaining intact telomeric DNA is referred to as a Telomere Restriction Fragment (TRF). Three µg of DNA was digested per sample at 4U of enzyme mix per µg of DNA for 12-16 hours at 37° C. To verify restriction enzyme digestion, samples (2 µl) were analysed by agarose gel electrophoresis (1.5%) by comparing to a 100 bp molecular weight marker (Figure 4.2).

Figure 4.2: Representative 1.5% Agarose gel analysis of *Hinf* I / *Rsa* I digestion of DNA samples from CMT7 cells transfected with pCINeoDNhTERT. Lane 1: 100 bp marker, lane 2: passage (P) 12, lane 3: P18, lane 4: P21, lane 5: p23, lane 6: Negative control (empty pCINeo) P13, lane 7: Negative control (empty pCINeo P15)



4.4.1.7.2 DNA fragment separation

4.4.1.7.2.1 Agarose gel electrophoresis and Southern blot

Separation of digested DNA was achieved by 0.8% agarose gel electrophoresis in 1X TAE buffer using highly pure agarose (Sigma, UK) following a standard protocol(The DIG system user's guide for filter hybridisation 2003) (Southern 1979). A DIG molecular weight marker was used (23.1-0.12 kb, Roche UK). Equal amounts of sample were run in each lane and each sample was run in duplicate. Individual samples were made up to 20 µl using dH_20 and 2 µl of 10X DNA loading buffer. Samples were run at 5 V/cm for between 4 and 5 hours until the loading dye front was approximately 2 cm from the base of the gel.

Southern transfer (Southern 1975) of the digested DNA was carried out by capillary action using 20X SSC as a transfer buffer following a standard protocol (Current protocols in molecular biology) to a high quality positively charged nylon membrane (Amersham, UK). Gels were pre-treated first by submersion in 0.25M HCl for 5 – 10 minutes; completion of this step was indicated by a change in colour of the bromophenol blue loading dye from blue to yellow. Following a rinse in dH_20, gels were submerged in a neutralising solution (0.5M Tris-HCl, 3M NaCl at pH 7.5) for 30 minutes with a change of solution after 15 minutes. All pre-treatment steps were carried out at room temperature and with gentle agitation using a rocking table (Luckham, UK).

Gels were inverted before Southern transfer to reduce the likelihood of irregularities in the upper gel surface resulting in uneven DNA transfer. Transfer was carried out overnight, and the DNA was then UV cross linked with 120 mJoules of energy using a trans-illuminator (Sigma, UK) to the nylon membrane. After twice washing the membrane with 2X SSC the blot was ready for probe hybridisation and chemiluminescent detection.

4.4.1.7.3 Hybridisation and chemiluminescent detection

All hybridisation steps were carried out using a Hybaid Maxi hybridisation oven (Hybaid, UK) and standard hybridisation flasks on a rotary mount. Pre-hybridisation was carried out at 42° C for 45 minutes using 25 ml of DIG Easy Hyb solution (Roche, UK). This solution was then discarded and replaced with 10 ml of DIG Easy Hyb containing 2 µl of digoxigenin (DIG) labelled telomere probe ($TTAGGG_7$). Hybridisation was carried out at 42° C for 3 hours.

Following hybridisation, 2 stringency washes were carried out; the first consisted of two washes in 2X SSC, 0.1% SDS for 5 minutes each at room temperature. This was followed by 2 washes in 0.2X SSC, 0.1% SDS at 50° C for 20 minutes each. Gentle agitation of the membrane was performed during both stringency washes and throughout the detection procedure.

Membranes were rinsed in a washing solution (0.3% w/v Tween® 20, 0.1M maleic acid, 0.15M NaCl, pH 7.5) for 5 minutes at room temperature followed by a 30 minute incubation at room temperature in freshly prepared blocking buffer (Roche, UK) dissolved in maleic acid buffer solution (0.1M maleic acid, 0.15M NaCl, pH7.5). Blots were then incubated with a DIG-specific antibody (750 units/ml anti-digoxigenin, Fab fragments) covalently coupled to alkaline phosphatase (Anti-DIG-AP) (Roche, UK). The Anti-DIG-AP working solution was prepared to a final concentration of 75 mU/ml (1:10,000) in blocking buffer (Roche, UK). The antibody solution was centrifuged for 5 minutes at 13,000 rpm before careful pipetting from the surface of the liquid to avoid background signal being generated by aggregated antibody.

Incubation in the antibody solution was followed by two further 15 minute washes at room temperature in wash buffer (Roche, UK), followed by a 5 minute incubation in detection buffer (100 mM Tris-HCl, pH 9.5, 100mM NaCl). Excess detection buffer was briefly blotted from the membrane by placing it DNA side up on a piece of 3MM Whatman filter paper. The blot was then placed on an acetate sheet and approximately 3ml of substrate solution placed drop-wise onto the DNA side before overlaying with another acetate. This system used the chemiluminescent alkaline phosphatase substrate CSPD® (25 mM Disodium 4-chloro-3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1]decan)-4-yl) phenyl phosphate) (Roche,UK) diluted 1:100 in detection buffer. Any bubbles were carefully removed from between the sheets and the composite was incubated at room temperature for 10 minutes. Excess substrate solution was then squeezed out and the two plastic sheets sealed with sellotape. The sealed membrane was incubated at 37° C for a further 10 minutes to aid the chemiluminescent signal. The membranes were then used to generate autoradiographs. Exposure time varied between 5 and 20 minutes to achieve optimum image quality for analysis.

4.4.1.7.4 Analysis of autoradiographs

Autoradiographs were analysed by densitometry by using TotalLab v2.01 software. Each smear was divided into equally sized segments, and a densitometric reading taken from each. Mean TRF (Kb) values were determined from the densitometric readings according to the formula:

$$\text{TRF (Kb)} = \frac{\Sigma (Od_i L_i)}{\Sigma (Od_i)}$$

Where Od_i is the chemiluminescent signal and L_i is the length of the TRF fragment at position i. The calculation takes into account the higher signal intensity from larger TRF fragments because of multiple hybridisation of the telomere-specific probe. Duplicate analyses were carried out on each sample.

4.4.2 Inhibition of canine telomerase with Telomestatin

4.4.2.1 Cell Lines

For Telomestatin assays, the D17 (canine osteosarcoma) and CMT7 (canine mammary tumour) telomerase-positive cell lines, along with the telomerase-negative K9SF cells were used. Culture conditions were as described in 2.2.1: Growth and manipulation of mammalian cells.

4.4.2.2 Telomestatin treatment

Telomestatin was provided as a kind gift from Professor Kazuo Shin-ya, Institute of Molecular and Cellular Biosciences, University of Tokyo, at a concentration of 10 mM in DMSO. Telomestatin was stored at -20° C until ready for use.

4.4.2.2.1 Optimisation of telomestatin concentration

In order to optimise the concentration of telomestatin to be used in further experiments, cells were plated in 6 well plates at a cell density of 5 x 10^5 cells per well in 5 ml of complete medium. Cells were placed in an incubator at 37° C for 4 hours to settle, then withdrawn and telomestatin added before cells were replaced in the incubator.

For optimisation, D17 and CMT7 cells were incubated in telomestatin at the following concentrations in order to assess the telomestatin concentration required to give optimal telomerase inhibition: 0.1 μM, 1.0 μM, 2.0 μM, 5.0 μM and 10 μM. As a negative control, cells were also incubated with an identical volume of DMSO only. Cells were incubated in the presence of DMSO for 7 days, after which they were trypsinised, centrifuged, washed in PBS and cell pellets stored for TRAP analysis. All experiments were carried out in duplicate.

4.4.2.2.2 Prolonged treatment with telomestatin

Following the initial optimisation, D17, CMT7 and K9SF cells were plated at a concentration of 5 x 10^5 cells in 25 cm^2 flasks with 2 μM telomestatin for a total of 21 days. As negative controls, cells were also incubated in an equivalent volume of DMSO and without the presence of any additive. At 7 days, 14 days, and 21 days cells in all flasks were trypsinised, and divided into 1 x 10^6, 2 x 10^5 and 2 x 10^6 cell aliquots for FACS analysis, TRF analysis and TRAP assays respectively. In addition, cells were reseeded at each timepoint at a density of 5 x 10^5 cells per flask for continued incubation with telomestatin.

4.4.2.3 Growth Rates

Cells were counted at each passage and the population doubling calculated as detailed in 4.4.1.5: Growth Rates.

4.4.2.4 Detection of apoptosis

Apoptosis was detected in cells treated with telomestatin using the Annexin-V – FLUOS kit (Roche, UK). In the early stages of apoptosis, various changes occur at the cell surface, one of which is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidylserine (Vermes *et al.* 1995). This protein can therefore be used as a probe for PS exposure upon the outer leaflet of the cell membrane and is therefore able to detect apoptotic cells (Homburg *et al.* 1995; Koopman *et al.* 1994; Verhoven *et al.* 1995). Since necrotic cells also expose PS due to loss of membrane integrity, the simultaneous application of propidium iodide (PI) discriminates these cells by also staining DNA. In cells which are in the early stages of apoptosis, annexin is taken up, but not PI.

During all pipetting procedures prior to Annexin V staining, cells were handled gently to avoid excessive disruption of cell membranes and consequent false positive results. Annexin V staining solution was made up by diluting 2 µl of Annexin-V-FLUOS and 2 µl of Propidium Iodide solution in 100 µl of incubation buffer (Roche, UK) per sample. Cells were trypsinised for no more than 5 minutes, and 1 x 10^6 cells withdrawn. These cells were washed twice with cold PBS and centrifuged at 200g for 5 minutes. The cells were then resuspended in 100 µl of Annexin V and incubated for 15 minutes in the dark at room temperature. After this incubation, 500 µl of incubation buffer (Roche, UK) was then added to each sample and placed on ice prior to FACS analysis which was performed immediately. Samples were analysed using a FACS flow cytometer (Beckman-Coulter Inc., Miami) with an excitation wavelength of 488 nm and a 515 nm bandpass filter for fluorescein detection and a filter >600 nm for PI detection. Analysis was performed using Epics XL™ (Beckman-Coulter, Miami) software.

4.4.2.5 Telomerase repeat amplification protocol

TRAP assays were performed on 2 x 10^5 cell pellets as described in 2.2.10.1: Telomerase repeat amplification protocol.

4.4.2.6 Telomere restriction fragment (TRF) analysis

TRF telomere length analysis was performed on genomic DNA prepared from cell pellets as described in section 4.4.1.7: Telomere Length Analysis.

4.4.3 Statistical Analysis

For comparison of telomere lengths in cells treated with DN-hTERT, the Wilcoxon Signed Rank Test was used. For each cell line paired values were taken at each passage number with positive values for pCINeo-DN-hTERT transfected cells and negative values for pCINeo (negative control) transfected cells. A p value of <0.05 was taken as the limit of statistical significance.

4.5 Results

4.5.1 Inhibition of canine telomerase with the dominant-negative mutant DNhTERT

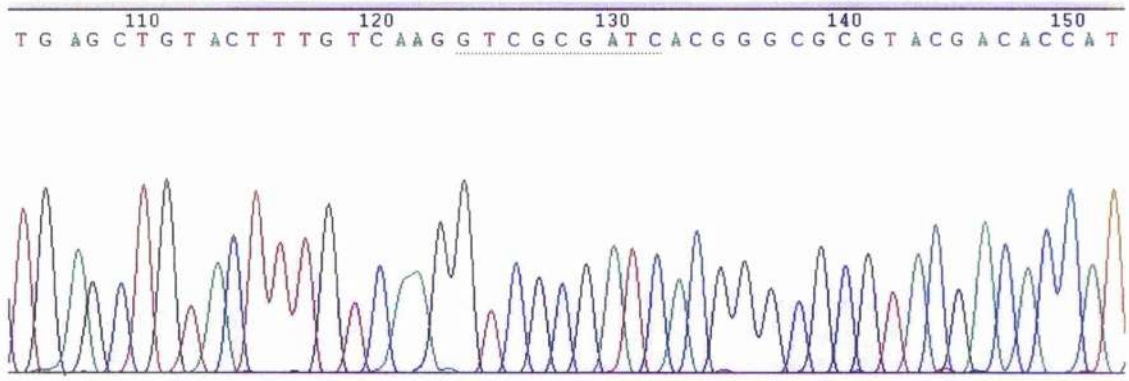
4.5.1.1 Generation of pCINeo-DNhTERT construct

Restriction digestion was performed using the *EcoR*I and *Sal*I enzymes to subclone DNhTERT from the pBabePuro vector into pCINeo. The 3.6 Kb fragment comprising DNhTERT was successfully removed from pBabePuro and ligated into pCINeo using T4 DNA Ligase with both 1:1 and 1:3 vector:insert ratios. Following transformation into One Shot™ TOP10 cells, pCINeo containing DNhTERT was then purified and sequenced to confirm the correct insert was present (Figure 4.3, Figure 4.4).

4.5.1.2 Generation of stable transfectants

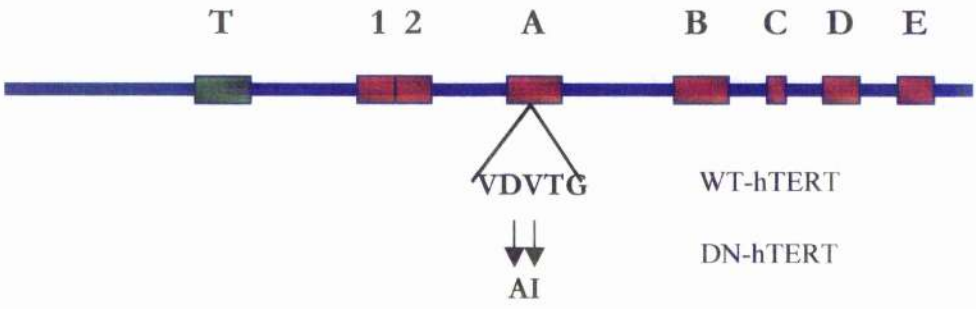
Following synthesis of the DNhTERT construct, stable transfectants containing pCINeo-DNhTERT and empty pCINeo (negative control) were established in both the CMT7 and D17 cell lines. Following the addition of G418 to flasks and reseeding at different dilutions, the 1:10 flask was selected as having the most appropriate cell density in both cell lines. Initial selection of cells was performed with a G418 concentration of 400 μg/ml which then was increased to 600 μg /ml. Stable transfectants were also generated in the K9SF cells using the pCINeo-DNhTERT construct. Untransfected (wild-type) K9SF cells were also maintained as negative control. Despite repeated attempts, it was not possible to generate a stable transfectant using the empty pCINeo vector in K9SF cells due to slow growth and problems with fungal contamination.

Figure 4.3: Chromas file sequencing of pCINeoDNhTERT using DNHT001F Primer and comparison with wild type hTERT sequence. Mutated site is underlined. Sequence from wild type (WT) hTERT in the region of the mutation from genbank accession No. AF015950 shown boxed for comparison.



Sequence of WT-hTERT in the mutated region from Genbank file AF015950 together with translation

Figure 4.4: Illustration of position of telomerase specific (T) and reverse transcriptase motifs (1,2, A-E) within hTERT mRNA transcript, together with translation of mutated sequence in RT motif A used to generate the DNhTERT construct.



4.5.1.3 Confirmation of transfection

Following the generation of stable transfectants, cells were harvested for RT-PCR confirmation of transfection. RNA extraction was performed using RNAWiz® (Ambion, UK) and cDNA synthesis was performed using random hexamers. PCR amplification using the DNHT001F/R primer pair revealed a 450 bp band in the pCINeo-DNhTERT but not negative control cells (Figure 4.5). This band was gel purified and sequenced using the DNHT001F and DNHT001R primers. Sequencing confirmed that the pCINeo-DNhTERT-transfected cells contained the mutated sequence.

4.5.1.4 Growth rate analysis

Growth rates of all cell lines were followed over 50 population doublings to assess the effect of the DNhTERT construct on the growth of telomerase-positive D17 and CMT7 cells. These are shown in Figure 4.6. No significant difference was seen between the pCINeo-DNhTERT-transfected cells and negative controls. Interestingly, in the K9SF cells, transfection with pCINeo-DNhTERT resulted in a marked increase in growth rate (Figure 4.7). In addition, the morphology of fibroblasts altered, and rather than assuming a spindle shape, cells became more rounded with altered growth. With further passaging, cells also showed a tendency to abandon growth in a monolayer and to form 'clumps' or 'spheroids' of cells. Examples are shown in Figure 4.8. Untransfected wild type cells were maintained up to passage 9 (PD6) before growth slowed and cells exhibited signs of senescence.

Figure 4.5: Representative 1.5% agarose gel electrophoresis of RT-PCR confirmation of transfection of D17 and CMT7 cells with pCINeo-DNhTERT. A: PCR amplification of cDNA using DNHT001F/R primers, B: PCR amplification of cDNA using CycloF/R primers

Lane:

1 D17 transfected with pCINeo-DNhTERT

2 D17 transfected with empty pCINeo

3 CMT7 transfected with pCINeo-DNhTERT

4 CMT7 transfected with empty pCINeo

5 – 8 Negative controls: RNA template used for amplification in place of cDNA

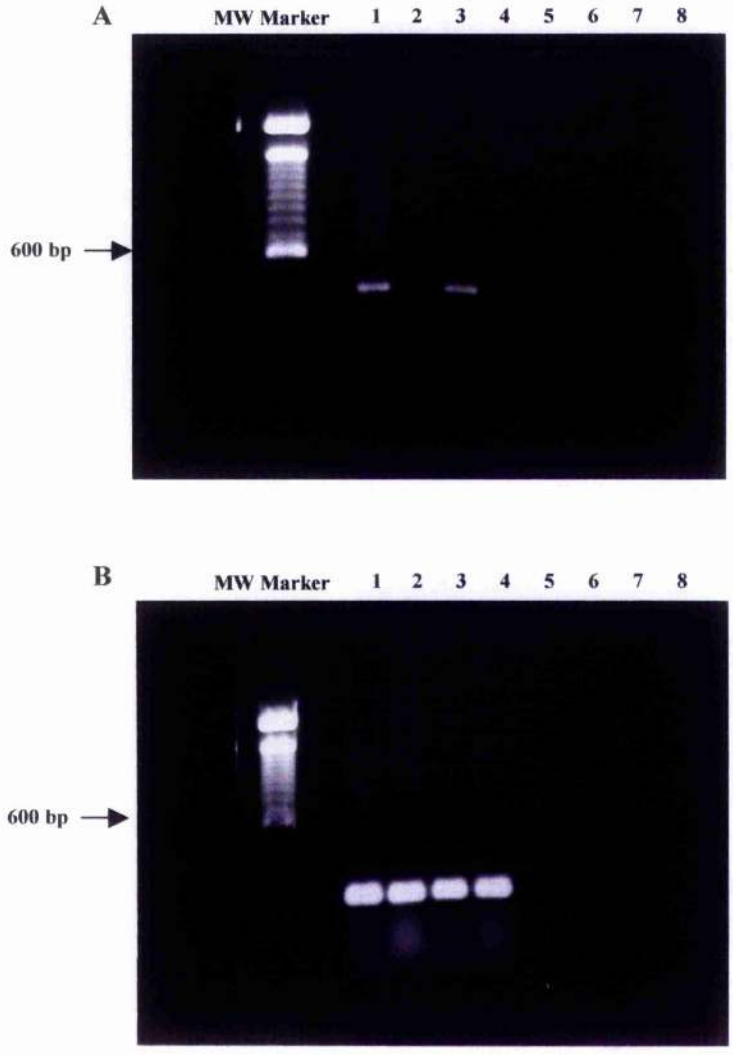


Figure 4.6: Growth rates of CMT7 and D17 Cells transfected with either pCINeoDNhTERT (DNHT) or empty pCINeo (NC).

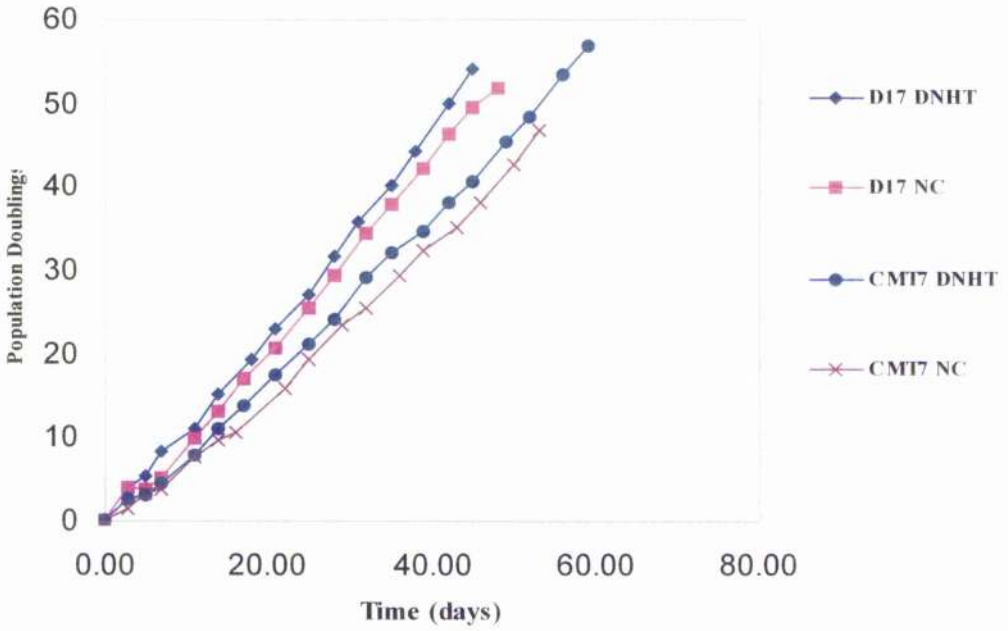


Figure 4.7: Growth rate of K9SF cells transfected with pCINeoDNhTERT. Also shown is the growth rate of untransfected (WT) K9SF cells. These cells were maintained up to PD 6 before growth ceased and signs of senescence appeared.

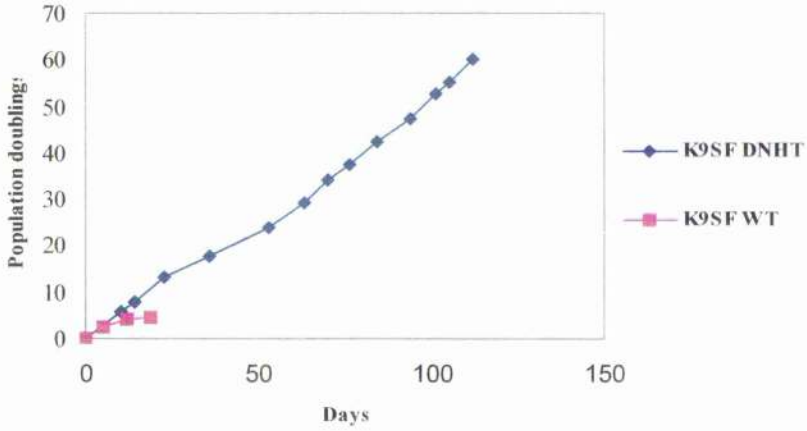
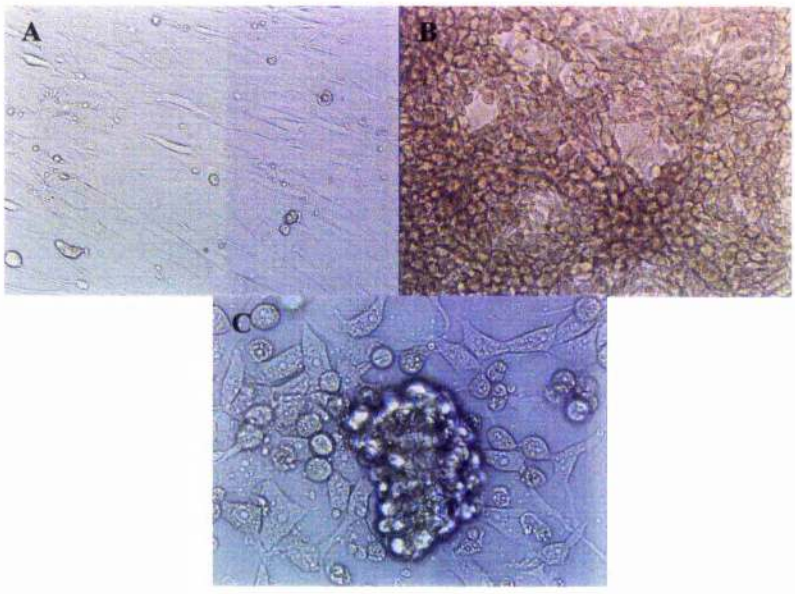


Figure 4.8: Illustration of morphological features of K9SF stable transfectants.

Note spindle shaped appearance and growth pattern of wild-type K9SF cells (A) compared with more rounded cell morphology and disordered growth of K9SF cells transfected with pCINeoDNhTERT (B and C). In addition, later passage cells showed a tendency to grow as spheroids rather than in monolayers (C). Magnification: A: x 10, B: x 10, C: x40.



4.5.1.5 Telomerase activity

TRAP assays were performed on CMT7, D17 and K9SF cells following the establishment of stable cell lines after approximately 25 population doublings had elapsed to evaluate the telomerase activity in each cell line following transfection with either pCINeoDNhTERT construct or empty pCINeo (negative control). CMT7 and D17 cells transfected with pCINeoDNhTERT were shown to have telomerase activity at similar levels to negative control cells transfected with empty pCINeo. Interestingly, K9SF cells transfected with pCINeoDNhTERT also showed strong telomerase activity with higher levels of activity than the CMT7 and D17 cells. These results are shown in Table 4.2 and Table 4.3 respectively.

Table 4.2: Telomerase repeat amplification protocol results of CMT7 and D17 cells transfected with pCINeoDNhTERT (DNHT) or pCINeo (NC)

A_S is the absorbance of the sample, A_{S0} is the absorbance of the heat inactivated version of each sample, ΔA_S is the corrected absorbance of each sample (calculated by subtracting the mean of all heat inactivated samples ($\bar{A}_{S0}$)from A_S), $\Delta A_S/\bar{A}_{S0}$ is the ratio of sample to background activity and RTA is the telomerase activity relative to the positive control (ΔA_S/Corrected absorbance of positive control). Samples were considered telomerase-positive if the corrected absorbance (ΔA_S) was greater than twice the background activity, and if $\Delta A_S/\bar{A}_{S0}$ was greater than 2.0. The results shown are the mean of at least two experiments, and the assay gave a mean background absorbance of 0.033 and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS})$ value of 0.34 after 10 minutes of colour development using the low activity positive control.

Cell Line	A_S	A_{S0}	ΔA_S	$\Delta A_S/\bar{A}_{S0}$	RTA.	Result
D17 DNHT	0.18	0.03	0.15	4.5	0.44	POS
D17 NC	0.18	0.04	0.15	4.4	0.44	POS
CMT7 DNHT	0.44	0.03	0.41	12.5	1.2	POS
CMT7 NC	0.29	0.04	0.26	7.7	0.76	POS

Table 4.3: TRAP results for K9SF cells transfected with pCINeoDNhTERT

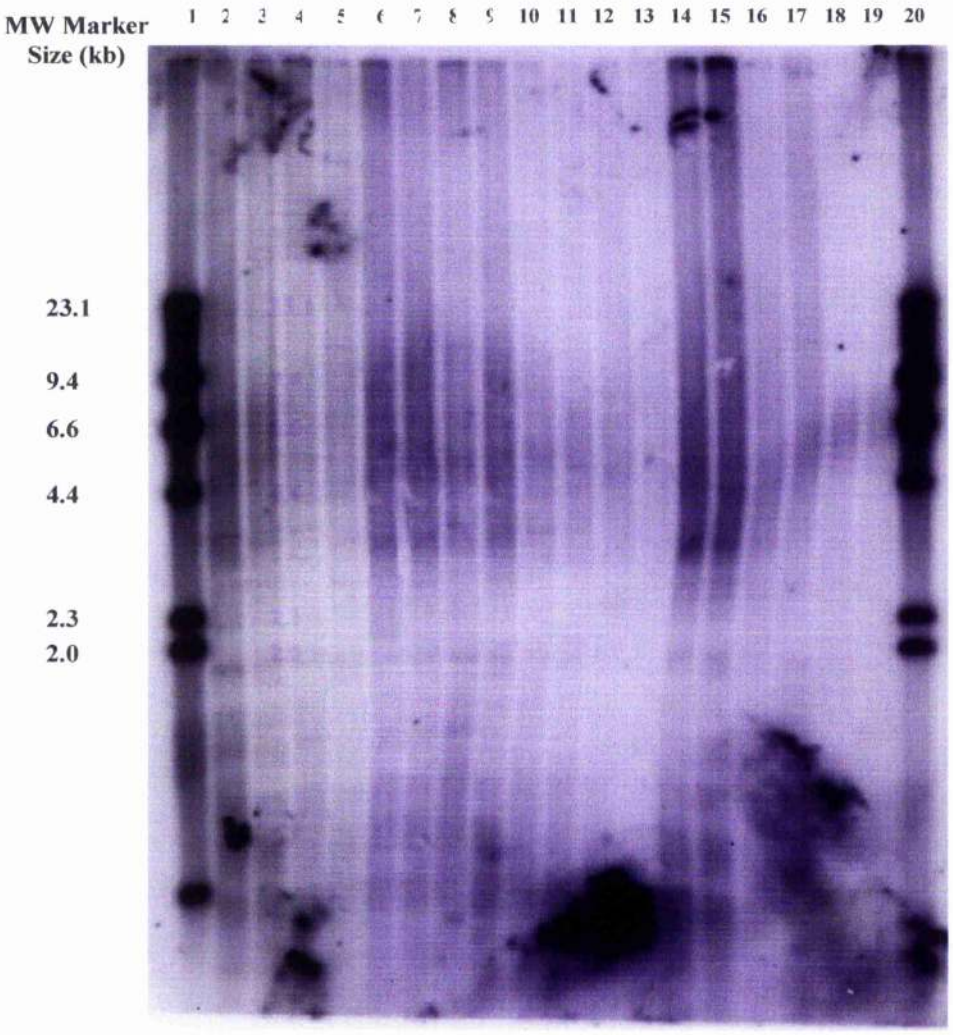
A_S is the absorbance of the sample, A_{S0} is the absorbance of the heat inactivated version of each sample, ΔA_S is the corrected absorbance of each sample (calculated by subtracting the mean of all heat inactivated samples ($\bar{A}_{S0}$)from A_S), $\Delta A_S/\bar{A}_{S0}$ is the ratio of sample to background activity and RTA is the telomerase activity relative to the positive control (ΔA_S/Corrected absorbance of positive control). Samples were considered telomerase-positive if the corrected absorbance (ΔA_S) was greater than twice the background activity, and if $\Delta A_S/\bar{A}_{S0}$ was greater than 2.0. The results shown are the mean of at least two experiments, and the assay gave a mean background absorbance of 0.105 and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$) value of 0.51 after 10 minutes of colour development using the high activity positive control.

Cell Line	A_S	A_{S0}	ΔA_S	$\Delta A_S/\bar{A}_{S0}$	RTA	Result
K9SF DNHT	3.44	0.10	3.35	31.9	6.57	POS

4.5.1.6 Telomere Length Analysis

TRF analysis of telomere length was performed on D17 and CMT7 cell lines. Samples for TRF analysis were harvested every 3-4 passages over the course of the experiment. A representative picture of typical TRFs is shown in Figure 4.9. Table 4.4 shows the mean TRF analysis of all tumour cell lines. No significant change in the mean telomere length of any cell line measured was seen over the course of the experiment. Using the Wilcoxon Signed Rank Test, no statistical difference was noted between telomere length of pCINeo-DNhTERT transfected cells and negative controls for either the CMT7 (p=0.62) or D17 (p= 0.25) cell lines.

Figure 4.9: Representative example of TRF Autoradiograph. CMT7 cells transfected with either pCINeoDNhTERT or empty pCINeo. Samples are run in duplicate in adjacent lanes.

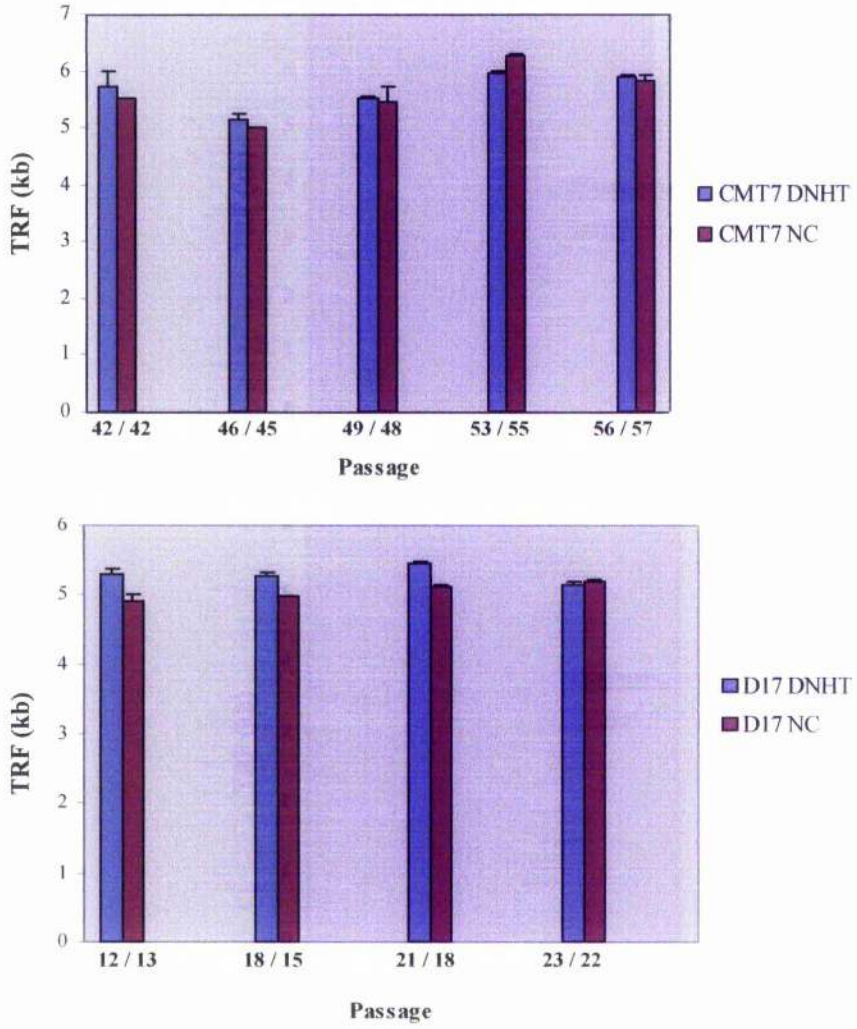


Lane	Construct	Passage
1,20	Molecular Weight marker	
2-3	pCINeo DNhTERT	42
4-5	pCINeo DNhTERT	46
6-7	pCINeo DNhTERT	49
8-9	pCINeo DNhTERT	53
10-11	pCINeo DNhTERT	56
12-13	pCINeo	42
14-15	pCINeo	45
16-17	pCINeo	48
18-19	pCINeo	51

Table 4.4: TRF analysis of CMT7 and D17 cells transfected with DNhTERT and negative controls

Cell Line	Construct	Passage Number	Mean TRF length (kb)
CMT7	DNhTERT	42	5.73
CMT7	DNhTERT	46	5.15
CMT7	DNhTERT	49	5.51
CMT7	DNhTERT	53	5.95
CMT7	DNhTERT	56	5.90
CMT7	NC	42	5.52
CMT7	NC	45	4.99
CMT7	NC	48	5.46
CMT7	NC	51	6.25
CMT7	NC	55	5.82
D17	DNhTERT	12	5.30
D17	DNhTERT	18	5.26
D17	DNhTERT	21	5.44
D17	DNhTERT	23	5.14
D17	NC	13	4.91
D17	NC	15	4.97
D17	NC	18	5.12
D17	NC	22	5.19

Figure 4.10: Graphs showing mean TRF lengths of CMT7 (A) and D17 (B) cells transfected with pCINeoDNhTERT (DNHT) and empty pCINeo (NC). Each graph represents the mean of two samples, each run in duplicate.



4.5.2 Inhibition of canine telomerase with Telomestatin

4.5.2.1 Optimisation of telomestatin concentration

In order to assess the optimal concentration to treat D17 and CMT7 cells for prolonged treatment with telomestatin, cells were seeded into 6-well plates at a density of 5 x 10^5 cells per well and treated for 7 days with varying concentrations of telomestatin in DMSO. Cells treated with 5 μM and 10 μM of telomestatin were found to have undergone cell death within 3 days of the addition of telomestatin, with all cells rounding up and lifting off the base of the wells. Following trypsinisation and staining with trypan blue at 7 days, staining confirmed that all of these cells were non-viable. All cells treated with telomestatin and negative controls were prepared for TRAP assays as described in section 4.4.1.6.1: Sample preparation.

4.5.2.1.1 TRAP Assays

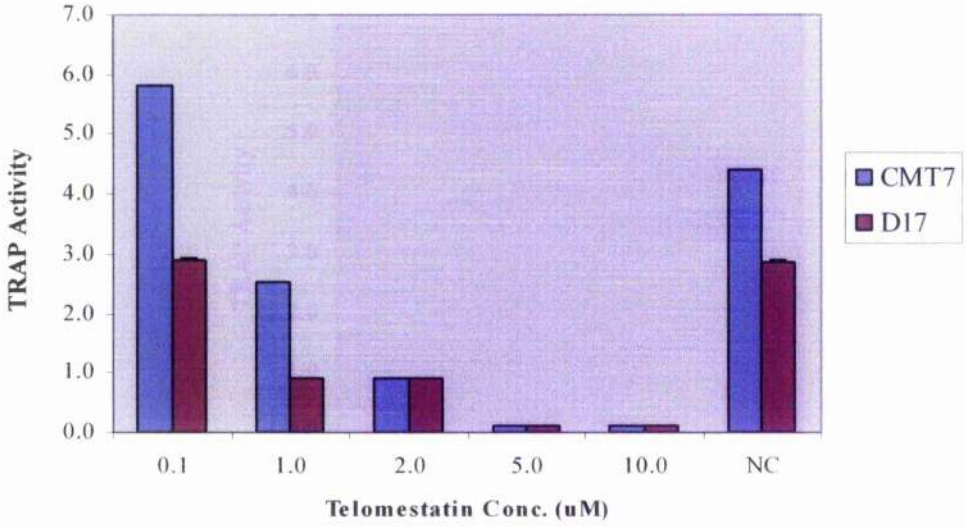
TRAP assays were performed on all cells following 7 days of treatment with telomestatin at varying concentration. The results of these are shown in Table 4.5, Figure 4.11. TRAP assays showed that in the 0.1 μM and negative control cells, no significant reduction in telomerase activity was seen. Cells treated with 5 μM and 10 μM telomestatin were found to have no detectable telomerase activity. Cells treated with 2 μM telomestatin showed approximately an 80% decrease in telomerase activity for CMT7 cells and approximately 67% decrease for D17 cells as compared with 0.1 μM-treated and negative control cells. Interestingly, the telomerase activity in D17 cells was consistently lower than that of CMT7 cells at all telomestatin concentrations with the exception of 2.0 μM.

Table 4.5: TRAP assay results of treatment of CMT7 and D17 cells with telomestatin at concentrations of 0.1, 1.0, 2.0, 5.0, 10.0 μM and negative control (NC) cells for 7 days.

A_S is the absorbance of the sample, A_{S0} is the absorbance of the heat inactivated version of each sample, ΔA_S is the corrected absorbance of each sample (calculated by subtracting the mean of all heat inactivated samples ($\bar{A}_{S0}$)from A_S), $\Delta A_S/\bar{A}_{S0}$ is the ratio of sample to background activity and RTA is the telomerase activity relative to the positive control (ΔA_S/Corrected absorbance of positive control). Samples were considered telomerase-positive if the corrected absorbance (ΔA_S) was greater than twice the background activity, and if $\Delta A_S/\bar{A}_{S0}$ was greater than 2.0. Using these criteria, samples were designated positive (POS) or negative (NEG). The results shown are the mean of at least two experiments, and the assay gave a mean background absorbance of 0.081 and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS}$ value of 0.34 after 10 minutes of colour development using the low activity positive control.

Cell Line	Telomestatin Concentration (μM)	A_S	A_{S0}	ΔA_S	$\Delta A_S/\bar{A}_{S0}$	RTA	Result
CMT7	0.1	0.51	0.08	0.43	5.3	1.26	POS
CMT7	1.0	0.25	0.07	0.18	2.2	0.53	POS
CMT7	2.0	0.14	0.07	0.07	0.89	0.21	NEG
CMT7	5.0	0.07	0.08	-0.01	0.1	-0.03	NEG
CMT7	10.0	0.07	0.08	-0.01	0.1	-0.03	NEG
CMT7	NC	0.39	0.07	0.32	4.0	0.94	POS
D17	0.1	0.35	0.1	0.25	3.1	0.74	POS
D17	1.0	0.15	0.09	0.06	0.7	0.18	NEG
D17	2.0	0.14	0.07	0.07	0.89	0.21	NEG
D17	5.0	0.07	0.08	-0.01	0.1	-0.03	NEG
D17	10.0	0.07	0.09	-0.02	0.1	-0.06	NEG
D17	NC	0.24	0.07	0.17	2.1	0.50	POS

Figure 4.11: Graph showing TRAP activity of CMT7 and D17 cells treated with telomestatin at concentrations of 0.1, 1.0, 2.0, 5.0, 10 μM and untreated (NC) cells.



4.5.2.2 Prolonged treatment with telomestatin

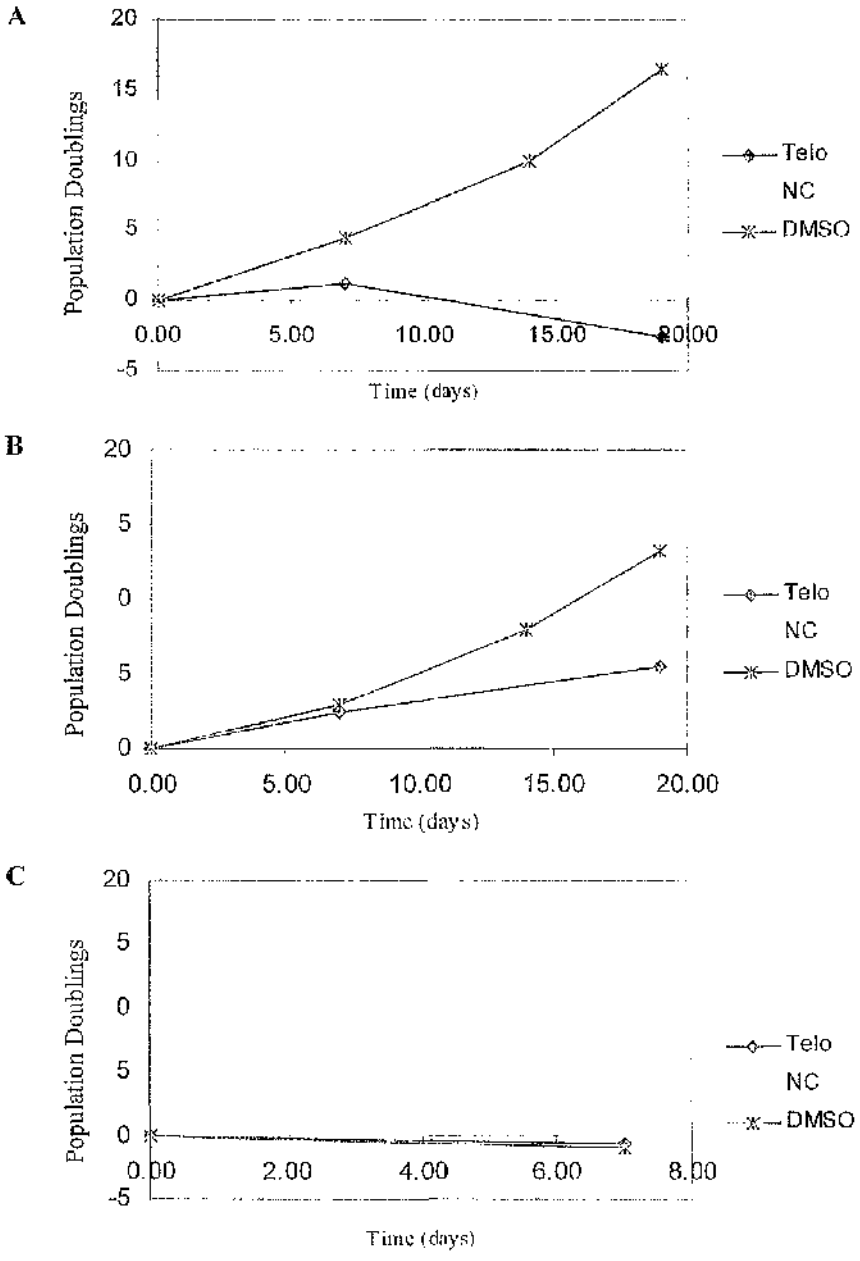
Following initial optimisation, the concentration of telomestatin that resulted in greatest telomerase activity reduction without causing cell death at 7 days was selected for longer term treatment of D17 and CMT7 cells. Cells were therefore seeded into 25 cm^2 flasks at a density of 5 x 10^5 cells per flask and treated with telomestatin at a concentration of 2.0 μM for the duration of the experiment. In addition, control cells were cultured in the absence of telomestatin, and with the same volume DMSO as telomestatin as a second negative control. K9SF cells were also maintained with the same conditions as a further negative control. All samples were maintained in duplicate. At the end of 7 days, cells were trypsinised, counted to assess growth rate, and re-seeded at a density of 5 x 10^5 cells per flask. In addition, aliquots of 2 x 10^6, 1 x 10^5 and 2 x 10^5 cells were removed for TRF analysis, apoptosis and TRAP assays respectively. The same process was repeated at 14 days. A final trypsinisation was performed at 20 days and the samples retained for TRF, apoptosis and TRAP studies.

4.5.2.2.1 Growth Rates

Growth rates of all cells are shown in Figure 4.12. Both CMT7 and D17 telomestatin-treated cells showed slower growth than negative controls (untreated and DMSO-treated cells), although this was much more prominent in D17 than CMT7 cells. When flasks were examined prior to trypsinisation at day 14, it was found that telomestatin-treated CMT7 and D17 cells had not reached confluence, and for this reason these flasks were not trypsinised at 14 days but rather at 20 days. These cells were rinsed, and fresh medium with telomestatin was added. Negative control flasks, however, were trypsinised at both 14 and 20 days. At 20 days, a significant proportion of D17 cells were found to have undergone apoptosis, with cells rounding up and no longer adherent to tissue culture flasks. This had also occurred in CMT7 cells, although a greater percentage of cells were still adherent.

K9SF cells were found to grow very slowly in the culture conditions used. For this reason, flasks of these cells were only maintained for 7 days. However, all flasks showed similar growth rates. At the end of 7 days these flasks were trypsinised and samples removed for apoptosis studies.

Figure 4.12: Growth rates of CMT7, D17 and K9SF cells following treatment with 2.0 uM telomestatin. A: D17 growth rates, B: CMT7 growth rates, C: K9SF cell rates. Telo: telomestatin-treated cells. NC: negative control (untreated) cells. DMSO: negative control (DMSO-treated) cells. Each graph shows the mean for two independent experiments.



4.5.2.2.2 Telomerase activity

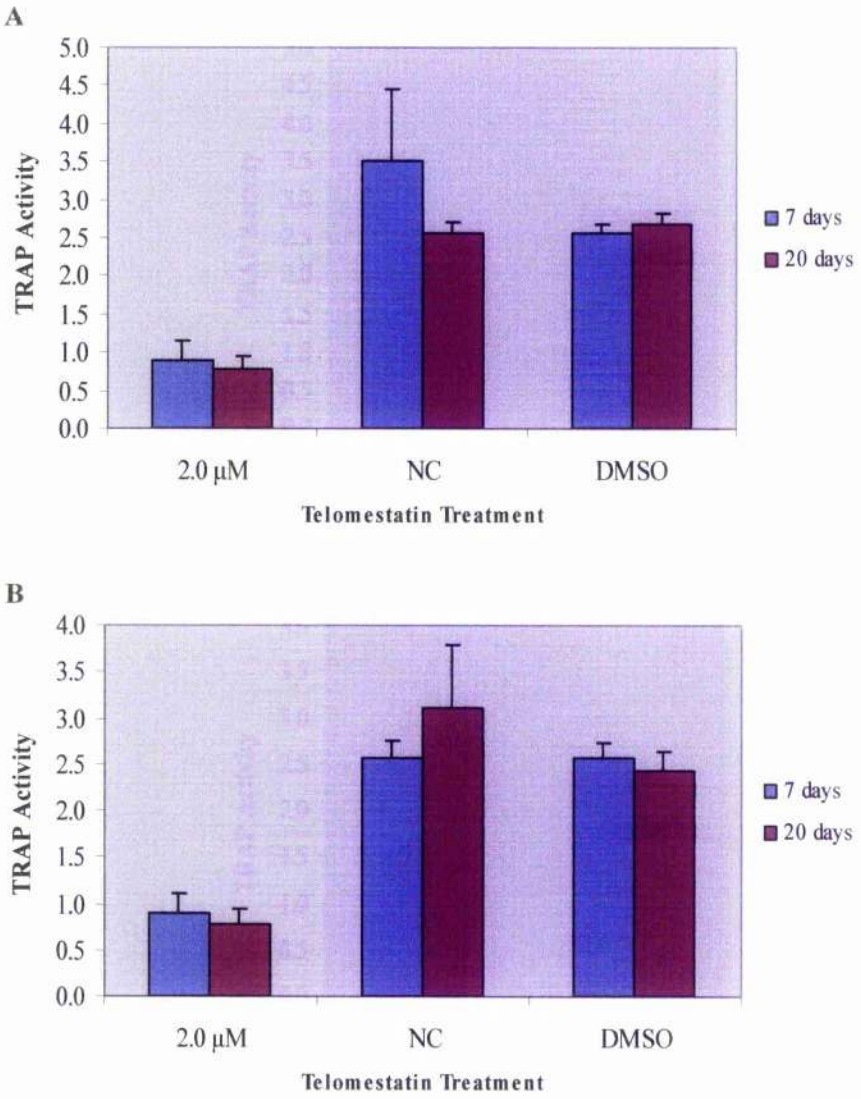
TRAP assays were performed on D17 and CMT7 cells at 7 days and at 20 days, due to the pronounced slowing of growth that occurred. TRAP results showed that all telomestatin-treated cells were negative for telomerase activity at 7 days and at 20 days, with a reduction in TRAP activity of 65% and 75% at day 7 for D17 and CMT7 cells respectively (vs untreated negative controls) and with a reduction of 68% and 65% at day 20. In contrast, all negative control cells were positive at these time points (Table 4.6, Figure 4.13).

Table 4.6: TRAP activity of CMT7 and D17 cells treated with telomestatin at 2.0 μM for 20 days.

A_S is the absorbance of the sample, A_{S0} is the absorbance of the heat inactivated version of each sample, ΔA_S is the corrected absorbance of each sample (calculated by subtracting the mean of all heat inactivated samples ($\bar{A}_{S0}$)from A_S), $\Delta A_S/\bar{A}_{S0}$ is the ratio of sample to background activity and RTA is the telomerase activity relative to the positive control (ΔA_S/Corrected absorbance of positive control). Samples were considered telomerase-positive if the corrected absorbance (ΔA_S) was greater than twice the background activity, and if $\Delta A_S/\bar{A}_{S0}$ was greater than 2.0. Using these criteria, samples were designated positive (POS) or negative (NEG). The results shown are the mean of at least two experiments, and the assay gave a mean background absorbance of 0.074 and a $(A_{TS8}-A_{TS8,0})/A_{TS8,IS})$ value of 0.27 after 10 minutes of colour development using the low activity positive control.

Cell Line	Time (days)	Telomestatin Concentration (μM)	A_S	A_{S0}	ΔA_S	$\Delta A_S/\bar{A}_{S0}$	RTA	Result
CMT7	7	2.0	0.14	0.07	0.07	0.89	0.26	NEG
CMT7	7	NC	0.33	0.07	0.26	3.51	0.96	POS
CMT7	7	DMSO	0.27	0.07	0.20	2.56	0.74	POS
CMT7	20	2.0	0.14	0.08	0.06	0.77	0.22	NEG
CMT7	20	NC	0.27	0.08	0.19	2.57	0.70	POS
CMT7	20	DMSO	0.29	0.08	0.21	2.68	0.78	POS
D17	7	2.0	0.14	0.07	0.07	0.89	0.26	NEG
D17	7	NC	0.29	0.09	0.20	2.56	0.74	POS
D17	7	DMSO	0.30	0.10	0.20	2.56	0.74	POS
D17	20	2.0	0.14	0.08	0.06	0.77	0.22	NEG
D17	20	NC	0.31	0.07	0.24	3.11	0.89	POS
D17	20	DMSO	0.27	0.08	0.19	2.43	0.70	POS

Figure 4.13: Graph showing telomerase activity of CMT7 (A) and D17 (B) cells treated with telomestatin at 2.0 μM for 20 days. NC: negative control cells, DMSO: negative control cells treated with DMSO.



4.5.2.2.3 Telomere length analysis

To assess whether the reduction in telomerase activity was associated with telomeric attrition, duplicate samples from CMT7 and D17 cells were analysed to examine the effect of telomerase inhibition on telomere length. The mean TRF value was obtained by averaging the mean TRFs obtained from two independent experiments, with each sample run out in duplicate. Results are shown in Table 4.7, Figure 4.14. In addition, a representative autoradiograph is shown in Figure 4.15.

TRF analysis showed that telomere length of negative control (untreated) CMT7 cells was 4.5 and 4.3 kb after 7 and 20 days respectively. Telomere lengths of negative control (DMSO-treated) CMT7 cells at equivalent timepoints were 3.99 and 4.33 kb. In contrast, the telomere length of CMT7 cells treated with telomestatin was shorter, at 3.15 and 3.0 kb after 7 and 20 days respectively. The mean telomere length of negative control (untreated) D17 cells was 5.34 and 4.87 kb in length after 7 and 20 days respectively. The mean telomere length of negative control (DMSO-treated) D17 cells was 5.47 and 4.87 kb in length after 7 and 20 days respectively. In contrast, the mean telomere length of telomestatin-treated D17 cells was 3.00 and 2.95 kb after 7 and 20 days. These results show that telomestatin treatment resulted in telomere shortening in both CMT7 and D17 cell lines. In CMT7 cells, telomeres shortened by 1.45/1.62 Kb at day 7/20 relative to negative controls – a change of approximately 30-40%. In D17 cells, telomeres shortened by 2.01/1.35 Kb at day 7/20 relative to negative controls (untreated) – a change of approximately 30-40%. Therefore, in both cell lines telomere shortening occurred to a similar degree. However, no further shortening appears to have occurred after day 7 since there is minimal difference between the telomere lengths at the two timepoints.

Table 4.7: TRF Analysis of CMT7 and D17 cells treated with telomestatin at 2.0 μM for 20 days

Cell Line	Time (days)	Telomestatin Concentration (μM)	A_S	Mean TRF length (kb)
CMT7	7	2.0	0.14	3.15
CMT7	7	NC	0.33	4.50
CMT7	7	DMSO	0.27	3.99
CMT7	20	2.0	0.14	2.68
CMT7	20	NC	0.27	4.30
CMT7	20	DMSO	0.29	4.13
D17	7	2.0	0.14	3.00
D17	7	NC	0.29	5.01
D17	7	DMSO	0.30	5.47
D17	20	2.0	0.14	2.95
D17	20	NC	0.31	4.30
D17	20	DMSO	0.27	4.13

Figure 4.14: Mean telomere length of CMT7 (A) and D17 (B) cells treated with telomestatin.

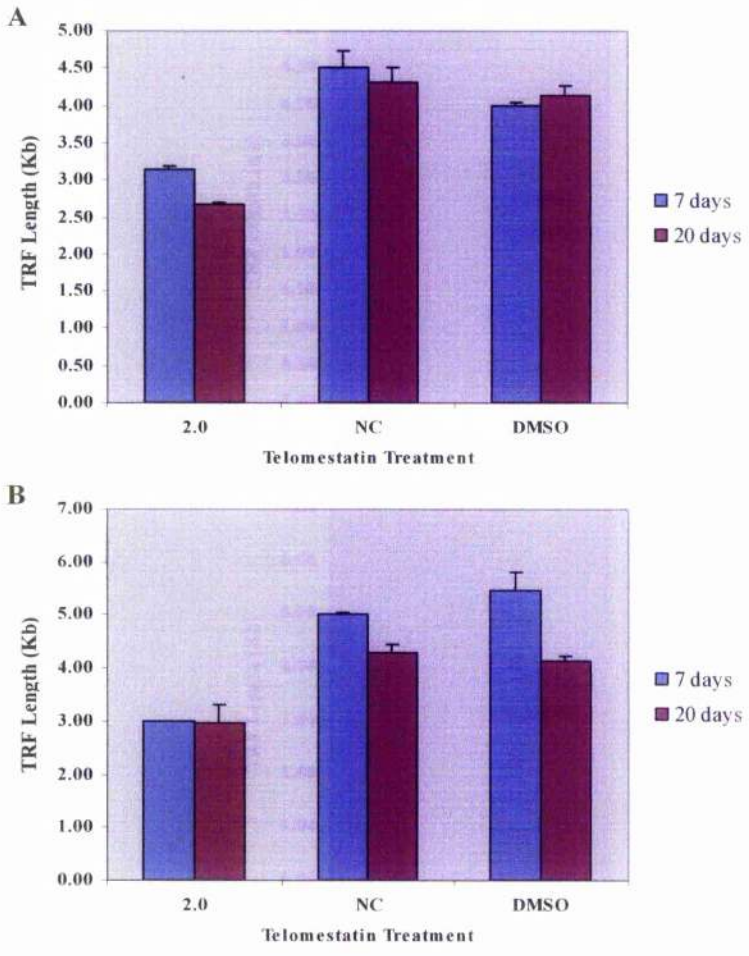
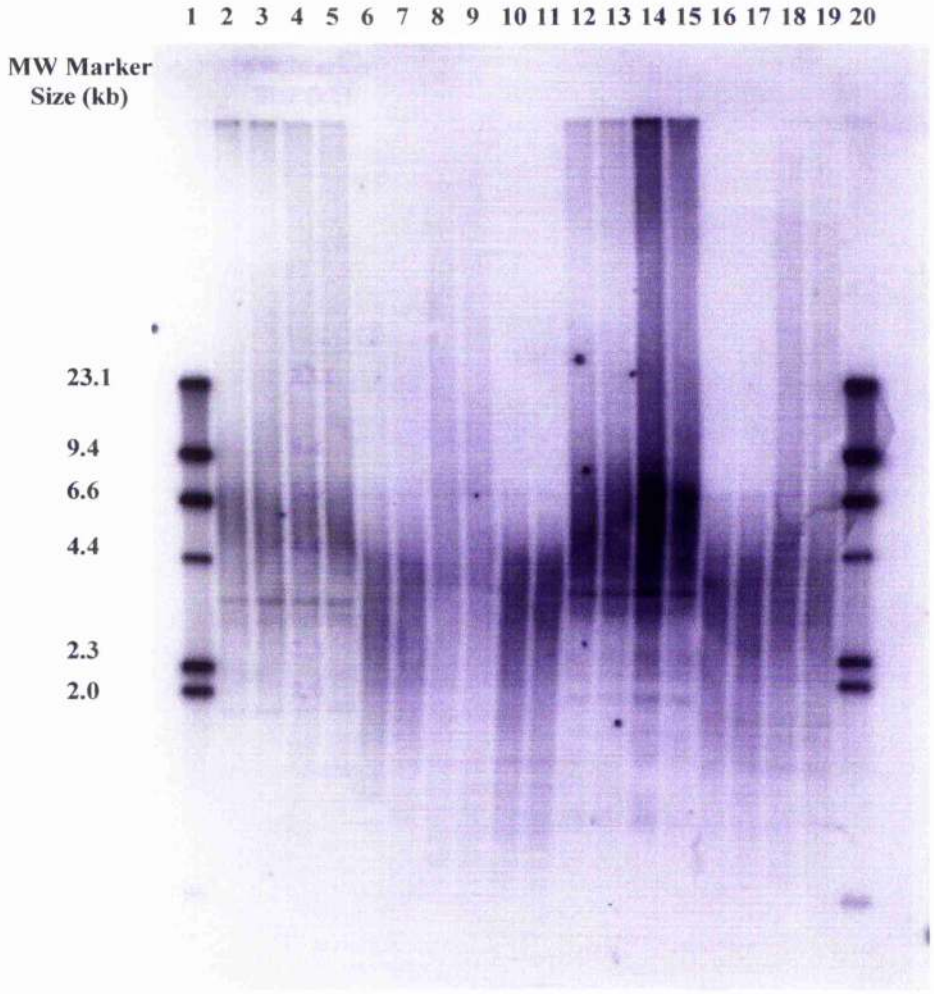


Figure 4.15: Representative TRF autoradiograph of CMT7/D17 cells following treatment with telomestatin at 2.0 μM for 20 days

Lane	Cell Line	Treatment	Time
1,20	Mass marker		
2-3	D17	Negative control (untreated)	7 days
4-5	D17	Negative control (untreated)	20 days
6-7	D17	Telomestatin	20 days
8-9	D17	Negative control (DMSO)	20 days
10-11	CMT7	Telomestatin	7 days
12-13	CMT7	Negative control (untreated)	7 days
14-15	CMT7	Negative control (untreated)	7 days
16-17	CMT7	Telomestatin	20 days
18-19	CMT7	Negative control (DMSO)	20 days



4.5.2.2.4 Apoptosis following prolonged treatment with telomestatin

To assess the ability of telomestatin to induce apoptosis in telomerase-positive cells, annexin and PI staining were performed on CMT7, D17 and K9SF cells following 7 days' exposure to 2 μM of telomestatin, and on CMT7 and D17 cells following 20 days' exposure to telomestatin. Apoptosis was assessed by counting the percentage of cells positive for annexin staining but negative for PI staining. The results of annexin staining for all cells are shown in Table 4.8 and Figure 4.16, together with representative graphs in Figure 4.17. At 7 days, D17 cells treated with telomestatin showed higher levels of apoptosis than negative controls (both untreated and DMSO-treated cells), with mean annexin staining of 3.2% as compared with 1.05% for untreated cells and 1.2% for DMSO-treated cells. CMT7 cells showed mean annexin staining of 3.75% as compared with 1.86% for untreated cells and 1.65% for DMSO-treated cells. Canine skin fibroblasts showed similar levels of apoptosis in all samples, with annexin staining percentages of between 8.0 and 8.9% for telomestatin, untreated cells and DMSO-treated cells. However, cell death, as assessed by the percentage of cells staining positively for both annexin and PI was found in all flasks, as assessed by the number of cells staining negative for both annexin and PI (Table 4.8). This was particularly marked in K9SF cells.

At 20 days, the levels of apoptosis in D17 cells treated with telomestatin were significantly higher than at 7 days, with 18.65% of cells displaying annexin staining as compared with 0.25% of negative controls and 0.1% of DMSO-treated cells. By comparison, CMT7 cells showed less apoptosis, although still higher than at 7 days, with 6.4% of telomestatin-treated cells annexin-positive as compared with 0.5% of negative controls and 0.4% of DMSO cells. In all CMT7 flasks at both 7 days and 20 days, a significant proportion of cells had undergone either late apoptosis or cell death, with only 74 – 80% of cells showing survival. D17 flasks showed some cell death at 7 days, with mean survival of approximately 80 – 87%, but in both negative control flasks at 20 days, mean survival was 95.3% and 96.4% for non-treated and DMSO-treated flasks respectively. However, in the telomestatin-treated D17 and CMT7 cells, mean survival at 20 days was considerably lower than negative control cells, with only 34.6 % and 58.9% cell survival in D17 and CMT7 cells respectively.

Table 4.8 Apoptosis and survival in D17, CMT7 and K9SF cells after 7 and 20 days exposure

Cell Line	Time (days)	Telomestatin Concentration (μM)	Apoptosis (%)	Survival (%)
D17	7	2.0	3.2	80.4
D17	7	NC	1.05	87.15
D17	7	DMSO	1.2	86.4
D17	20	2.0	18.65	34.6
D17	20	NC	0.25	95.25
D17	20	DMSO	0.1	96.4
CMT7	7	2.0	3.75	73.85
CMT7	7	NC	1.86	75.3
CMT7	7	DMSO	1.65	73.7
CMT7	20	2.0	6.4	58.9
CMT7	20	NC	0.5	80.5
CMT7	20	DMSO	0.4	74.1
K9SF	7	2.0	8.9	39.9
K9SF	7	NC	8	44.4
K9SF	7	DMSO	8.4	50.6

Figure 4.16: Graph showing apoptosis (%) in D17 and CMT7 cells treated with telomestatin (Telomestatin), negative control – untreated (NC) and negative control - DMSO-treated (DMSO) cells following 7 and 20 days' exposure.

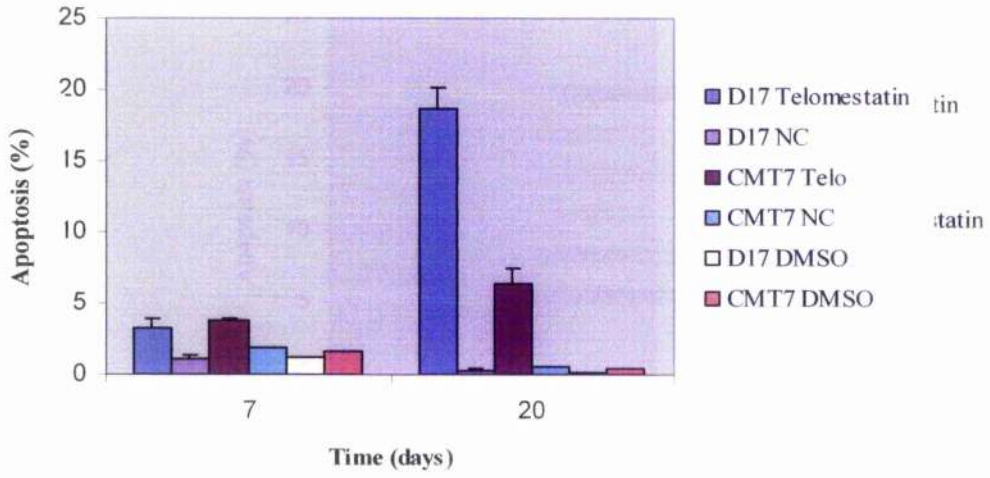


Figure 4.17: Representative FACS analysis of Annexin and PI staining in CMT7 cells at 7 and 20 days

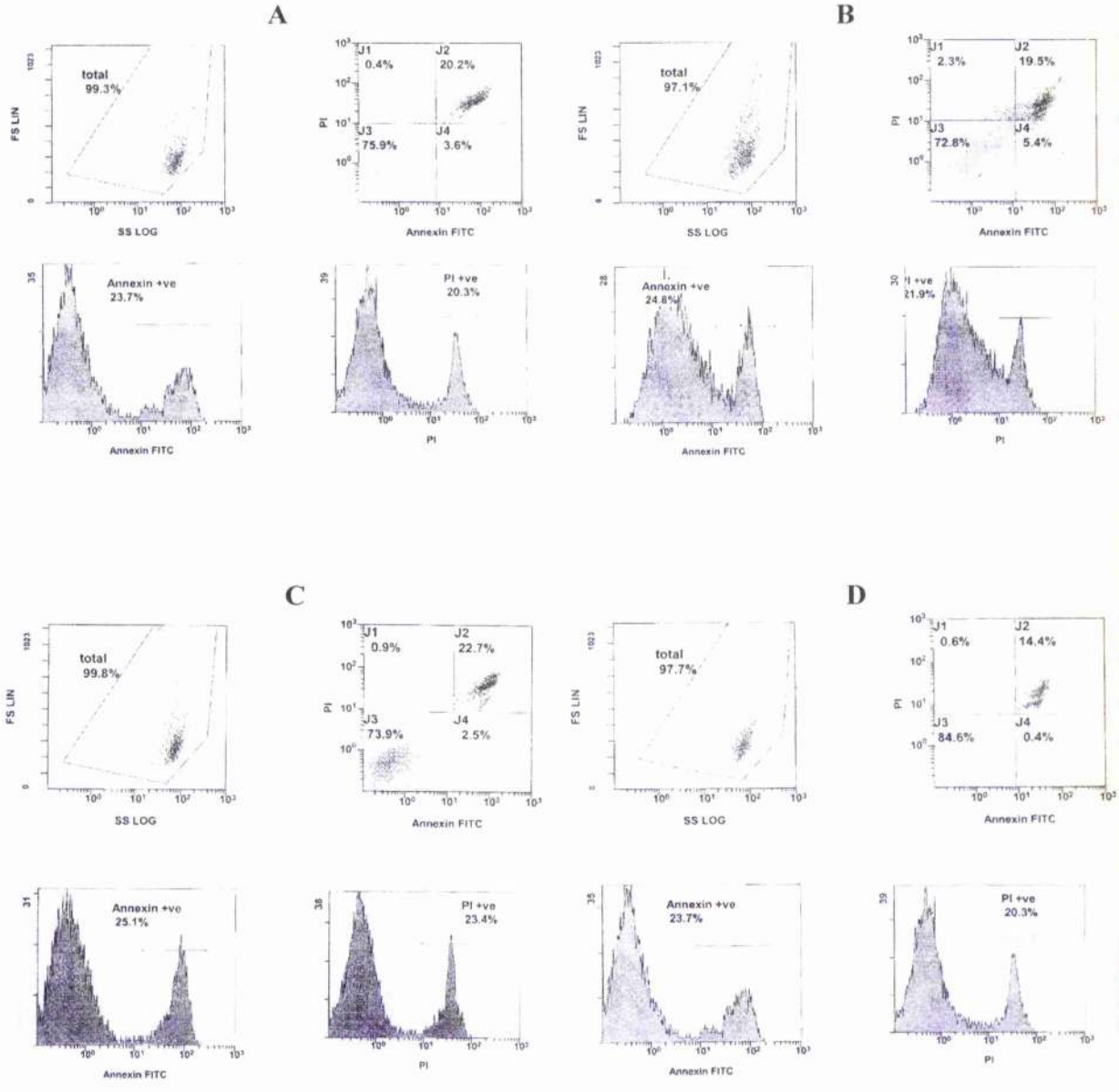
A: Telomestatin-treated cells following 7 days exposure

B: Telomestatin-treated cells following 20 days exposure

C: Negative control (untreated) cells following 7 days exposure

D: Negative control (untreated) cells following 20 days exposure

J1: Annexin-negative and PI-positive (dead cells), J2: Annexin-positive and PI-positive (late apoptotic/dead cells), J3: Annexin-negative and PI-negative (live cells), J4: Annexin-positive and PI-negative (early apoptotic cells).



4.6 Discussion

The aims of this chapter were to investigate two forms of telomerase inhibition in telomerase positive canine cancer cell lines. The first of these, DN-hTERT is the dominant negative mutant of the human TERT reverse transcriptase catalytic subunit. The second, telomestatin, is a naturally occurring G-quadruplex ligand, which functions by binding telomeric DNA in a G-quadruplex and preventing access of telomerase.

4.6.1 Inhibition of Canine Telomerase with DNhTERT

To evaluate the effects of inhibition of the dominant negative mutant DNhTERT, we synthesised the construct pCINeo-DNhTERT and used it to generate stable transfected cell lines in the telomerase-positive CMT7 and D17 cell lines. As negative controls, we also generated stable transfectants in these cell lines using empty pCINeo. In addition, we generated stable transfectants in telomerase-negative K9SF cells using the pCINeo-DNhTERT construct, but were unsuccessful in our attempts to stably transfect these cells with empty pCINeo.

When we examined the growth rate of transfected cells over time, we found that the pCINeo-DNhTERT construct had no impact on the growth rate of either CMT7 or D17 cells (Figure 4.6). In addition, TRAP assays performed on these cells showed that telomerase activity was still present, despite the confirmation of successful transfection (Table 4.2, Figure 4.5). In addition, no significant change in telomere length was found in these cells over the course of 50 population doublings (Figure 4.10). These findings suggest that the human dominant negative mutant of TERT, DNhTERT, whilst abrogating telomerase activity in human telomerase-positive cells, does not have an inhibitory effect on canine telomerase. The reasons for this remain to be determined. Although canine and human TERT share a total homology at the amino acid level of 67%, similar enough to allow hTERT to function in canine cells (Nasir *et al.* 2004), it may be that the homology in the region of the mutation in Motif A (62% across the reverse transcriptase region) is sufficiently different to prevent mutations in this region from creating a dominant negative effect. Alternatively, it may be that dominant negative splice mutants either do not occur in the dog or arise from splice sites not located in motif A. However, given the similarity between the two molecules, it seems reasonable to expect that, given its important for reverse transcriptase functioning, alterations to motif A of canine TERT should interfere with the ability of TERT to function and may also result in dominant negative mutants as they do in humans. An important experiment to try to confirm this would be to mutate canine TERT in the region of motif A (firstly by deleting motif A altogether) and examining the effects of such mutations in telomerase-positive cells. However, the generation of these mutants requires obtaining wild type canine TERT cDNA, a hurdle which has yet to be overcome.

Interestingly, the growth rate of the K9SF telomerase-negative cells transfected with pCINeo-DNhTERT was significantly increased over untransfected K9SF cells. In addition, these cells became telomerase positive and appeared to change their morphology and growth pattern, with less-ordered growth and the ability to grow out of a single monolayer (Table 4.3, Figure 4.8). An important caveat must be attached to this last observation however. When further passages of wild-type fibroblasts were grown over a longer period of time, in one case a single flask appeared to develop an area of cells with similar morphology (results not shown). Thus, it is possible that the cells may have undergone spontaneous immortalisation. This seems unlikely since RT-PCR confirmation of transfection revealed the presence of a 450 bp band when amplified using the DNHT001F/R primer pair, and also since the change in growth pattern and morphology appeared to coincide with the point of transfection (Figure 4.7), whereas the single transformed flask occurred only after several further passages of these cells had elapsed.

Our findings would appear to suggest that the human DNhTERT mutant functions to restore telomerase activity in telomerase-negative canine cells. More significantly, the presence of DNhTERT appears to alter the morphology and growth pattern of these cells, inducing a potentially malignant phenotype. An alternate explanation is that in these cells, DNhTERT activity, in allowing more rapid growth and division, also allowed other mutations to occur that led to the development of a malignant phenotype. In either case, these findings would appear to be different from the situation in human telomerase-negative fibroblasts, in which the induced expression of hTERT allows unlimited cell divisions, but does not change the growth pattern or morphology of cells (Bodnar *et al.* 1998). An important question that arises from this is whether this effect is simply the result of reactivated telomerase activity or whether structural differences between human and canine TERT result in other changes related to a cancerous phenotype in dog cells. Several further experiments need to be performed to isolate these. Firstly, it would be valuable to repeat this experiment in a different telomerase-negative canine cell line, to confirm our findings. In addition, the transfection of these cells with wild type human TERT as well as the DNhTERT mutant should be performed to determine whether any difference in morphology or growth pattern is due to the mutated sequence in DNhTERT. If these morphologic changes are confirmed, it is then possible to assess whether the induced phenotypic changes are indeed similar to those acquired by cancer cells, by examining growth in low-serum conditions, and also by examining growth in agar to determine whether these cells acquire invasive potential. Secondly, a vital experiment is to transfect canine telomerase-negative skin fibroblasts with wild type canine TERT to examine the effects of immortalisation on these cells. This last experiment, however, again relies on obtaining the full length canine TERT cDNA, which has so far proved technically challenging.

It should be noted that we were unable to create a stable transfectant using empty pCINeo in

telomerase-negative K9SF cells. Untransfected fibroblasts from this cell line grow slowly, and undergo senescence at approximately passage 8-11. Transfections were performed at passage 4-5. In addition, the transfection process resulted in significant cell death and slowed growth. Following the initiation of selection with G418, further cell death in the pCINeo-transfected cells combined with the naturally slow growth rate meant that cells did not reach confluence despite being maintained for a period of 4 weeks. Furthermore, technical problems with persistent fungal contamination meant that flasks had to be discarded. As a result, despite repeated attempts, stable transfectants could not be generated to act as negative controls, and untransfected wild-type cells were used instead.

4.6.2 Inhibition of canine telomerase with telomestatin

Telomestatin showed considerably more promise as a telomerase-inhibitor in canine cells compared to DNhTERT. To evaluate the effect of telomestatin on telomerase activity, we initially incubated CMT7 and D17 cells with telomestatin at varying concentrations for 7 days and monitored telomerase activity by performing TRAP assays. This experiment showed that concentrations of 1.0 and 2.0 μM successfully reduced telomerase activity, with the greater reduction occurring at a concentration of 2.0 μM (Figure 4.11). Treatment of cells with either 5.0 μM or 10.0 μM telomestatin also reduced telomerase activity, but resulted in the death of all cells within 2 days. For this reason, longer-term inhibition studies were performed using a telomestatin concentration of 2.0 μM.

Treatment of CMT7 and D17 cells with 2.0 μM telomestatin led to a reduction in growth rate in both cell lines. In addition, TRAP assays, TRF analysis and apoptosis studies showed that telomestatin treatment led to decreased telomerase activity (Table 4.6), telomere shortening (Table 4.6), and cell death (Figure 4.16) that occurred from approximately 7-10 days after initiating treatment. Interestingly, the magnitude of telomerase inhibition, growth rate slowing and apoptosis were all greater in D17 cells than in CMT7 cells. This may be due to the fact that D17 cells naturally possess lower levels of telomerase activity than CMT7 cells, and consequently are less resistant to the effects of telomestatin. Telomere lengths, however, were roughly similar in both cell lines, with telomere lengths in treated D17 cells falling to 3.0/2.95 at 7 and 20 days respectively, as compared with 3.15/2.68 in CMT7 cells. There appeared to be no change in telomere length between 7 and 20 days for either cell line. This would appear to suggest that apoptosis occurred in both cell lines independently of telomere length, since apoptosis at 20 days was considerably higher in both cell lines than at 7 days. Similar results have been reported in the human literature, and support the theory that apoptosis in cells treated with G-quadruplex ligands occurs through uncapping of the telomeres and change in telomere structure, rather than as a result of exhaustion of telomere reserve (Pennarun *et al.* 2005; Shammas *et al.* 2004). An alternative explanation, however, is that a telomere length of approximately 3.0 kb represents the minimal

viable telomere length for these two cell lines, and that any cells with a telomere shorter than this underwent apoptosis. If this were the case, the number of cells undergoing apoptosis would have accumulated as more and more cells reached the critical limit of telomere length. Given that critical minimal lengths in human cell lines can approach 2.0 kb, this seems perhaps unlikely (Tauchi *et al.* 2003). However, in order to prove the means by which the canine cell lines entered apoptosis, several other experiments should be performed. Firstly, a number of other telomerase-positive cell lines with telomere lengths of varying size should be examined to determine whether the length of time between onset of telomerase inhibition and apoptosis is related to telomere length. Secondly, it would be important to evaluate levels of p21 and p27 expression in these cells to examine whether DNA damage response pathways are upregulated, as would be consistent with an uncapping model, and to examine whether that upregulation occurs independently of telomere length. Similarly, other factors involved in the DNA damage response pathway, such as ATM, should be examined, as they are known to be upregulated with telomestatin treatment (Tauchi *et al.* 2003).

The change in growth rate in all cells was an interesting finding. Both cell lines appeared to have an initial lag phase of approximately 7 days in the case of D17 cells and slightly longer than this in the CMT7 cells. Some authors have reported that the effects of telomestatin occur after an initial lag phase of 5-7 days (Shammas *et al.* 2004), but this time varies between reports, with some cell lines requiring exposure for up to 30 days to show a loss in proliferative capacity (Tauchi *et al.* 2003). The initial telomere length of CMT7 cells was approximately 1.0 kb longer than that of D17 cells, which could account for this difference if apoptosis was induced by exhaustion of telomeric reserve. However, the rate of telomere loss seems to have been more rapid than this would suggest, since both cell lines had mean telomere lengths of approximately 3.0 kb at 7 days, a shortening rate of approximately 2-3 kb within the first 7 days. In addition, this rate of shortening is much more rapid than that reported in human cell lines, where a loss of 1.0 kb over 39 days was reported in response to exposure of SW26 cells to telomestatin (Kim *et al.* 2003a). However, there does seem to be disagreement over the amount of telomere shortening induced by telomestatin, with other authors suggesting considerably greater telomeric attrition (Tauchi *et al.* 2003). In order to confirm this, our experiments could be repeated with more frequent evaluation of telomere length. This would also allow a more precise determination of the timepoint at which growth inhibition occurred in both cell lines.

The annexin and PI staining appeared to show increasing levels of apoptosis with prolonged exposure to telomestatin. Consistent with our other findings, the level of apoptosis in D17 cells appeared to be considerably higher at 20 days than in CMT7 cells. For this reason it would be important to repeat this experiment with a longer duration of treatment for CMT7 cells to see if similar levels of apoptosis are reached with longer treatment. An important caveat should be attached to these results, however, as evidenced by the high levels of cell death that occurred in all

flasks. Up to 30% of untreated D17 cells and 20% of untreated CMT7 cells at 7 days were found to have undergone either late apoptosis or cell death as shown by positive staining for both annexin and PI. These figures were even higher for K9SF cells. The annexin-V staining protocol is designed primarily for non-adherent cells in suspension. During trypsinisation and counting of adherent cells, some trauma occurs, leading to exposure of PS on the outer membrane leaflet, with the result that some normal cells can show positive staining for annexin (van Engeland *et al.* 1998). To minimise this, trypsinisation was restricted to no longer than 5 minutes, and all procedures were performed slowly to minimise disruption of cell membranes during trypsinisation and staining. In addition, we performed an initial experiment in untreated CMT7 and D17 cells to assess the baseline level of annexin-positive staining that occurs, and found this to be generally less than 1% (results not shown). However, this does not account for the high number of annexin and PI-positive stained (ie late apoptotic or dead) cells present in negative control flasks. In addition, the level of early apoptosis present in telomestatin-treated cells was considerably higher than in negative control cells. It is possible that other factors in the culture conditions were not optimal, with the result that cells in all flasks underwent cell death. Alternatively, it is possible that handling of cells resulted in such severe membrane disruption that PI-positive staining became apparent, although this seems unlikely. Another approach to resolving this dilemma would be to investigate other means of assaying apoptosis (eg TUNEL-staining) or alternatively to stain cells without trypsinisation and examine and count both annexin- and PI-positive staining using fluorescence microscopy.

4.6.3 Potential problems with therapeutic strategies based on telomerase Inhibition

The value of telomerase inhibition as a clinical means of treating cancer must be tempered by the fact that successful inhibition, theoretically, leads only to the restoration of a mortal phenotype to tumour cells, without affecting other characteristics of cancer growth. Initial studies using the dominant negative mutant of TERT in humans seemed to support this, with growth inhibition occurring only after telomeric reserve in treated cells had been exhausted (Hahn *et al.* 1999a; Zhang *et al.* 1999). In some locations, such as the brain, further expansion of tumour size during this 'lag phase' following telomerase inhibition may not be possible without causing the death of the patient, rendering this approach on its own unfeasible in many situations. Indeed, in U251-MG glioblastoma cells, this lag phase constitutes approximately 30 doublings, or one month (Kondo *et al.* 1998b). However, studies inhibiting telomerase using other means such as ribozymes directed at hTERT or oligodeoxynucleotides directed at hTERT mRNA have suggested that growth inhibition of tumour cells may occur more rapidly, due to other effects (Kraemer *et al.* 2003; Saretzki *et al.* 2001). For instance, expression of mutant hTR in human telomerase-positive cells leads rapidly to a decrease in cell viability and an increase in apoptosis, independent of p53 status and initial telomere length (Guiducci *et al.* 2001; Kim *et al.* 2001; Marusic *et al.* 1997). The fact

that such cell death occurs without exhaustion of telomeric reserve and only in telomerase-positive cells is likely to be the result of a change in the physical state of the telomere, with uncapping leading to the activation of DNA damage response genes such as p21 (Abdul-Ghani *et al.* 2000). Most studies examining G-quadruplex ligands also support this mode of action, with apoptosis occurring rapidly and independently of initial telomere length. These findings suggest that G-quadruplex ligands may be worth investigating in the clinical setting. It should be noted that two other factors may also allow the use of telomerase inhibitors in the clinical setting. Firstly, if the physical bulk of tumour cells can be reduced through surgical excision or other means, it may be that telomerase inhibition can be employed subsequently to limit tumour regrowth. Secondly, it may be that direct inhibition of telomerase could be employed alongside other telomerase-targeted therapies, such as TERT-promoter driven gene expression (discussed in Chapter V). The combination of different approaches targeting telomerase, involving both direct inhibition and telomerase-targeted gene expression, holds great promise and may have synergistic effects on tumour cell killing.

Another potential problem concerns the development of resistance to telomerase inhibitors. This may occur in several ways, but one important ways involves the developing of alternative mechanisms for maintaining telomere length, such as the ALT pathway. Importantly, a relatively high proportion of glioblastomas have been shown to maintain telomere length using the ALT pathway (Carroll *et al.* 1999). However, the mechanism by which telomestatin and other G-quadruplex ligands work suggest that they may still be able to interfere with telomere maintenance in ALT cells due to their interaction and stabilisation of telomere ends. Indeed, work with the ALT-possessing SAOS-2 cell line indicates that these cells still undergo apoptosis following treatment with G-quadruplex ligands (Pennarun *et al.* 2005) Thus, telomestatin is worth investigating even in ALT cells.

One important question that remains to be answered in particular with respect to the use of telomestatin is whether adequate concentrations can be achieved at the tissue level with systemic administration. In the brain, controlled, continuous delivery could potentially be achieved using recently developed delivery systems such as osmotic minipumps combined with stereotactic techniques (Zhu *et al.* 1996). However, to date no studies have examined the pharmacokinetics of telomestatin with systemic dosage. Experience with another G-quadruplex ligand, BRACO19, which was used in one study at a dose rate of 2 mg/kg for 2-3 cycles of 5 daily doses given intraperitoneally in a mouse xenograft model suggest that systemic administration should be feasible (Kelland 2005). Thus, although pharmacokinetic data still needs to be established, telomestatin represents a potentially valuable way of targeting telomerase-positive tumours, and further work examining its use in dogs appears to be warranted.

CHAPTER V

SEQUENCING AND CHARACTERISATION OF THE CANINE TERT PROMOTER

5.1 Abstract

Several anticancer therapies have been developed using either tissue-specific or cancer-specific gene promoters to drive the expression of transgenes. The only truly cancer-specific promoters currently known are the promoters of the TR and TERT genes involved in telomerase activity. Since telomerase activity is dependent principally on regulation of the TERT gene, research has focused on the activity of this gene in normal and cancer tissues. Upregulation of the TERT gene depends primarily on transcriptional activity of its promoter, although post-transcriptional modification of the TERT transcript and methylation of the TERT promoter may also play a role. The human TERT promoter is GC-rich, has no TATA box and contains multiple binding sites for transcription factors including Sp1, c-Myc and p53. Using this promoter, several groups have developed a number of promoter-driven therapies to induce apotosis or cell death by other means both *in vitro* and *in vivo* in nude mouse models, with considerable success. Some groups have also utilised the TR promoter in a similar fashion with good results.

The aims of this chapter were to sequence and characterise the canine TERT promoter and TR promoter as a prelude to developing TERT and TR promoter-driven therapies. We successfully sequenced approximately 5.4 Kb of the canine TERT promoter and created deletion constructs to identify the core promoter responsible for activity in telomerase-positive cells following luciferase assays. The core promoter was identified as a region 314 bp upstream of the ATG start codon, and conveyed approximately 50% of the activity of a positive control vector in CMT7 and D17 cells. Importantly, inclusion of the initial part of the TERT gene with the core promoter reduced activity by approximately 75%, suggesting that important negative regulatory factors exist within the canine TERT gene. Transfection of other telomerase-positive cells with the core promoter construct showed promoter activity varied from between approximately 8% to 50% of the activity of the positive control. In addition, transfection of telomerase-negative fibroblasts with all deletion constructs showed that the activity of the TERT promoter was restricted to telomerase-positive cells. A number of potentially important putative transcription factor binding sites were identified within the core promoter, as well as within the TERT gene itself, although there were also significant differences between the canine promoter and its human counterpart. In addition to sequencing and characterising the TERT promoter, initial sequencing and analysis of the canine TR promoter was performed. In this experiment, genome walking was used to generate approximately 700 bp of the canine TR promoter sequence amplified from genomic DNA. The most remarkable finding in the TR promoter sequence was the presence of multiple (TAAAA)n repeats. Although the significance of these repeats is currently unknown, there is some evidence from the human literature to suggest that they are important for transcriptional regulation of some genes. Together, these experiments, by elucidating the canine TERT and TR promoters, have provided an important tool that can be used in the design of future telomerase-specific promoter-driven therapies.

5.2 Introduction

Although telomerase inhibition has shown promise as an anti-cancer strategy, it has limitations which have prompted the investigation of other strategies using telomerase. In particular, the possibility that telomerase could be used as a target to guide gene expression specifically in cancer cells has excited considerable interest. This introduction will examine the principles underlying such targeted gene expression and the ways in which it has been used to date in designing cancer treatments for humans as a background to the development of similar strategies in dogs.

5.2.1 Promoter-driven gene therapy

Gene therapy involves the transfer of exogenous genes (transgenes) into the somatic cells of a patient to elicit a desired effect. Early work investigated gene therapy for the treatment of inherited diseases in people, and this strategy is particularly suited to the treatment of specific conditions involving the absence of a single gene and its product. A good example of this is the recently-published treatment of autosomal recessive α-mannosidosis in cats (Vite *et al.* 2005). Gene therapy may also be used to treat cancer, either by replacing the function of defective tumour suppressor genes or by introducing genes capable of killing tumour cells. The challenge facing all forms of cancer therapy is to selectively kill cancer cells whilst sparing normal cells. Conventional anti-cancer treatments such as radiotherapy and chemotherapy are usually designed to kill rapidly dividing cells, but this can include normal cells which are undergoing mitosis. This leads to a dose-limiting toxicity in normal tissue and limits their use. However, gene therapy strategies might avoid this toxicity if they can be designed to specifically deliver their products to cancer cells.

It has become clear that gene expression is regulated by a complex interplay of factors that act in a cell-type-specific manner to produce a wide range of effects. The diversity of these effects arises from the interaction between tissue-specific transcriptional control elements present in the various cell types (Robson & Hirst 2003). These transcriptional control elements, or promoters, can be exploited to drive the transcription of a therapeutic gene either in a tissue-specific or tumour-specific manner.

Strategies using tissue-specific promoters employ the promoters of genes that are switched on only in certain tissues. Expression of transgenes under the control of these promoters generally results in the constitutive expression of genes throughout the target tissue. One of the main limitations of this sort of promoter is that transgene expression may lead to cytotoxic effects in normal tissue as well as tumour tissue derived from the same cells. For this reason, the use of these promoters must necessarily be restricted to tissues in which damage is not critical for the survival of the host, for example prostate, melanocytes or thyroid (Robson & Hirst 2003). Notwithstanding this limitation,

tissue-specific gene expression has been examined using several tissue specific promoters, including the tyrosinase (melanocytes), prostate-specific antigen (prostate), glial fibrillary acidic protein (glial cells), Nex (neural crest-derived cells), albumin (liver), thyroglobulin (thyroid), and ovarian specific (ovaries) promoters (reviewed in (Robson & Hirst 2003)).

The ideal tumour-specific promoter should be highly active in tumour cells and have no activity in normal cells. This encompasses a vast number of promoters, but they have been divided into subgroups depending on their characteristics (Harrington *et al.* 2000). Tumour-specific promoters can be described as 1) cancer-specific (i.e. specific for the malignant process without being influenced by the tissue type), 2) tumour-type-specific (i.e. specific to the type of tumour, and therefore the cell-type from which it arises), 3) tumour microenvironment-related (i.e. promoters which respond to factors within the tumour microenvironment such as hypoxia) and 4) tumour vasculature related promoters (i.e. promoters which are more active in the tumour vasculature as compared with normal vasculature). Whilst there are a number of promoters within the other groups, the promoters of only a single enzyme, telomerase, can be considered to be cancer-specific (Robson & Hirst 2003), and this chapter will focus on the design of gene therapy strategies in dogs based on these promoters. However, an essential prerequisite for the design of such therapies is a basic understanding of the regulation of telomerase activity in normal and cancer cells. Therefore, a brief review of our current understanding of human telomerase regulation will be covered before reviewing the telomerase promoter-based therapies currently under development as a comparison for the design of such therapies in dogs.

5.2.2 Regulation of telomerase activity in humans

Several strands of evidence point to the fact that the regulation of telomerase expression depends primarily on the activity of the TERT gene. Firstly, although expression of both the TR and TERT genes are necessary for telomerase activity *in vitro*, TR is widely expressed in somatic tissues where TERT is undetectable in most somatic cells (Beattie *et al.* 1998; Meyerson *et al.* 1997; Weinrich *et al.* 1997). Secondly, levels of TERT mRNA expression in humans temporally parallels changes in telomerase expression during cell differentiation and neoplastic transformation (Takakura *et al.* 1998; Wu *et al.* 1999). Thirdly, ectopic expression of hTERT is sufficient to restore telomerase activity in some (although not all) telomerase-negative cell lines, including human foreskin fibroblasts, mammary epithelial cells, retinal pigment epithelial cells, and umbilical endothelial cells (Bodnar *et al.* 1998; Counter *et al.* 1998; Vaziri & Benchimol 1998; Weinrich *et al.* 1997). These three findings together suggest that regulation of the TERT gene is the major factor responsible for controlling telomerase expression.

In general, regulation of the TERT gene occurs through three mechanisms: regulation of gene transcription, post-transcriptional modifications and promoter methylation. A brief description of these three mechanisms follows.

5.2.2.1 Transcriptional Regulation

When SV40-transformed HA1 cells are maintained in culture, telomerase activity is not found during their extended lifespan, but is found after cells have gone through proliferative crisis and become immortalised. Cong *et al* found that transfection of these cells with a construct containing the hTERT promoter driving a luciferase reporter gene resulted in luciferase activity in cells only after proliferative crisis (Cong *et al.* 1999). This experiment showed that telomerase activity coincided with hTERT promoter activity, and thus showed that telomerase expression is principally regulated at the level of gene transcription.

5.2.2.1.1 The hTERT core promoter region

Transcription of any gene is regulated via the upstream regulatory, or promoter, region of the gene – the region of DNA extending in a 5' direction from the ATG start codon. Defining the region of the TERT promoter that conveys greatest activity was an important step in understanding the regulation of TERT prior to the development of promoter-driven therapies in humans. Wick *et al* reported that following promoter fragment linkage to the luciferase reporter gene, insertion into a plasmid vector and transient transfection of HEK-293 cells, the promoter fragment 43 – 1125 bp upstream of the ATG start codon conferred greater luciferase expression than fragments of longer length extending further upstream (Wick *et al.* 1999). However, within this region, Cong *et al* reported that a smaller, core region extending from 330bp upstream of the ATG start codon to the first 37bp of exon 2 conveyed the greatest activity (Cong *et al.* 1999). Most other authors refer to this as the core promoter of the TERT gene. However, in addition to core activity being contained in the upstream region, several authors have also reported the presence of detectable regulatory activity within exons 1 and 2 and intron 1 of the hTERT gene (Renaud *et al.* 2003).

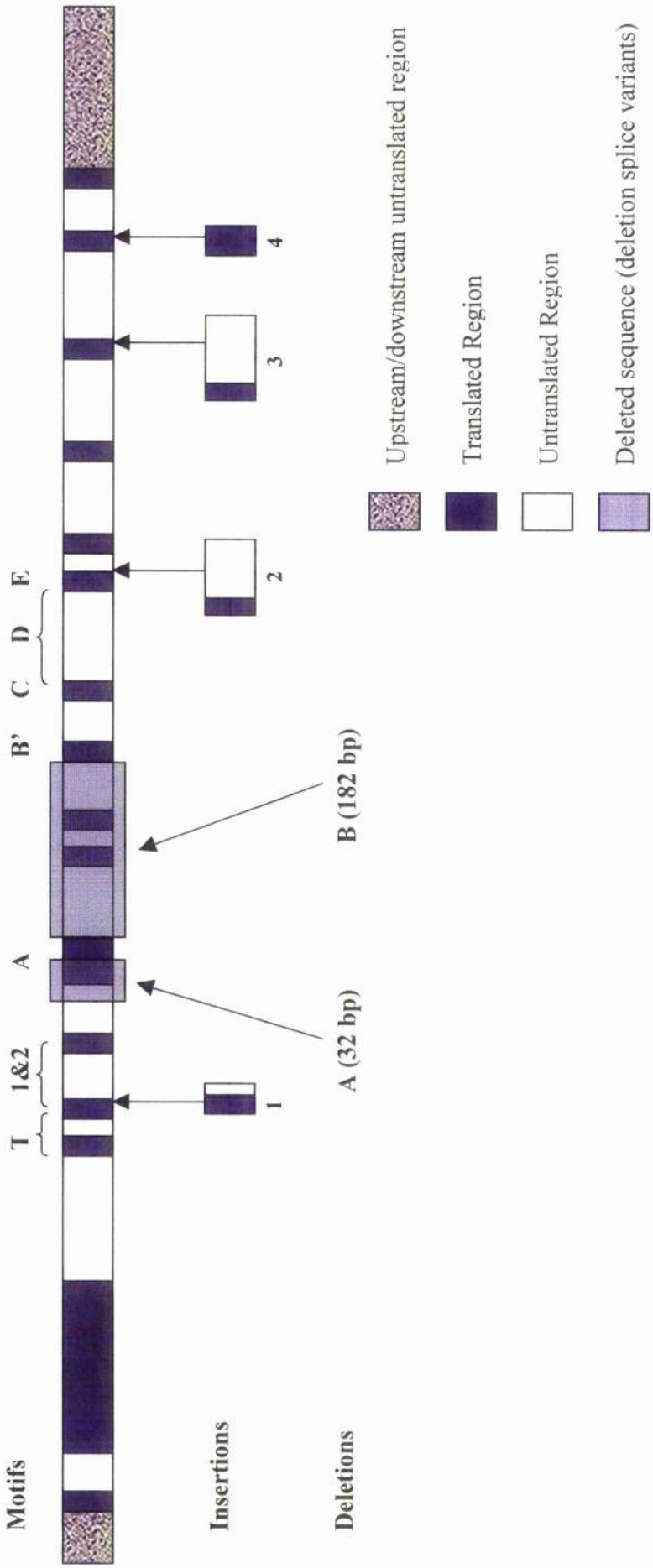
5.2.2.2 Post-transcriptional modification of hTERT

Telomerase has been widely reported to be expressed in a cell and age-specific manner. In higher eukaryotes, such tissue and development-dependent regulation of gene expression often involves alternate splicing of mRNA transcripts (Cong *et al.* 1999). In addition to full length hTERT mRNA, several authors have described the existence of six mRNA splice variants (Kilian *et al.* 1997; Wick *et al.* 1999) (Figure 5.1). The variants contain variations in the region comprising the RT domains and also in the C-terminal region. Alignment between respective cDNA sequences

and the genomic hTERT sequence has shown that all the hTERT variants identified can be explained by the use of alternative splice sites in the hTERT genomic sequence (Wick *et al.* 1999). Deletion variants DEL1 and DEL2 are characterised by the deletion of specific sequences. Variant DEL1, also known as α-β+, α-del or Adel, comprises a 36 bp in-frame deletion and leads to the partial loss of RT motif 3, most likely resulting from the use of an alternative 3' splice acceptor sequence in exon 6. The DEL2 variant, also known as α+β-, β-del or Bdel, arises when the normal 5' splice donor and 3' splice acceptor sequences of introns 6, 7 and 8 are not utilised. As a result, exon 6 is directly fused to exon 9, causing an ORF shift with a translation stop codon present in exon 10. The DEL1+2 (α-β-) variant results from the combination of DEL1 and DEL2.

By contrast, a second group of variants is defined by the insertion of intron sequences which lead to the introduction of premature translation termination codons. The variant INS1 encodes an alternative 3'-located splice site instead of the normally used 5' splice donor sequence of intron 4, resulting in an insertion of the first 38 bp of intron 4 between exons 4 and 5. Similarly, INS2 is the result of insertion of intron 11 sequences following the use of an alternative 5' splice donor sequence in intron 11, although the exact alternative 5' splice donor sequence used has not been determined. INS3 is generated by the use of an alternative 3' splice acceptor sequence, causing a 159 bp insertion of intron 14 sequence between exons 14 and 15 leaving the 3' part of intron 14 unspliced. Finally, a fourth insertion variant, INS4 , was identified from the human leukaemic cell line K562. In this variant, the first 600 bp of intron 14 replaces exon 15 and the 5' part of exon 16. Splicing occurs through the use of an alternative internal 5' splice donor sequence in intron 14 and an alternative 3' splice acceptor sequence in exon 16, leading to an hTERT variant with an altered C-terminus.

Figure 5.1: Genomic Organisation of the hTERT gene. RT motifs (T, 1, 2, A ,B', C, D, E) and alternate splicing sites are shown together with upstream and downstream untranslated regions.



There appears to be some uncertainty as to the role splice variants play in regulating telomerase activity. Yi *et al.* examined the presence of splice variants in a number of telomerase-positive immortal cell lines and found that there is surprisingly little variation in the proportion of alternatively-spliced forms of hTERT in different cell lines (Yi *et al.* 2001). In all cells with endogenous telomerase activity, approximately 5% of the total hTERT mRNA exists as the wild type version, and most cell lines have only 1- 30 copies of total hTERT mRNA per cell (Yi *et al.* 2001). Approximately 80-90% of the total hTERT mRNA exists as α+β- (DEL2) form, and 5-15% in α-β- (DEL1+2), and <1% in α-β+ (DEL1) form. However, the fact that there is an approximately 20-fold variation in telomerase activity between these cell lines and that these proportions remain relatively consistent suggests that telomerase activity is not regulated by the presence of splice variants (Yi *et al.* 2001). Several studies have also examined the presence of splice variants in tumour tissues of varying origin, including meningiomas, liver tumours, ovarian, uterine and breast (Cabuy & de Ridder 2001; Kotoula *et al.* 2002; Ulaner *et al.* 2000; Zaffaroni *et al.* 2002). In all telomerase-positive tissues so far examined, wild-type (α+β+) hTERT mRNA is found only together with other variants. However, the α+β- (Bdel or DEL2) variant has been reported as the sole transcript expressed in some tissues (Cabuy & de Ridder 2001; Kotoula *et al.* 2002; Ulaner *et al.* 1998; Ulaner *et al.* 2000; Wick *et al.* 1999; Zaffaroni *et al.* 2002). Kotoula *et al* reported that in gliomas, telomerase activity is associated with predominant expression of the wild-type transcript (Kotoula *et al.* 2004). However, a third of these tumours also possess relatively high levels of the α+β- variant. The α-β+ variant was found in all low grade (I-III) grade tumours, the majority of which were telomerase-negative. However, in no tumour was this variant predominantly expressed. In addition, it was found in 60% of high grade (IV) tumours. Overall, the total hTERT mRNA level was only associated with the presence of the wild-type splice variant. Although this picture is somewhat complicated, overall it does not appear that telomerase activity regulation is heavily influenced by splice variants in these tumours.

5.2.2.3 hTERT promoter methylation

Several groups have noted the existence of highly GC-rich sequence within the core promoter. Two CpG islands have been identified, 900 bp and 4600 bp upstream of the start codon in exon 1 (Wick *et al.* 1999). A large CpG island, with a GC content >70% and an observed/expected CpG ratio >0.6 extends from bp –900 into exon 2. A smaller CpG island, with GC content >60% and observed/expected CpG ratio >0.6 extends from bp –4300 to –4600. No TATA- like sequences are present in the putative transcription start region.

Given the presence of these CpG islands and the highly GC-rich status of the hTERT promoter, several authors have investigated the potential for methylation as a means of regulating hTERT

activity. The addition of methyl groups to cytosine residues alters and restricts the access of transcription factors to binding sites within the promoter and thus represses promoter activity. Several tumour suppressor genes use this mode of gene silencing (Herman 1999; Zingg & Jones 1997). At this stage, the significance of methylation as a means of regulating hTERT gene expression remains unclear, with conflicting reports of the impact of methylation on gene activity. hTERT promoter methylation has been observed in most hTERT-negative normal cells and approximately one third of hTERT-positive cells, suggesting different means of regulating hTERT expression, with methylation dependent and -independent pathways both existing (Dessain *et al.* 2000; Devereux *et al.* 1999; Guilleret *et al.* 2002). In normal human oral keratoinocytes and fibroblasts, the absence of telomerase activity is associated with a hypermethylated hTERT promoter (Shin *et al.* 2003). Furthermore, when telomerase-positive human teratocarcinoma (HT) and HL60 myeloid leukaemia cells are induced to differentiate into telomerase-negative cells, hTERT promoter methylation gradually increases prior to a drop in telomerase activity, suggesting that in these cells promoter methylation is a means by which the hTERT gene is downregulated (Liu *et al.* 2004). However, telomerase-positive Lan-1, HeLa and Col15 cells possess hypermethylated hTERT promoters, and treatment with a demethylating agent in these cells reduces methylation and hTERT expression (Guilleret & Benhattar 2003). Thus, whilst methylation may still be an important means by which telomerase is regulated in these cells, methylation has the opposite effect – ie to activate, rather than silence hTERT gene expression. In addition, whilst methylation of the hTERT gene promoter is seen more commonly in cervical cancer than in normal cervical tissue, the same does not hold true for ovarian cancer, and methylation status does not correlate with gene expression in either tumour type (Widschwendter *et al.* 2004). Collectively these data suggest that methylation may play a role in regulating hTERT gene expression, but the manner in which this occurs and its significance are both likely to vary between tumour and cell types.

5.2.3 Characterisation of the human TERT gene promoter

5.2.3.1 Transcription factor binding sites within the core promoter

Using transcription element search software, several authors have reported the existence of canonical and non-canonical motifs for the binding of numerous transcription factors. These transcription factors can be divided into those that enhance hTERT expression and those that repress it. In addition, the function of some transcription factors is currently poorly understood. Those activating transcription factors with binding sites located in the hTERT promoter include c-Myc, Sp1 and oestrogen (Kyo *et al.* 2000; Misiti *et al.* 2000; Takakura *et al.* 1999; Wick *et al.* 1999). Binding sites for repressor products have also been elucidated, including WT1 (Wilm's tumour suppressor gene protein), MZF-2, p53 and Mad1. The location of all known binding sites

together with the putative function of their associate binding factors is listed in Table 5.1. Within the most proximal 900bp of the promoter, Wick *et al* reported the presence of five Sp1 binding sites, one c-Myc binding site, one AP-2 binding site and one CCAC box (Wick *et al.* 1999). In addition, a CCAAT box and a second c-Myc binding site are located 1788 and 3993 bp upstream of the start codon respectively. The five Sp1 binding sites and one c-Myc binding site are contained within the promoter region 43 – 1125 bp upstream of the ATG codon which conferred greatest promoter activity. The clustering of Sp1 binding sites is common in promoters of genes that do not contain TATA promoter sequences (Wick *et al.* 1999). Additionally, the oncoprotein c-Myc has been shown to induce hTERT expression in HMEC and normal fibroblast cells (Wang *et al.* 1998).

Table 5.1: Characterisation of hTERT promoter transcription factor binding sites

Transcription Factor	Activator / repressor	Position in hTERT promoter relative to ATG
AP2	Activator	-620,-559,-322,-228,-167,-151,-139,-131,-127,-113,-108,-5,+12,+98,+129,+193,+315
AP4	Activator	-853,-618,-531,-509,-315,-192,-56,-118,+289
CCAC	Activator	-827
c-Ets-2	Activator	-247,-29
c-Myb	Activator	-894,+242
c-Myc	Activator	-242,-34
CREB/ATF/AP1	Activator	-728
ER	Activator	-2754
ER/AP1	Activator	-794
IK2	Activator	-815,-647,-377,-227,+121,-175
Mad1	Repressor	-242,-34
MAZ	Activator	-117
MAZ/Sp1	Activator	-151,-108
MyoD	Activator	-183,+214,–319
MZF-2	Repressor	-764,-696,-620,-594
NF1	Activator	-929,-841,-458,-232,-96,-17,+68,+149,+343
NF-E2/Sp1	Activator	-206
NFkB/T3Rα/NMYC	Activator	-669
p53	Repressor	-1954,-1317
PR	?	-477
Sp1	Activator	-953,-806,-358,-323,-262,-206,-188,-168,-151,-133,-113,-108,-84,+254
Sp1/ER	Activator	-950
WT1	Repressor	-358

5.2.3.1.1 Activators of hTERT gene expression

5.2.3.1.1.1 c-Myc

Correlation between c-Myc oncogene expression and telomerase activity was first identified before characterisation of the hTERT promoter region had been established. Wang *et al* induced telomerase expression in telomerase-negative human mammary epithelial cells using directed c-Myc expression to levels similar to those measured in breast carcinoma cell lines (Wang *et al.* 1998). These cells showed a 50-fold increase in hTERT mRNA following c-Myc vector transfection. Later, characterisation of the hTERT promoter identified several c-Myc binding sites which account for this finding. The c-Myc oncoprotein forms a complex with the Max protein that binds as a heterodimer to activate gene transcription (Blackwood & Eisenman 1991). This dimer recognises and binds the consensus sequence 5'-CACGTG-3', known as an 'E-Box' (Kyo *et al.* 2000; Wu *et al.* 1999). An additional, related sequence, 5'-CA(C/T)GCG-3', also binds the c-Myc/Max heterodimer, potentially providing further regulation through binding at these non-canonical sites (Wu *et al.* 1999). Overall, of the 29 c-Myc complex binding sites that have been identified in the region of the hTERT gene, 18 are canonical (Wu *et al.* 1999). Of these, much research has focused on the two E-boxes located centrally within the hTERT minimal promoter (Greenberg *et al.* 1999; Horikawa *et al.* 2002; Takakura *et al.* 1999). Gene reporter analysis using the luciferase gene and mobility shift assays have suggested direct activation of hTERT by c-Myc at these E-boxes (Greenberg *et al.* 1999; Horikawa *et al.* 2002; Takakura *et al.* 1999; Wang *et al.* 1998; Wu *et al.* 1999). Additionally, studies revealing that c-Myc-induced up-regulation of hTERT occurs in the absence of new protein synthesis provide further evidence of direct activation of hTERT by c-Myc (Greenberg *et al.* 1999).

The relative importance of the two E-boxes for full activity of the hTERT promoter has been debated. Removal of the proximal E-box (at position –34) results in a 10-fold decrease in promoter activity, similar to expression levels for promoterless constructs (Greenberg *et al.* 1999). This finding, together with the observation that the distal E-box is not conserved in the mouse TERT promoter, suggest that c-Myc activation of hTERT is mediated predominantly through the proximal E-box (Greenberg *et al.* 1999). However, luciferase assay results following distal E-box deletion (at position –242) also reveal significant reduction in activity in all cell types studied (Horikawa *et al.* 2002; Kyo *et al.* 2000; Takakura *et al.* 1999). These apparently contradictory results suggest that a synergistic system may operate in the human TERT promoter, with occupation of both E-boxes by c-Myc necessary for maximal activity. Additionally, in RD rhabdomyosarcoma cells induced to differentiate into muscle cells, both E-boxes appear to be essential for regulating telomerase activity (Ma *et al.* 2003). However, some studies have also suggested that E-box-dependent regulation of the hTERT promoter can occur independently of c-Myc (Ducrest *et al.* 2001).

5.2.3.1.1.2 Sp1

The co-activity of Sp1 and c-Myc in regulating hTERT expression has been examined in some depth (Kyo *et al.* 2000). Co-transfection of a c-Myc or c-Myc/Max expression vector together with an Sp1 expression vector results in an increase in hTERT transcription of up to seven fold, depending on cell type (Kyo *et al.* 2000). In the absence of Sp1, however, only marginal activation occurs, suggesting that Sp1 is important for full activity of c-Myc (Kyo *et al.* 2000). Although Sp1 has traditionally been considered to be a constitutively-expressed factor, expression has been reported to vary up to 100-fold in different tissues (Saffer *et al.* 1991). In addition, the findings that cancer cells express high levels of c-Myc and Sp1 while normal cells do not, and that levels of Sp1, Myc and hTERT all rise in a co-ordinated fashion during SV40-induced transformation of fibroblasts, both suggest it acts as an important co-operative factor with c-Myc (Kyo *et al.* 2000). Similarly, the decrease in telomerase activity in murine muscle cells during differentiation is associated with decreased levels of Sp1 (Nozawa *et al.* 2001). In addition, some reports have suggested that Sp1 may also co-operate with oestrogen to activate hTERT transcription in ovary epithelial cells (Kyo *et al.* 1999; Misiti *et al.* 2000).

5.2.3.1.1.3 Oestrogen

Whilst most somatic cells do not exhibit telomerase activity, a few important cell types do. These include cells with high proliferative potential, such as endometrial cells (Tanaka *et al.* 1998). Telomerase activity during the proliferative phase of the uterine cycle has been shown to correlate with rising oestrogen levels (Kyo *et al.* 2000; Tanaka *et al.* 1998). Moreover, *in vitro* treatment of breast cancer cells and normal ovarian epithelium with oestrogen induces a rise in hTERT mRNA levels and telomerase activity (Kyo *et al.* 1999; Misiti *et al.* 2000). Two potential oestrogen response elements (EREs) exist within the hTERT promoter. The ERE located 2754bp upstream of the ATG start codon binds oestrogen and its receptor (ER), and luciferase reporter assays using this sequence show a 10-fold increase in promoter activity following treatment with oestrogen (Kyo *et al.* 1999). However, this effect is not seen in ER-negative cells, suggesting that the regulatory role of oestrogen is limited to ER-positive cells.

The ER site located 950bp upstream of the ATG contains an Sp1 recognition sequence adjacent to an ER half site (ER/Sp1) constituting a motif that functions as an ERE. Although some groups have found this potential ERE to be less important for function than the more distal site, mutation at this site drastically reduces hTERT promoter activity, suggesting an important role for direct activation of hTERT by oestrogen at this site as well (Kyo *et al.* 1999; Misiti *et al.* 2000).

5.2.3.1.2 Repressors of hTERT gene expression

5.2.3.1.2.1 WT1

Many of the studies characterising the hTERT promoter have found evidence for repressors of promoter function in a region between 300 and 700 bp upstream of the ATG (Horikawa *et al.* 1999; Takakura *et al.* 1999). Within this area is a binding site for the Wilms' Tumour 1 tumour suppressor gene product (WT1) (Oh *et al.* 1999). Previously, the anti-oncogenic WT1 protein had been shown to be involved in growth regulation of kidney cells (Coppes *et al.* 1993; Englert 1998). However, WT1 is expressed in a very limited range of tissues, with additional expression in gonad and spleen, suggesting that WT1-mediated control is regulated in a tissue-specific manner. As confirmation of this, gene reporter assays in which mutation of the WT1 binding site is performed affects promoter activity only in those cells known to express WT1 (Horikawa *et al.* 2002).

5.2.3.1.2.2 p53

The possibility that the anti-oncogenic protein p53 might be involved in regulating hTERT transcription was first raised by the discovery of an inverse relationship between p53 levels and telomerase activity. Kanaya *et al* found that overexpressing p53 in SiHa cervical cancer cells resulted in significant repression of hTERT transcription (Kanaya *et al.* 2000). Two binding sites, located at 1954 and 1317 bp upstream of the ATG appeared to be responsible for this finding. Importantly, the repressive effects of exogenous p53 preceded the cell growth inhibition traditionally associated with p53 activity, suggesting a possible additional pathway for cell proliferation control by p53, perhaps through the direct repression of hTERT gene expression. Interestingly, as with c-Myc and oestrogen, Sp1 is also likely to play a co-operative role in p53-based regulation, since mutation of Sp1 binding sites completely eliminates the repressive effects of p53 (Kanaya *et al.* 2000).

5.2.3.1.2.3 MZF-2

Although WT1 has been implicated in the negative regulation of hTERT, the tissue-specific nature of this protein intensified the search for other repressors. Four binding sites for the myeloid-specific zinc finger protein 2 (MZF-2) within a region 200 bp upstream of the hTERT core promoter (Fujimoto *et al.* 2000). Promoter constructs with their 5' boundary within this area (ie –800 to –600 bp) characteristically have lower activity than other constructs (Horikawa *et al.* 1999; Takakura *et al.* 1999). However, MZF-2 levels have been found to remain relatively stable during cellular differentiation (the time at which telomerase activity is repressed), casting doubts on the activity of MZF-2 alone being responsible for this repression (Fujimoto *et al.* 2000). Therefore, the

importance of MZF-2 as a factor controlling hTERT gene expression in most cell types remains uncertain.

5.2.3.1.2.4 Mad1

The E-box motifs bind a dimer of transcription factors that can either contain c-Myc and Max or Mad1 and Max (Gunes *et al.* 2000). The Max transcription factor is ubiquitously expressed in many cells, and dimerisation with c-Myc produces a transcription factor capable of activating the hTERT promoter, whilst dimerisation with Mad1 produces a transcription factor capable of repressing the hTERT promoter. In undifferentiated cells, as well as most neoplastic and transformed cell lines, high levels of c-Myc expression are generally present, while Mad1 expression is generally minimal (Gunes *et al.* 2000). Conversely, in cells with minimal expression of hTERT, such as normal somatic cells, the situation is reversed and c-Myc levels are low while Mad1 levels are high (Gunes *et al.* 2000; Oh *et al.* 1999). This antagonism between c-Myc and Mad1 in the control of hTERT expression suggests that hTERT expression relates directly to the relative levels of c-Myc and Mad1 (Oh *et al.* 1999). Indeed, ectopic expression of c-Myc in normal somatic cell lines with Mad1-mediated repression leads to activation of hTERT gene expression, presumably by outcompeting endogenous Mad1 levels in dimerisation with Max (Oh *et al.* 1999). Thus, it may be that reversible control of hTERT gene expression involves the interplay between c-Myc and Mad1 at one or both of the E-box sequences in the hTERT core promoter.

5.2.3.1.3 Regulatory factors within the hTERT gene

Whilst several authors confirmed the extent of the core hTERT promoter, some evidence began to emerge that the hTERT gene itself influences the activity of hTERT transcription. Takakura *et al*, in their characterisation of the promoter, created a deletion construct extending from 19 to 78 bp of exon 1 downstream of the ATG start codon (Takakura *et al.* 1999). This construct displayed significantly less activity than the smallest of all other deletion constructs (containing 31 bp of promoter upstream of the ATG as well as 78 bp of the exon). Following this, Renaud *et al* examined the activity of promoter constructs containing the minimal promoter together with up to 1000 bp of the hTERT gene (Renaud *et al.* 2003). Surprisingly, whilst the core promoter was confirmed to exist within the 297 bp upstream of the ATG, the addition of exon 1 and intron 1 sequence decreased the transcriptional activity of the promoter to 10% of the original. Interestingly, as more of the gene was included in these constructs, the transcriptional activity of the promoter fell by up to 100-fold, with the greatest reduction found in constructs with 1000 bp of exon 2 in addition to exon 1 and intron 1 (Renaud *et al.* 2003). Thus, structural elements within the hTERT gene appear to be important for repression of transcription.

5.2.4 Telomerase promoter-driven therapy

The finding that up to 95% of human tumour cells contain telomerase activity has prompted extensive investigation into telomerase promoter-driven gene expression as a cancer-targeting strategy designing promoter-driven gene expression using the promoters involved with telomerase activation. Since hTERT expression seems to be the major determinant of telomerase activity, the majority of this work has focused on using the hTERT gene promoter. However, some authors have also employed the hTR promoter (Bilsland *et al.* 2003; Plumb *et al.* 2001).

The ability to drive gene expression selectively within telomerase-positive cells has been employed in a number of ways. A number of pro-apoptotic genes have been used, including members of the caspase family, both initiators (caspase-8) and effectors (caspase-6) in the caspase cascade (Takeuchi *et al.* 2004). In malignant glioma cells, using a 378bp fragment of the core hTERT promoter to drive these genes results in apoptosis in 20-30% of cells, with caspase-6 being a slightly stronger inducer of apoptosis than caspase-8 (Takeuchi *et al.* 2004). This effect is also reproduced in a nude mouse model in which targeted expression of either gene results in significant tumour volume shrinkage that persists for up to 3 weeks following treatment (Komata *et al.* 2001a; Komata *et al.* 2002b). In other cell lines, caspase-8 expression under the control of the hTERT promoter can also induce apoptosis in up to 80% of cells, and result in a 50% shrinkage of tumour volume in a similar nude mouse model (Koga *et al.* 2000). Similarly, the hTERT promoter has been used to direct expression of the pro-apoptotic *Bax* gene *in vitro* and *in vivo* in tumour cells, the systemic administration of which normally causes significant liver toxicity (Gu *et al.* 2000). Another pro-apoptotic gene which has been investigated is the FADD gene, which causes selective cell apoptosis in transfected cells (Koga *et al.* 2001; Komata *et al.* 2001b).

A similar approach is to direct the expression of genes capable of causing cell death by means other than apoptosis. A number of genes, including the bacterial nitroreductase and herpes simplex virus thymidine kinase (HSV-TK) genes, are capable of converting harmless prodrugs into a toxic byproduct that causes cell death. An advantage of this approach over the pro-apoptotic gene approach is that the toxic by-products, if they diffuse readily between cells, can cause significant cell death through a 'bystander effect'. A number of groups have investigated the ability of the HSV-TK gene to cause selective cell death in telomerase-positive cells. HSV-TK converts gancyclovir (GCV) into a cytotoxic metabolite, resulting in cell death. The hTERT promoter has been used to drive expression of HSV-TK in telomerase-positive cells in several systems, including carcinoma cells, ovarian and cervical cancer cells, and in osteosarcoma cells, and has shown promising results both *in vitro* and *in vivo* (Majumdar *et al.* 2001; Song *et al.* 2003; Takeda *et al.* 2003). Targeted expression of the bovine αGal epitope, a major xeno-antigen in human cells, has been used to induce antibody-mediated cell lysis in telomerase-positive pancreatic carcinoma cells (Sawada *et al.* 2002). Similarly, targeted expression of the diphtheria toxin A-chain (DT-A) gene

in telomerase-positive bladder and hepatocellular carcinoma cells results in significant cell death *in vitro* (Abdul-Ghani *et al.* 2000).

A slightly different approach also makes use of telomerase promoters to target telomerase positive cells, but rather than driving the expression of a gene of interest, the promoters are used to drive replication of a normally-quiescent viral vector. In these circumstances, replication of the virus can proceed to such an extent that the resultant viral load causes lysis of telomerase-positive cells. Such 'conditionally-replicating' oncolytic viruses have been created usually by replacing the E1A internal promoter of adenoviral vectors with the hTERT promoter (Wirth *et al.* 2003). Kim *et al* went further by modifying the hTERT promoter through the addition of an extra E-box and Sp1 binding site before using it to drive replication (Kim *et al.* 2003b). Both wild-type and modified promoters have shown promising results both *in vitro* and *in vivo*, with several groups reporting significant reductions in tumour size following treatment with this strategy (Huang *et al.* 2003; Kim *et al.* 2003b; Wirth *et al.* 2003). The advantage of this approach is that viral infectivity is not affected by these modifications, so that infection of tumour cells with the virus leads to viral spread throughout the tumour, but replication and subsequent cell death only occurs within those cells possessing telomerase activity.

Although the majority of strategies using the telomerase promoter have used the hTERT promoter to drive either gene expression or viral replication, some groups have also investigated the hTR promoter as a means for driving gene expression (Boyd *et al.* 2001). Interestingly, although hTR is expressed in a greater variety of normal somatic cells than hTERT, the hTR promoter seems to drive gene expression with similar specificity to the hTERT promoter and in some cases is slightly stronger (Bilsland *et al.* 2003). Using the hTR promoter, Bilsland *et al* showed significant reduction of tumour size following injection with an adenovirus expressing bacterial nitroreductase under the control of the hTR promoter in xenografted nude mice (Bilsland *et al.* 2003). Similar to the HSV-TK construct, bacterial nitroreductase converts the prodrug CB1954 into a readily diffusible, active cytotoxic agent, again providing a useful bystander effect.

5.3 Aims of the Chapter

The overall aim of this chapter was to begin developing telomerase promoter-driven therapies for canine cancer. In order to do this, our first aim was to sequence and characterise the upstream regulatory region of the canine TERT promoter, identifying potential transcription factor binding sites within the promoter along with the transcription start site. Secondly, using promoter deletion constructs, we set out to identify the minimal promoter region responsible for activity in telomerase-positive cells to enable the development of future telomerase-targeted therapeutic strategies. Thirdly, given the potential shown by the hTR promoter in specifically driving gene expression in human tumour cells, we also aimed to sequence and characterise the canine TR gene promoter.

5.4 Materials and Methods

5.4.1 Genomic Library Screening

The promoter sequence of canTERT was initially isolated by Dr S Campbell by lambda library screening using a canine genomic DNA Lambda FIX® II Library (Clontech, UK). A radiolabelled 450bp PCR fragment comprising exon 2 of the canTERT gene (Nasir *et al.* 2004) was used as the probe. A single colony of the XL1-Blue MRA strain was used to inoculate 50ml LB broth. Aliquots of Library suspension (containing 2.5 x10^5 pfu of bacteriophage) were mixed with 1.7 ml bacterial cell suspension and incubated for 15 minutes at 37°C. NZY top agar was mixed with the infected cell preparation and spread evenly over 4 NZY agar plates before incubation at 37°C for 8 hours. Plaques were then transferred to nitrocellulose membranes, and membranes were hybridised overnight followed by exposure to film. Developed radiographs were aligned in duplicate to identify spots corresponding to unique plaques which were subsequently submerged in SM buffer/chloroform at 4°C overnight. Phage DNA was isolated from one positive clone using standard protocols. The phage DNA contained an insert of approximately 8.0Kbp.

Lambda phage DNA was sequenced using an automated ABI 310 genetic analyser (PE Applied Biosystems, UK) using the T7 primer. Based on the sequence generated using the T7 primer, a further primer was designed and used to sequence the phage DNA. This was repeated until the entire upstream regulatory sequence of canTERT was identified. From these analyses, raw unanalysed sequence data of approximately 8Kbp was determined (provided in Appendix B).

5.4.2 Confirmation of the canine TERT promoter sequence

In order to confirm the sequence obtained from phage DNA, five forward (sense) primers and three reverse anti-sense primers were designed as described in section 2.2.6.1: Primer design, and supplied by Sigma-Genosys (Table 5.2) for PCR amplification from genomic DNA. The primers were based on initial sequence of the canTERT promoter obtained from genomic library sequencing, and were located as illustrated in Figure 5.2. Each primer of 25-28 nucleotides in length required 40-60% GC content. Using these criteria, sense oligonucleotide primers (cTERTprom-5522F, cTERTprom-1572F, cTERTprom-907F, cTERTprom-677F, can3F, cTERTprom-172F) and anti-sense oligonucleotide primers (cTERTprom-628R, LG9R, LG7R, cTERTprom+487R) were designed.

Figure 5.2: Location of forward (blue) and reverse (pink) primers used for sequencing and generation of deletion constructs

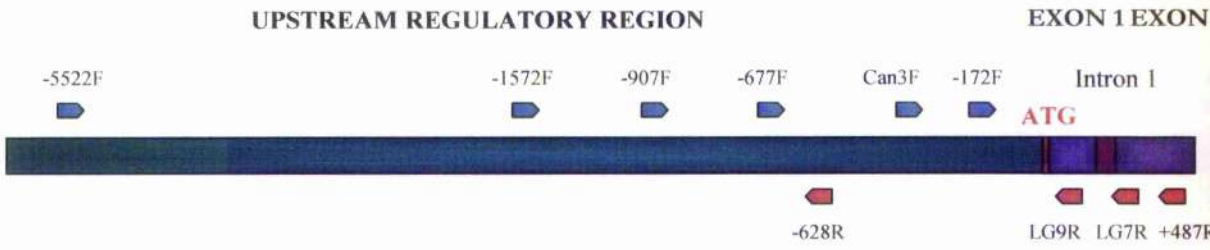


Table 5.2: Details of primers used in sequencing the canine TERT promoter and generation of deletion constructs

Primer Identification	Position (Start/end relative to ATG)	Oligonucleotide primer sequence (5′-3′)	Tm (°C) & GC (%) content
cTERTprom-5522F	-5506/-5474bp	GGCTCTCGAGCTCGAGTCTGGGTGACTCCAAA	66.4°C : 59%
cTERTprom-1572F	-1557/-1535bp	GCTCAAGCCAACAACATCAATT	65.0°C : 60%
cTERTprom-907F	-890/-867bp	GTCCTGTTCAAAGCGTCATGATGAA	69.2°C : 61 %
cTERTprom-677F	-665/-644bp	CTTTTTATCTCCTTTCGTGGAC	60.5°C : 60 %
cTERTprom-172F	-172/-150bp	CCGGGCCGGAGAGCAGAGGACG	80.2 C : 77%
Can3F	-314/-290bp	CTGTGACAGGGACAAACCCAAGGAC	75.0°C : 60 %
cTERTprom-628R	-612/-577bp	CGGTTTTCTCGAGCTCGAGCCCTACTTGCCAGGGA	75.0°C : 60 %
LG9R	+38/+66bp	AGCACCTCCCGGTAGCGGCCCCGCAGCA	75.0°C : 60 %
LG7R	+313/+333bp	CTCCTTGAGGCAGGACACCT	72.0 C : 60%
cTERTprom+487R	+457/+479bp	TTACCGTGTTGGGCAGGTAGCT	68.5 C : 55%

5.4.2.1 PCR amplification

PCR was performed using the primers detailed in Table 5.2 in order to isolate a 5.6kbp fragment of the canTERT promoter using genomic DNA isolated from PBMC (2µl) as a template in a final volume of 50µl containing 0.4µm of forward and reverse primers, 0.2mM of dNTPs, 1.5Mm $MgCl_2$ and 2 units of *Taq* DNA polymerase (Invitrogen, UK). Using a thermal cycler (Perkin Elmer) samples were subjected to an initial denaturation at 95° C for 2 minutes followed by 35 cycles of amplification, each cycle consisting of a denaturation step of 95° C for 30 seconds and an annealing temperature of 55-70° C (depending on the annealing temperature of the primer pair used) for 30 seconds followed by an elongation step of 72° C for 30 seconds per Kb of expected product. The final elongation step of 72° C for 10 minutes completed the reaction. PCR products (4 µl) were visualised by agarose gel electrophoresis (1.5%) gel by comparing the bands created to a 100 bp molecular weight standard.

5.4.2.2 PCR amplification - Troubleshooting

As initial attempts to clone the entire promoter fragment were unsuccessful, the following modifications and PCR sequencing kits were used as detailed below: HotstarTaq, Q solution, addition of 5% DMSO, Failsafe PCR mix (Cambio, UK), PCRx enhancer system. PCR products (4 µl) were visualised by agarose gel electrophoresis (1.5%) gel by comparing the bands created to a 100 bp molecular weight standard.

5.4.2.2.1 Modification of PCR cycle conditions

Initial PCR conditions used included an annealing temperature calculated to be 5° C below the lower of the two annealing temperatures of the primer pair used. Subsequent modifications of cycling conditions included lowering the annealing temperature to 55° C to decrease the specificity of primer binding. If PCR using the lower annealing temperature produced a band of the expected size along with other bands (due to non-specific priming), the annealing temperature was subsequently increased in 2° C steps with successive PCR attempts until specific priming occurred.

In addition to annealing temperature modifications, a modified 'hot start' step was performed to reduce mispriming events and primer-dimer formation at low temperatures. To perform a modified 'hot start', the reaction mix was chilled on ice until the thermal cycler had reached a temperature of 95° C before adding the reaction. In addition, the initial denaturation step was increased to 5 minutes.

In addition to the above-mentioned modifications, samples were also made up with the addition of Dimethylsulfoxide (DMSO) at 5% or glycerol at 10%.

5.4.2.2.2 HotStarTaq and Q solution

PCR amplification was performed using genomic DNA as a template (≤ 1 µg) in a final volume of 100 µl, containing 0.5µm of forward and reverse primers, 0.2mM of dNTPs, 10 µl of 10x PCR buffer (containing 15 mM $MgCl_2$), 20 µl of Q solution, and 2.5 units of HotStarTaq DNA Polymerase (Qiagen, UK). A master mix was prepared containing all components necessary for the total number of assays to be performed with the exception of the DNA template, and 100 µl aliquoted into each PCR tube. In addition, duplicate reactions were performed without Q-solution. DNA template was added to each tube immediately before the tubes were placed in the thermal cycler. Samples were then subjected to initial denaturation of 15 minutes at 95° C followed by 35 cycles of amplification, each cycle consisting of a denaturation step of 94° C for 30 seconds and an annealing temperature of 55-70° C (depending on the annealing temperature of the primer pair used) for 30 seconds followed by an elongation step of 72° C for 30 seconds per Kb of expected product. The final elongation step of 72° C for 10 minutes completed the reaction. PCR products (4 µl) were visualised by agarose gel electrophoresis (1.5%) gel by comparing the bands created to a 100 bp molecular weight standard.

5.4.2.2.3 Failsafe PCR mix

The Failsafe™ PCR Premix kit (Cambio, UK) contains a blend of thermostable DNA polymerases together with a set of twelve reaction premixes, designed to amplify difficult or long templates. The enzyme blend contains a 3' - 5' proofreading enzyme for high fidelity amplification, whilst the premixes contain a buffered salt solution together with all four dNTPs, varying amounts of $MgCl_2$ and FailSafe PCR Enhancer (with betaine). The presence of betaine is designed to improve the yield and specificity of amplification of many target sequences, especially those containing a high GC content or secondary structure. In addition, betaine may also enhance the PCR by protecting DNA polymerases from thermal denaturation. The twelve premixes are designed to rapidly optimise the PCR conditions required for maximal amplification of any given template, with the appropriate premix then being used for further amplification reactions once optimisation has been completed.

PCR was performed using genomic DNA as a template (5 µl) in a final reaction mix of 25 µl, containing 1 µl of each of the forward and reverse primers, 0.5 µl Failsafe PCR enzyme mix (1.25 units) and distilled water. A mastermix was made up sufficient for 14 reactions. Then 25 µl of each premix was aliquoted into each of 12 0.2 ml PCR tubes and 25 µl of the mastermix added to

each tube. PCR was then performed using the following conditions: initial denaturation at 98° C for 2 minutes, followed by 35 cycles of: denaturation at 95° C for 30 seconds, annealing at 55° C for 30 seconds and extension at 68° C for 30 seconds per Kb of expected product, with a final extension step of 68° C for 10 minutes. Samples (4 µl) were analysed by agarose gel electrophoresis (1.5%) by comparing the bands created to a 100 bp molecular weight standard.

5.4.2.2.4 KOD Hot Start Taq Polymerase

KOD Hot Start Taq (Novagen, UK) is an optimised buffer and *Taq* polymerase designed for amplifying GC-rich and complex templates. The *Taq* polymerase mix includes two antibodies that prevent polymerase and 3' to 5' exonuclease activity at ambient temperatures and thereby avoids mispriming events during reaction setup and initial temperature elevation. Genomic DNA template (2 µl) was amplified in a final volume of 50 µl containing 5 µl (1X) 10X PCR buffer, 0.2 mM dNTPs, 1 mM $MgSO_4$, 0.3 µM of each of the forward and reverse primers, and 1 unit of KOD Hot Start DNA Polymerase. The reaction was assembled in PCR tubes and transferred to the thermal cycler. PCR conditions consisted of an initial denaturation/polymerase activation step at 94° C for 2 minutes, followed by 35 cycles of denaturation at 94° C for 15 seconds, annealing at 60° C for 30 seconds and extension at 72° C for 30 seconds per Kb of expected product. Samples (4 µl) were analysed by agarose gel electrophoresis (1.5%) by comparing the bands created to a 100 bp molecular weight standard.

5.4.2.2.5 PCRx enhancer

The PCRx Enhancer System (Invitrogen, UK) is an optimised buffer and cosolvent system that simplifies PCR amplification of problematic and/or GC-rich templates. The PCRx Enhancer Solution offers higher primer specificity, broader optimal magnesium concentration range and broader optimal annealing temperature range. The Enhancer Solution lowers the maximum primer annealing temperature by approximately 2° C per 1X PCRx enhancer Solution concentration, but also widens the effective annealing temperature over a much broader range.

In the initial optimisation step, genomic DNA template (1 µl) was amplified in a final volume of 50 µl. A master mix sufficient for 30 µl per reaction for six reactions was made up initially, however, containing 5 µl (1X) of 10X PCRx Amplification buffer, 0.2 mM of dNTPs, 1.5 mM $MgSO_4$, 0.2 µM of forward and reverse primers, 1 µl of template DNA and 2.5 units of Platinum® *Taq* DNA polymerase (Invitrogen, UK). Thirty µl of the master mix was then aliquoted into each of six PCR tubes. Varying amounts of PCRx Enhancer Solution were then added to each tube to reach a final Enhancer Solution of 0X – 4X as detailed below.

Component	Final PCRx Enhancer Solution Concentration					
	0X	0.5X	1X	2x	3X	4X
Master Mix	30	30	30	30	30	30
10x Enhancer Solution	0	2.5	5	10	15	20
Distilled Water	20	17.5	15	10	5	0

PCR tubes were then placed in the thermal cycler and an initial denaturation at 95° C for 2 minutes was performed, followed by 35 cycles of denaturation at 95° C for 30 seconds, annealing at 55° C for 30 seconds and extension at 68° C for 30 seconds per kb. A final extension step at 68° C for 10 minutes was then performed. Products (4 µl) were then analysed by agarose gel electrophoresis.

5.4.2.3 Cloning into pCR2.1®TOPO vector

PCR products amplified from genomic DNA were cloned using methods described in section 2.2.3.8: Ligation using TA cloning methods.

5.4.2.4 Bioinformatics analysis

The search for putative transcription factor binding sites was performed using web-based motif-searching tools TFBIND (http://www.tfbind.hgc.jp), TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html), TESS (Transcription Element Search System: http://www.cbil.upenn.edu/tess/) and MOTIF (http://motif.genome.jp). Transcription factors were only considered for analysis if they had the following scores: $L_a \geq 12.0$, $L_a = 2.0$, $L_q = 1.0$, $L_i = 0$. CpG island analysis was performed using the web-based CpG Island Searcher (http://www.uscnorris.com/cpgislands/cpg.cgi).

5.4.2.5 Transcription Start Site Identification

To determine the Transcription Start Site (TSS) of the canTERT gene, the GeneRacer™ kit (Invitrogen, UK) was used for RNA ligase-mediated (RLM) rapid amplification of cDNA ends (RACE). This method selectively ligates an RNA oligonucleotide to the 5' ends of decapped mRNA. Reverse transcription using a primer specific to the gene sequence (GSP) or using an oligo(dT) primer specific to the polyA tail of mRNA can then be used to synthesise cDNA from the mRNA. This cDNA can then be PCR-amplified using a forward primer specific to the ligated oligonucleotide and a reverse GSP to generate products which can be sequenced to determine the 5' untranslated sequence.

In the first step, total RNA was treated with calf intestinal phosphatase (CIP) to dephosphorylate non-mRNA and truncated mRNA. Total RNA (5µg) previously extracted from C17 and D17 cells was added to 10X CIP buffer (1µl), RnaseOut™ (40U), CIP (10U) and the total volume made up to 10 µl with DEPC-treated water in a 1.5 ml eppendorf tube. This reaction mixture was mixed gently by pipetting and briefly vortexed before flash-spinning to collect the contents in the bottom of the tube. The sample was then incubated at 50° C for 1 hour.

Following incubation, samples were centrifuged briefly and placed on ice before RNA was precipitated. To the eppendorf tube, 90 µl of DEPC-treated water and 100 µl of phenol:chloroform were added and the samples vortexed vigorously for 30 seconds. Samples were then centrifuged at maximum speed for 5 minutes at room temperature. Following centrifugation, the aqueous phase was withdrawn from the top of the samples and transferred to a new eppendorf tube. To this was added 2µl of 10 mg/ml mussel glycogen and 10 µl of 3M sodium acetate, pH 5.2, and the tube was then inverted several times to mix. Then 220 µl of 95% ethanol was added and the sample vortexed briefly before being placed on dry ice for 10 minutes. To pellet RNA, the samples were then centrifuged at maximum speed for 20 minutes at 4° C. The supernatant was then removed, taking care not to disturb the resultant RNA pellet, and 500 µl of 70% ethanol was added. The tube was inverted several times and vortexed briefly before centrifugation at maximum speed for 2 minutes at 4° C. Supernatant was again carefully removed, and brief centrifugation performed to collect any residual ethanol in the bottom of the tube. This was carefully removed and the pellet left to air dry at room temperature for 1-2 minutes before being resuspended in 7 µl of DEPC-treated water.

In the second step, the mRNA cap was removed from intact, full length mRNA using tobacco acid pyrophosphatase (TAP). To the eppendorf containing RNA from the previous step was added 10X TAP buffer (1 µl), RnaseOut™ (40U) and TAP (0.5U). The mixture was mixed gently by pipetting and brief vortexing followed by a flash-spin to collect contents in the bottom of the tube. The sample was then incubated for 1 hour at 37° C, centrifuged briefly and placed on ice. RNA was then precipitated as described above.

Following decapping, the GeneRacer™ RNA Oligo was then ligated to the 5' end of mRNA in the third step. The oligo is designed to optimise ligation to decapped mRNA by virtue of the fact that it has minimal secondary structure to provide a free 3' end for efficient ligation and a string of adenines at the 3' end to increase ligation efficiency (Uhlenbeck & Gumport 1982). Seven µl of dephosphorylated, decapped RNA from step 2 was added to the tube containing lyophilised GeneRacer™ RNA Oligo (0.25 µg). The sample was pipetted up and down several times to resuspend the RNA oligo then flash-spun to collect the contents in the bottom of the tube. The sample was then incubated at 65° C for 5 minutes to relax the RNA secondary structure then placed on ice to chill for approximately 2 minutes before re-centrifuging to collect the contents in the

bottom of the tube. Then, 10X Ligase buffer (1 µl), 10 mM ATP (1 µl), RnaseOut™ (40U) and T4 RNA ligase (5U) were added to the tube which was then incubated for 1 hour at 37° C. The sample was then flash-spun to collect the contents in the bottom of the tube and placed on ice. RNA precipitation was then performed as previously, with the RNA pellet resuspended in 10 µl of DEPC-treated water rather than 7 µl.

In the fourth step, the mRNA was reverse-transcribed into cDNA using SuperScript™ III RT (Invitrogen, UK). To the RNA sample from step 3 was added 10 µM reverse primer (1 µl - consisting of either oligo(dT) or LG7R GSP), dNTP mix (1 µl), and sterile, distilled water (1 µl). The mixture was then incubated at 65° C for 5 minutes to remove any RNA secondary structure before being placed on ice for 1 minute and flash spinning to collect the contents in the bottom of the tube. Then First Strand Buffer 5X (4 µl), 0.1 M DTT (1 µl), RnaseOut™ (40U) and SuperScript™ III RT (200U) were added to the sample which was mixed by pipetting several times. The sample was then flash-spun to collect the contents in the bottom of the tube and incubated for 30 minutes at 50° C for Oligo(dT) or 55° C for GSP. The RT reaction was then inactivated by heating at 70° C for 15 minutes before being placed on ice for 2 minutes to chill. The sample was flash spun to collect the contents in the bottom of the tube, then 1 µl of RNaseH (2U) was added to the mixture which was then incubated at 37° C for 20 minutes to inactivate any remaining RNA. The sample containing cDNA was then stored at –20° C until ready for use in PCR amplification.

As a positive control, a sample containing total RNA from HeLa cells (Invitrogen, UK) was run in parallel to samples obtained from CMT7/D17 cells. Following the synthesis of cDNA, PCR amplification was then performed using the forward primer specific to the GeneRacer™ RNA oligo and the GSP (LG7R). Initial PCR was performed as described in 2.2.6: Amplification of DNA by the polymerase chain reaction, using Platinum HiFi Taq polymerase (Invitrogen, UK). Secondary PCR was also performed using the nested forward primer specific to the GeneRacer™ RNA oligo and a nested reverse GSP (LG9R).

5.4.3 Creation of canTERT promoter deletion constructs

Following confirmation of the canTERT promoter sequence, selected fragments were PCR-amplified using plasmid DNA as a template. The same primers used for confirmation of the canTERT promoter sequence, shown in Table 5.2 and Figure 5.2, were also used to amplify these fragments. Following successful amplification and cloning into pCR2.1®TOPO of a 1.6 kb-sized fragment of the promoter together with exon 1, intron 1 and 15 bp of exon 2 using the -1572F/LG7R primer pair, purified plasmid DNA derived from this construct was used as a template for further nested PCR using the following primer pairs: -1572F/LG9R, -907F/LG7R, -907F/LG9R, -677F/LG9R, can3F/LG7R and can3F/LG9R. PCR products were purified and cloned

into pCR2.1®/TOPO using methods described in 2.2.3.8: Ligation using TA cloning methods, before being sequenced with the M13F and M13R primers as described in 2.2.7: DNA sequence analysis.

5.4.3.1 Restriction enzyme digestion of vectors

All pCR®2.1TOPO/canTERT promoter constructs containing the canTERT promoter fragments were digested with *Xho* I and *Sac* I restriction enzymes. The luciferase reporter vector, pGL3-Basic (Promega, UK) was also digested with *Xho* I and *Sac* I restriction enzymes. More specifically, the pCR®2.1/canTERT promoter vectors (5µg) were digested for one hour with *Xho* I (5 units) in buffer D (10X) together with 0.3µl bovine serum albumin (BSA) in a 10µl total volume at 37° C, before adding *Sac* I (5 units) and (10X) Multi-Core™ buffer to a final volume of 20µl and continuing with incubation for a further 3 hours. The recipient pGL3-Basic luciferase reporter vector (5µg) was digested with *Xho* I (5 units) and *Sac* I (5 units) using the same protocol. Samples (4µl) were analysed on a TAE agarose gel (1%) by comparing the bands created to a 100bp DNA ladder to confirm digestion before purification and ligation.

5.4.3.2 Purification, ligation and transformation

Digested DNA samples (linearised pGL3-Basic and released canTERT promoter fragments) were purified using the QIAquick®Gel Extraction Kit (QIAGEN, UK) and eluted in 30µl of sterile water. The DNA was quantified (2µl) by gel electrophoresis on a TAE agarose gel (1%) by comparing the bands created to a Low DNA Mass™ Ladder (2µl).

The quantity of vector (pGL3-Basic) and insert (promoter fragment) for each ligation reaction was calculated according to the equation shown in section 2.2.3.7: Ligation of restriction digested DNA fragments. For each calculation the vector mass (X) was 150 ng and the insert size (Y) was approximately 2.1, 1.5, 1.2, 1.1, 0.9, 0.8, 0.7 and 0.3 kb for the canTERT promoter deletions (in descending size). Ligation was performed using molar ratios of 3:1 and 1:1 insert to vector. The cut ends of the inserts were ligated to complementary ends of the cut pGL3-Basic vector using T4 DNA ligase (Promega, UK) overnight at 16° C. The ligations (20ng) were transformed into 50µl of One Shot™ TOP10 competent cells which were heat shocked for 45 seconds at 42° C before adding 250µl SOC medium, shaking horizontally at 225 rpm at 37° C and then grown overnight at 37° C on LB/ampicillin agarose plates. Six to ten colonies were selected from each plate and transferred to LB broth for overnight culture at 37° C.

The DNA constructs were isolated from overnight cultures by the alkaline lysis method of plasmid DNA isolation following the QIAprep®PCR Spin Miniprep Kit (Qiagen, UK) and eluted in 50µl of

sterile water. The DNA samples were then assessed by spectrophotometry for quantification. pGL3/canTERT promoter constructs were screened with *Xho* I and *Sac* I restriction enzyme digestion as described in 2.2.3.11.2: Restriction analysis of small scale plasmid preparations. Since all the promoter inserts were directionally cloned it was not necessary to screen for correct orientation. Recombinant plasmids were sequenced and analysed as described in 2.2.7: DNA sequence analysis.

5.4.4 Luciferase reporter assays

The pGL3-Basic luciferase reporter vector was chosen to analyse the canTERT promoter deletion constructs since luciferase reporters offer an extremely sensitive system for measuring the activity of regulatory regions of DNA such as promoters. A variety of luciferase-based vectors are available enabling strictly controlled experiments including a negative control (promoter-less vector, pGL3-Basic), positive control (SV40-driven luciferase, pGL3-Control) and a *Renilla* luciferase vector (pRL-CMV) as an internal standardised control. This Dual-Luciferase®Reporter assay system has the unique ability to assay both the fire-fly and *Renilla* luciferase activities sequentially in the same sample to control for both cell number and transfection efficiency.

5.4.4.1 Cell lines

CML10 (canine melanoma), D17 (canine osteosarcoma), A72 (fibroblastoid tumour), GHK (canine kidney), CMT3 (canine mammary tumour), CMT7 (canine mammary tumour) and CMT8 (canine mammary tumour) are canine cell lines which have previously been shown to be telomerase positive (Nasir *et al.* 2001). CML 10, D17, A72 and GHK cell lines were cultured in DMEM (Gibco BRL, Grand Island, New York) supplemented with 10% fetal calf serum. CMT3, CMT7 and CMT8 cell lines were cultured in RPMI (Gibco BRL) supplemented with 10% fetal calf serum and L-glutamine. Canine primary skin fibroblasts (K9SF) were cultured in MEM-α (Gibco BRL) supplemented with 10% fetal calf serum, L-glutamine and epidermal growth factor (EGF).

5.4.4.2 Transfection of cells with luciferase reporter constructs

Transient transfections were performed using Lipofectamine™ in the presence of Plus™ reagent (Invitrogen, UK). For each assay, 1.5 x 10^5 cells were seeded into 6-well plates and transfected the following day, when the cells reached a confluence of approximately 40-50%. For comparison, the transcriptional activities of pGL3basic (negative control) and pGL3control (positive control) were also tested. All transfections were performed in triplicate, and the results of at least 2 independent experiments were averaged. Cells were transfected with 1μg of pGL3/canTERT promoter vectors containing the firefly luciferase gene. For each well, 1μg plasmid DNA was diluted in the presence

of 100µl serum free media and precomplexed with 6µl Plus Reagent for 15 minutes at RT. Four µl of Lipofectamine diluted in 100µl serum free media was then added to the precomplexed DNA and the solution incubated at RT for a further 15 minutes. Cells were incubated in the presence of the DNA-Plus-lipofectamine complexes at 37°C with 5% CO_2 for 3 hours. The medium was then replaced with fresh growth medium and cells incubated for a further 48 hours prior to harvesting. Co-transfection with the pRL-CMV vector (Promega, UK), containing the *Renilla* luciferase gene was used as an internal standardised control to assess transfection efficiency.

5.4.4.3 Luciferase assays

Dual-Luciferase®Reporter assays were performed in triplicate as described in 2.2.8.1: Genetic reporter systems. Both firefly and *Renilla* luciferase values were attained for each well. The mean of the 3 wells analysed for each sample was calculated, and luciferase values adjusted for cell concentration by standardising each sample against the mean protein concentration for all 3 wells. The corrected firefly luciferase value for each sample was then expressed as a percentage of the corresponding *Renilla* luciferase value, which served to correct for transfection transficiency. The final corrected luciferase values of all test samples as well as the negative control were then expressed as a percentage of the positive control (SV40 promoter-driven luciferase).

5.4.5 Sequencing of the canine TR promoter

5.4.5.1 PCR amplification of the canine TR gene

Initial sequence searches were performed by screening partial, unannotated canine DNA sequences obtained as part of the canine genome project via NCBI trace files (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi?). Trace files were screened using the sequence of the human TR gene obtained from Genbank (AF221907). BLAST searches revealed three trace files: 271822249, 239572746, 272209162, all of which showed good alignment with the hTR sequence and with each other. One of these, 271822249 contained 329 bp of upstream regulatory sequence.

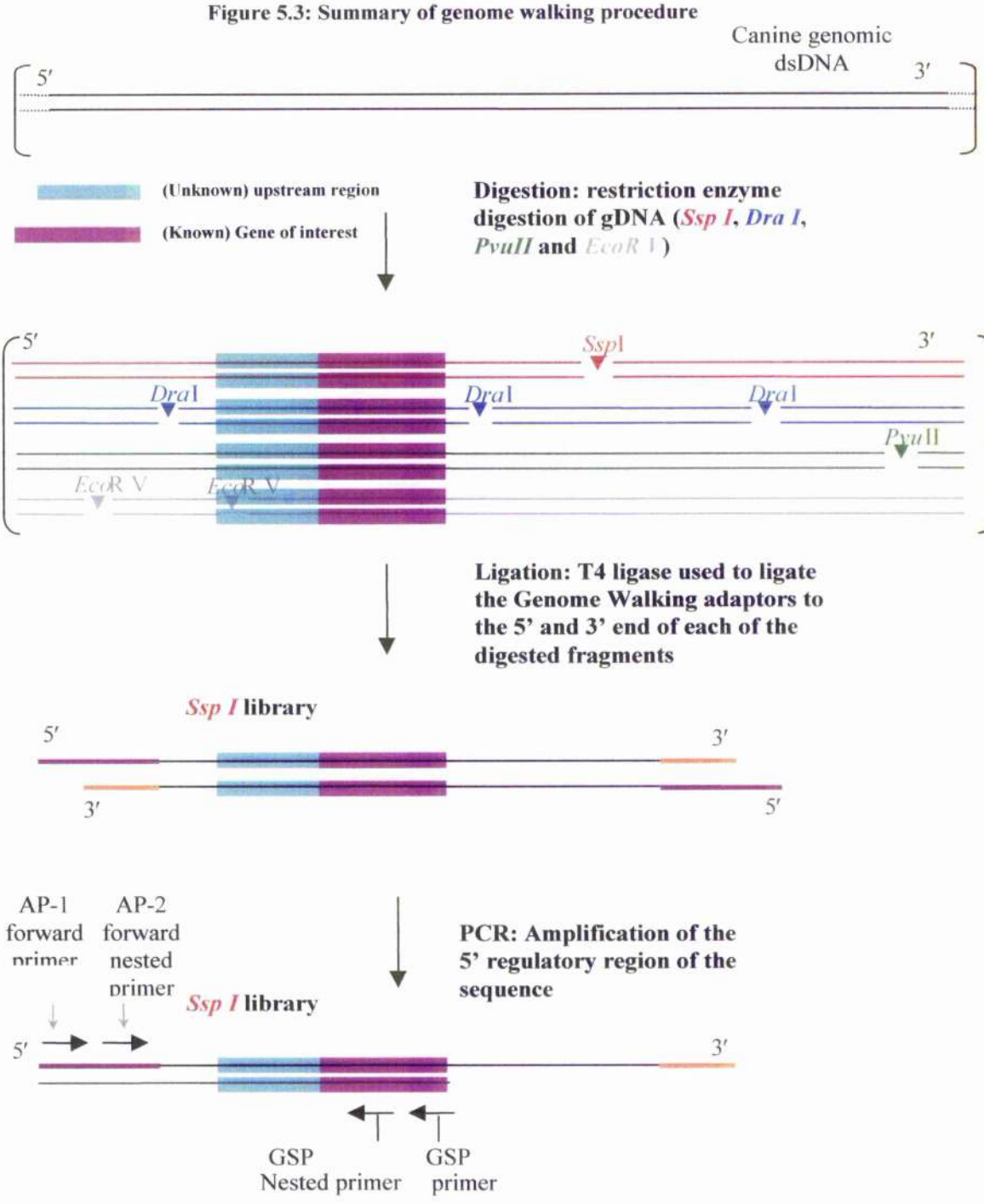
The sequence obtained from trace files was used to design primers for amplification and sequencing of the canine TR gene following genome walking. The details of these are shown in Table 5.3. Initial primers dogTR1F and dogTR1R were designed to amplify a 286 bp fragment of the canine TR gene from genomic DNA to confirm that the sequence obtained from trace files was amplifiable. PCR was performed using the following conditions: initial denaturation at 95° C for 2 minutes, followed by 35 cycles of denaturation at 95° C for 30 seconds, annealing at 55° C for 30 seconds and extension at 68° C for 30 seconds per kb. A final extension step at 68°C for 10

minutes was then performed. The products from this reaction were then analysed by agarose gel electrophoresis, cleaned and cloned into pCR2.1®TOPO as previously described prior to sequencing with the T7 forward primer.

5.4.5.2 Genome Walking

The Genome Walking procedure, shown in Figure 5.3, is explained in further detail below.

Figure 5.3: Summary of genome walking procedure



All genome walking performed during this part of the project was undertaken by Ms L. Gault. The GenomeWalker™ technique (BD Bioscence, UK) provides a method for walking up-stream towards promoters from a known DNA sequence (Siebert *et al.* 1995) and is a simpler and potentially faster approach than those protocols involving the generation and screening of gDNA libraries. In summary, canine gDNA was digested with four different restriction enzymes and GenomeWalker Adaptors were then ligated to 5′ ends of the cut fragments to form four libraries composed of gDNA fragments. These libraries were then used as templates for each of three genome walks, which consisted of two PCR amplifications using sense adaptor primers and anti-sense gene specific primers (GSPs) (Figure 5.3). The GSP sequences were designed based on the 5′ end of partial canine TR gene sequence located through trace file screening. PCR products thus generated were then cloned and sequenced allowing the design of new gene specific primers for the second and third genome walks.

5.4.5.2.1 Primer design

Two primers were designed for each genome walk as described in 2.2.6: Amplification of DNA by the polymerase chain reaction, and were synthesised and supplied by Sigma Genosys (Table 5.3). Each primer of 25-28 nucleotides in length required 40-60% GC content to ensure that primers would effectively anneal to the template at the recommended annealing and extension temperature of 67° C. Primer sequences were designed to prevent the folding and formation of intra-molecular hydrogen bonds and particular attention was paid to reducing the GC content at the 3′ end of the gene specific primers to prevent their binding to the 3′ end of the adaptor primers.

5.4.5.2.1.1 Canine TR primers

Using these criteria, two anti-sense oligonucleotide primers (GSP-1R, GSP-2R) were designed according to the 5′ region of the canine TR sequence obtained from screening trace files. On completion of the first genome walk it was possible to design a third anti-sense oligonucleotide primer (GSP-3R) which was used in combination with GSP-2R for the second genome walk along the canine TR promoter sequence (Figure 5.12).

5.4.5.2.1.2 GenomeWalker Adaptor primers

Forward primers, specific to the Adaptor sequence attached to the 5′ end of the gDNA fragments, were supplied with the kit and are referred to as Adaptor primer-1 (AP1) and nested Adaptor Primer-2 (AP2).

Table 5.3: Primer sequences for genome walking

Primer name	Oligonucleotide primer sequence 5'to 3'	Tm (°C) & GC (%) content
DogTR1F	TCCGTCTAACCCTAACTGAGCA	58.4° C : 52 %
DogTR1R	GGCTGACAGAGCCCAACTCTT	60.2° C : 57 %
GSP1R	TGATTGGCCGAAGCCGTCATCTCGCCTCT	82.6° C : 59 %
GSP2R	TTTCTCCACCCCCCTCCCTCTCCATC	77.3° C : 62 %
GSP3R	CTACGGTTCTACTTCCGGCCGTGAGGA	75.9° C : 59 %
AP1	GTAATACGACTCACTATAGGGC	58.4 °C : 45 %
AP2	ACTATACGGCACGCGTGGT	58.8 °C : 58 %

5.4.5.2.2 Preparation of canine genomic DNA

Canine gDNA was prepared from peripheral blood mononuclear cells (PBMCs) as described in 2.2.2: Preparation of canine genomic DNA from peripheral blood mononuclear cells.

5.4.5.2.3 PCR amplification of the canine TR gene from genomic DNA

In order to confirm the sequence of the canine TR gene, a 286 bp fragment was amplified using the primers dogTR1F and dogTR1R. PCR was performed using genomic DNA (2 µl) as described in 5.4.5.1: PCR amplification of the canine TR gene.

5.4.5.2.4 Digestion and purification of canine genomic DNA

Four blunt ended digestions of canine gDNA (2.5µg) were performed, each in a total volume of 100µl with the appropriate restriction enzyme *Stu* I, *Dra* I, *Pvu* II or *Eco*R V (80 units) and restriction enzyme buffer (1x) combination (Table 2.1 in Chapter 2). Reactions were incubated at 37° C overnight before samples (5µl) were analysed for complete digestion by TAE agarose gel (1%) electrophoresis. For DNA purification, phenol (95µl) was added to each reaction, vortexed and flash spun to separate the aqueous and organic phases. The upper aqueous layer from each tube was mixed with an equal volume of chloroform added, vortexed and briefly spun to separate the aqueous and organic phases. This time the DNA in the upper layer was precipitated with 2 volumes

of ethanol (95%), 1/10 volume 3M NaOAc (pH 4.5) and 20µg of glycogen. After vortexing and centrifugation at 15,000rpm for 10 minutes, supernatants were decanted and the pellet washed in 100µl ethanol (80%). After a final centrifuge at 15,000 rpm for 5 minutes and supernatant discarded the pellet was air-dried and dissolved in 20µl of Tris-EDTA (10/0.1, pH 7.5). The quality of gDNA was again assessed using TAE agarose gel analysis.

5.4.5.2.5 Ligation of digested canine gDNA to GenomeWalker Adaptors

The digested, purified DNA (4µl) was ligated to the GenomeWalker Adaptors (25µM) in a total volume of 8 µl with T4 DNA ligase (4 units) and ligation Buffer (1x) at 16° C overnight. The reactions were incubated at 70° C for 5 minutes before dilution to a total volume of 80 µl with Tris-EDTA (10/1 pH 7.4).

GenomeWalker Adaptor sequence

AP1

5' **GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT** 3'

AP2

5.4.5.2.6 PCR-based DNA Walking in GenomicWalker Libraries

The four different libraries were subjected to two rounds of PCR (primary and secondary) using a PE 2400 thermal cycler. For the first genome walk, the primary round of amplification used the adaptor-ligated gDNA as template with 0.2µM of sense primer 'Adaptor Primer 1' (AP1) and anti-sense 'Gene Specific Primer' (GSP-1R) (Table 5.3), 10X BD Advantage 2 PCR Reaction Buffer (40 mM Tris-HCl, 15 mM KOAc, 0.02% Triton X-100), dNTPs (10mM), and BD Advantage 2 Polymerase Mix (50x) (5 units of Tth DNA polymerase, 0.5µg of TthStart Antibody, 1% glycerol, 0.2 mM Tris-HCl pH 7.5 and 4.6 mM KCl) in a total volume of 50µl. Primary PCR conditions were held at 94° C for 25s, 72° C for 4 mins for 7 cycles, and 94° C for 25s, 67° C for 4 mins for 32 cycles, then 67° C for 4 mins. Primary PCR products were diluted 50 fold and 1µl was used as a template for secondary, nested PCR with 0.2µM of sense primer 'Adaptor Primer 2' (AP2) and anti-sense 'Gene Specific Primers' (GSP-2R) (Table 5.3) in a reaction mix as described for primary PCR. Secondary PCR conditions were 94° C for 25s, 72° C for 4 mins for 5 cycles, and 94° C for 25s, 67° C for 4 mins for 20 cycles, then 67° C for 10 mins.

The second genome walk used anti-sense 'Gene Specific Primer' (GSP-2R) in the primary round of PCR and nested primer (GSP-3R) in the secondary round of PCR. PCR conditions were altered slightly with the annealing temperature reduced from 72 to 69°C and 67 to 64°C in secondary PCR. In addition, 6% DMSO and 3% glycerol were added to the reaction mix. A negative control containing all PCR components (primers, dNTPs, Advantage 2 PCR buffer and Advantage 2 Polymerase Mix) except template was performed to ensure no contamination of the PCR reactions with extraneous DNA that might serve as a template for PCR amplification.

PCR products were purified and cloned into pCR2.1®/TOPO using methods described in 2.2.3.8: Ligation using TA cloning methods, before being sequenced with the M13F and M13R primers as described in 2.2.7: DNA sequence analysis.

5.5 Results

5.5.1 Sequence analysis of the canTERT promoter

Screening of a canine genomic DNA library with a 450bp canTERT probe yielded a single positive clone. Sequence analysis revealed that approximately 8Kb of sequences upstream of the canTERT ATG were present within this clone. Initial attempts to clone a 5.6 Kbp subfragment using both the cTERTprom-5522F/LG7R and cTERTprom-5522F/LG9R primer pairs were unsuccessful. However, a smaller subfragment of 4929 bps using cTERTprom-5522F/cTERTprom-628R was successfully amplified and cloned into pCR2.1®TOPO. Following multiple attempts to amplify the proximal upstream fragment, successful amplification of a fragment with the cTERTprom-1572F/LG7R primer was achieved using the PCRx™ Enhancer system (Invitrogen, UK). This fragment was also cloned into pCR2.1®TOPO. Sequencing of these two fragments at least 3 times allowed the generation of a composite sequence of the promoter region as far upstream as –5390 bp with an overlapping region between -600 and -1590 bp. Sequencing included the initial part of the canTERT gene (the ATG start codon together with exon 1 (218 bp), intron 1 (106 bp) and 15 bp of exon 2. The initial A nucleotide of the start codon was designated +1. The canTERT promoter sequence (5390bp) upstream of the ATG has been deposited in NCBI GenBank database: AY563633 and this sequence together with exon1, intron 1 and the small fragment of exon 2 is given in Appendix B.

5.5.1.1 CpG Analysis

Sequence analysis showed that the canTERT promoter is highly GC rich and has no detectable TATA or CAAT boxes. Whilst genomic DNA has an overall GC content of 40% and an observed/expected (O/E) CpG ratio of 0.25, within the 5390 bp fragment, 4 CpG islands were found, defined as areas larger than 200bp with a GC content of at least 60% and an O/E CpG ratio of greater than 0.6. The largest of these, with a GC content >80% and an O/E CpG ratio of 0.8, was identified extending from bp –339 into exon 2. A smaller island was identified with GC content >60% and O/E CpG ratio of 0.6 extending from bp –1280 to –1078. Further upstream, two other CpG islands, with GC contents >62% and >65% and O/E CpG ratios of 0.6 were identified extending from bp –4471 to –4267 and bp –5042 to -4766 respectively. This is shown in Figure 5.4.

5.5.1.2 Transcription Factor Binding Sites

Multiple putative transcription factor binding sites were identified within the promoter and are shown in Table 5.4. These include binding sites for the Oestrogen Receptor, MZF-2, a single E-box and multiple Sp1 binding sites. The sequence of 1.6 Kb of the promoter together with potential transcription factor binding sites is shown in Figure 5.5. A comparison of the canine and human TERT 5' promoter regions showed that many of the same transcription factor binding sites known to be involved in hTERT regulation are present within the canTERT promoter including AP1, c-ets-2, c-myb, NF1, Myo D, WT1, c-myc, GR/PR, MZF-2, ER/Sp1 and MAZ (Figure 5.6). The hTERT promoter also contains one PR and p53 binding site, which were not detected in the canTERT promoter. Interestingly, several binding sites unique to the canTERT were also identified including GR, c-ets-1, c-jun, myc-CF-1, SRY and MTF-1 (Figure 5.6).

5.5.1.3 Transcription Start Site

Using the GeneRacer™ (Invitrogen, UK) RACE kit, multiple attempts were made to obtain the 5' end of the transcription sequence. For reverse priming of RACE-ready RNA, both oligo(dT) and the gene-specific primer LG7R were used. Initial results revealed the existence of multiple, non-specific bands. Secondary and nested PCR were then performed using platinum HiFi Taq Polymerase (Invitrogen, UK) in addition to the PCRx Enhancer system, to try to amplify the 5' end of the untranslated region. However, all attempts were unsuccessful, yielding only multiple, non-specific bands (Figure 5.7).

Figure 5.4: CpG Island analysis of 5.5Kb of the canTERT promoter, including CpG islands in relation to ATG start codon, Exon 1/Intron 1/Exon 2 and location of forward primers used to generate deletion constructs.

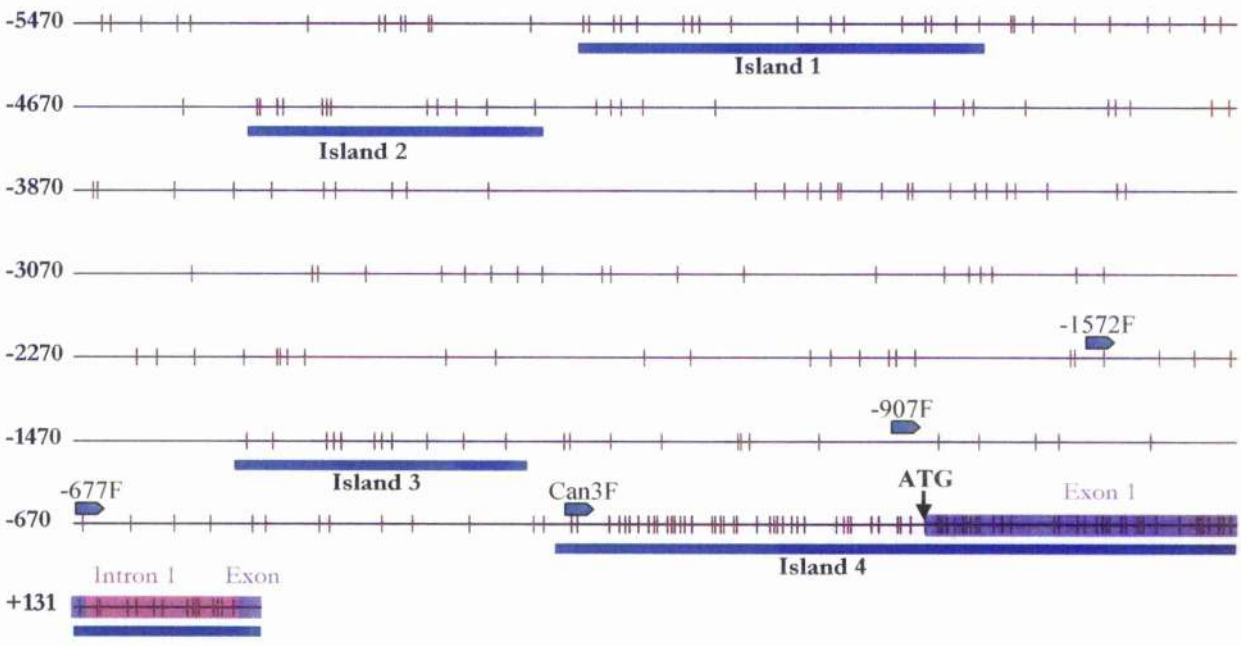


Table 5.4: Potential transcription factors present in the canTERT promoter

Transcription factor	Activator/Repressor of hTERT	Binding Sites (Nucleotide position)
Sp1	Activator (Cong *et al.* 1999; Horikawa *et al.* 1999; Takakura *et al.* 1999; Wick *et al.* 1999)	-1439,-1423,-1157,-1104,-908,-838,-800,-761,-752,-742,-726,-663,-605,-447,-404,-385,-285,-248,-197,-89,-67,-50,-38,-16
c-ets-2	Activator (Horikawa *et al.* 1999)	-795
c-Myc/Mad/Max (E-Box)	Activator (Cong *et al.* 1999, Gunes *et al.* 2000; Horikawa *et al.* 1999; Oh *et al.* 2000; Takakura *et al.* 1999; Wick *et al.* 1999)	-1397
c-Myb	Activator (Cong *et al.* 1999)	-927,-858
GR/PR	?	-1393,-1294,-891,-491
GR	?	-993,-600,-474,-305
AP1	Activator (Cong *et al.* 1999)	-1314,-774,-326
SRY	?	-1306
Myc-CF1	?	-1134,-254
MTF1	?	-1111
NF1	Activator (Cong *et al.* 1999)	-1047,-513
c-jun	?	-1008
MZF-2	Repressor (Fujimoto *et al.* 2000)	-605
ER/Sp1	Activator (Kyo *et al.* 1999, Misiti *et al.* 2000)	-1061,-774,-547,-326
MyoD	Activator (Cong, Wen, & Bacchetti 1999)	-266
WT1-KTS	Repressor (Hasu *et al.* 2001)	-254
MAZ	Activator (Horikawa *et al.* 1999; Takakura *et al.* 1999)	-49
c-ets-1	?	-1480, -1163

Figure 5.5: Sequence of approximately 1.6Kb of the canTERT promoter together with potential transcription factor binding sites (boxed). CpG islands are shown underlined, and the ATG start codon in bold.

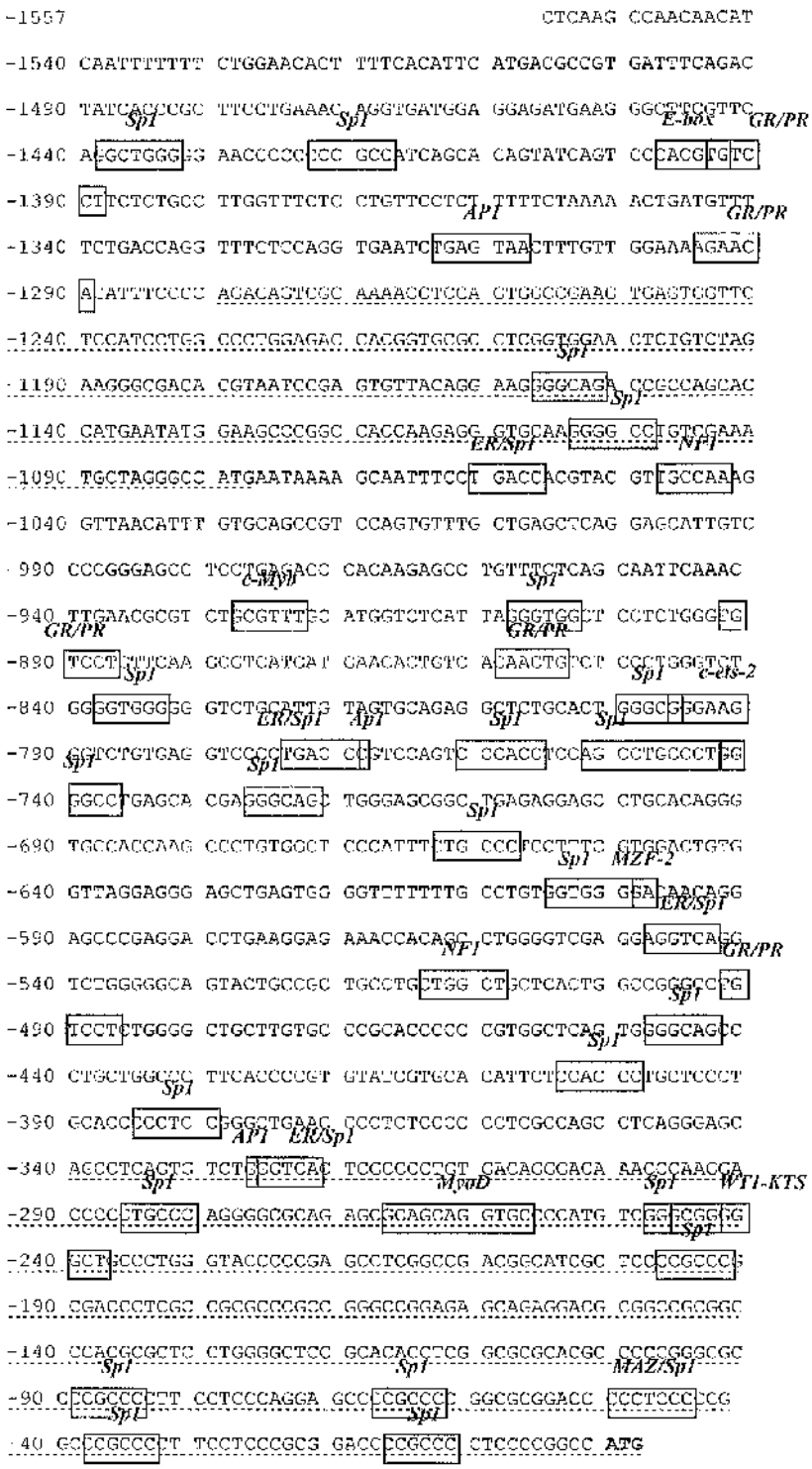
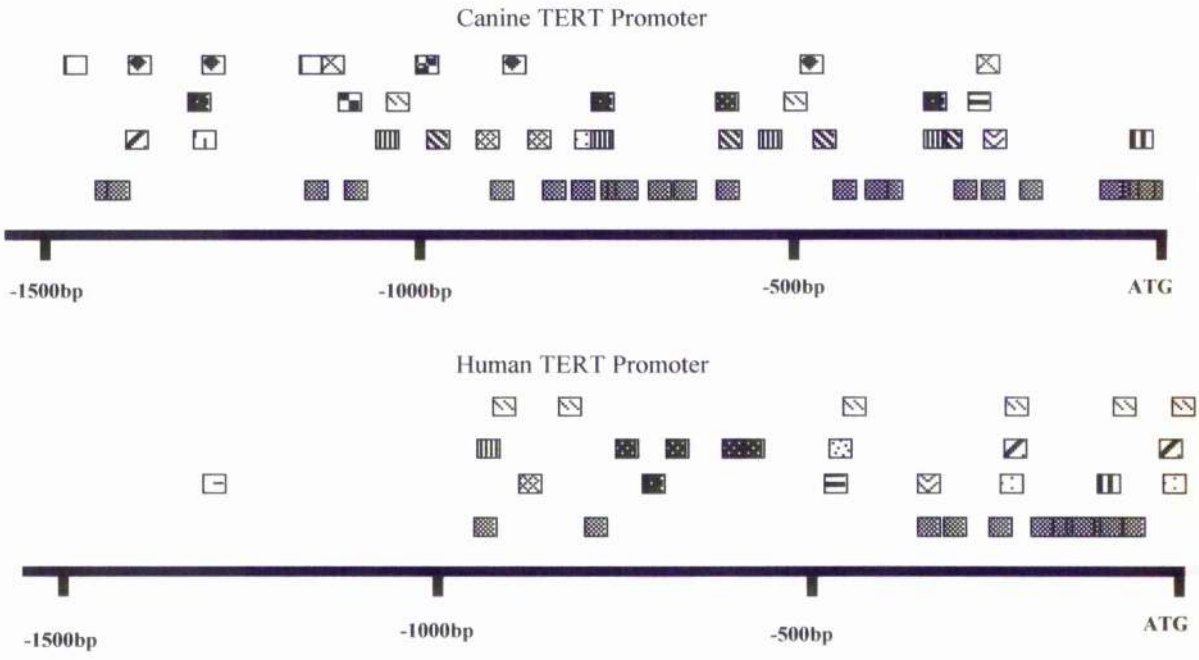


Figure 5.6: Comparison of canTERT promoter with hTERT promoter



Putative Transcription Factor Binding Sites

Binding sites common to both human and canine TERT promoters

- Sp1
- c-Ets-2
- c-Myc
- c-Myb
- GR/PR
- MAZ
- AP1
- MZF-2
- ER/Sp1
- MyoD
- NF1
- WT1

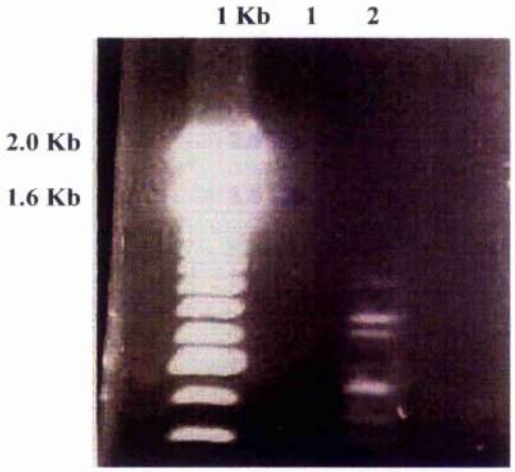
Binding sites present in human but not canine TERT promoter

- PR
- p53

Binding sites present in canine but not human TERT promoter

- GR
- c-Ets-1
- c-jun
- Myc-CF1
- SRY
- MTF-1

Figure 5.7: Representative example of attempts to amplify the transcription start site using the GeneRacer protocol. RT-PCR: Lane 1 D17, Lane 2 CMT7. Note lack of product from D17 cells and multiple bands suggesting non-specific priming in CMT7 cell sample. Approximate size of the two largest bands in the 1 Kb mass ladder are shown.



5.5.2 Identification of the canTERT core promoter

In order to identify those areas of the canTERT promoter that convey strong activity and in order to identify potential repressing and activating transcription factor binding sites, deletion constructs were created from the 5.6 Kb promoter initially sequenced. Initial PCR amplification of a 1929 bp fragment using the cTERTprom-1572F/LG7R primer pair was successfully accomplished using the PCRx Enhancer system with genomic DNA template. This fragment, termed CTERTPROM1, was cloned into pCR2.1®TOPO and sequenced. Using this initial construct as a template, successive PCR reactions were performed with the nested primer pairs: cTERTprom-1572F/LG9R, cTERTprom-907F/LG7R, cTERTprom-907F/LG9R, cTERTprom-677F/LG9R, can3F/LG7R and can3F/LG9R. Following cloning into pCR2.1®TOPO and sequencing, these were termed CTERTPROM2, CTERTPROM3, CTERTPROM4, CTERTPROM5, CTERTPROM6 and CTERTPROM7 respectively. Constructs CTERTPROM1, CTERTPROM3 and CTERTPROM6 contained sequence upstream of the ATG together with exon 1, intron 1 and 15 bp of exon 2. All of these constructs are represented in Figure 5.8.

The ability of these constructs to direct expression of the luciferase reporter gene was assayed by transient transfections in D17 and CMT7 telomerase-positive cells. Luciferase assays showed that CTERTPROM1 was active in both cell lines, with 9.1% and 8.3% of the activity of pGL3control in

CMT7 and D17 cells respectively (Figure 5.9). CTERTPROM2, which included the 1557 bp upstream of the ATG but omitted structural gene sequence, displayed a twofold increase in activity relative to CTERTPROM1, with 21.9%/20.3% of the activity of the pGL3control vector in CMT7/D17 cells. CTERTPROM3 and CTERTPROM4, containing 890 bp upstream of the ATG with and without TERT gene sequence respectively, both displayed similar activity to that of CTERTPROM2, with 17.3%/18.7% and 20.0%/22.4% of pGL3control in CMT7/D17 cell lines. CTERTPROM5 included 665 bp of the promoter but no gene sequence. CTERTPROM6 and CTERTPROM7 included 314 bp of the promoter with and without gene sequence, respectively. CTERTPROM5 and CTERTPROM6 displayed similar activity to CTERTPROM2, with 18.3%/15.5% and 15.5%/13.7% of the activity of pGL3control in CMT7/D17 cells respectively. However, CTERTPROM7, containing the most proximal 314 bp of the promoter, displayed significantly greater activity than all other constructs, with 54.9%/49.4% of the activity of pGL3control, and a near fourfold increase in activity when compared with CTERTPROM6/CTERTPROM1 (Figure 5.9). The sequence of CTERTPROM7, the core promoter together with 65bp of exon 1, is shown in Figure 5.10.

Figure 5.8: Illustration of deletion constructs

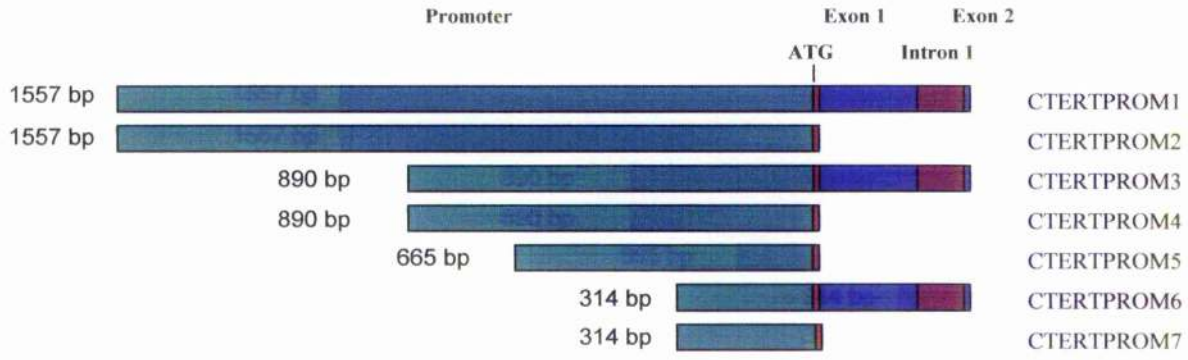


Figure 5.9: Luciferase activity of canTERT promoter deletion constructs expressed as a percentage relative to pGL3control in telomerase-positive CMT7 and D17 cells

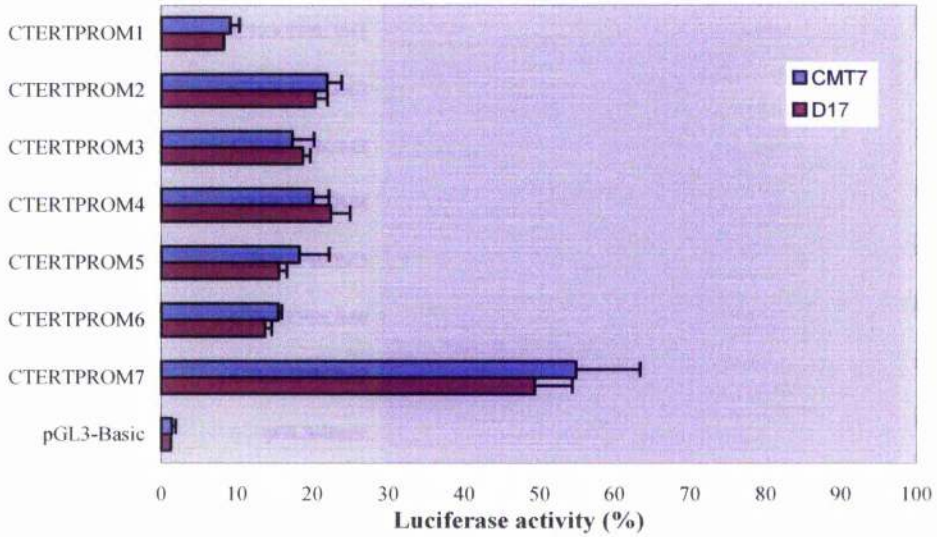
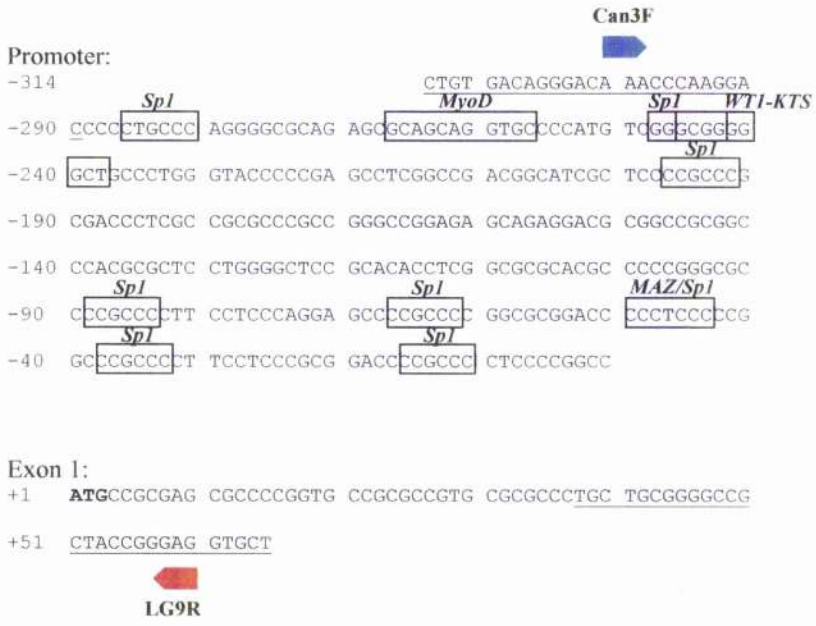


Figure 5.10: Sequence of core promoter (CTERTPROM7) including exon 1, showing binding sites for Can3F and LG9R (underlined) and putative transcription factors (boxed). Start codon shown in bold.



5.5.2.1 Specificity of activity of the canTERT core promoter

The ability of the minimal core promoter to direct expression specifically in telomerase-positive cells was then investigated in several telomerase-positive cell lines: CML10 (canine melanoma), A72 (fibroblastoid tumour), MDCK (canine kidney), GHK (canine kidney), CMT3 (canine mammary tumour) and CMT8 (canine mammary tumour). In addition, specificity of activity was assessed by transfecting telomerase-negative canine primary fibroblasts. The level of activity of CTERTPROM7 varied in each cell line, with maximal activity of 52.5% of the positive control in CML10 cells. However, the A72 and CMT3 cell lines displayed lower activity, with 13.9% and 10.3% of the activity of the pGL3control respectively. GHK and CMT8 cells showed intermediate levels of canTERT promoter, with 27.7% and 22.7% of the pGL3control respectively (Figure 5.11). Transfection of canine primary skin fibroblasts resulted in minimal activity with all constructs, as shown in Figure 5.12.

Figure 5.11: Activity of CTERTPROM7 promoter fragment in different cell lines expressed as percentage of activity of positive control pGL3Control vector. Activity of negative control (pGL3Basic with no insert) shown in maroon.

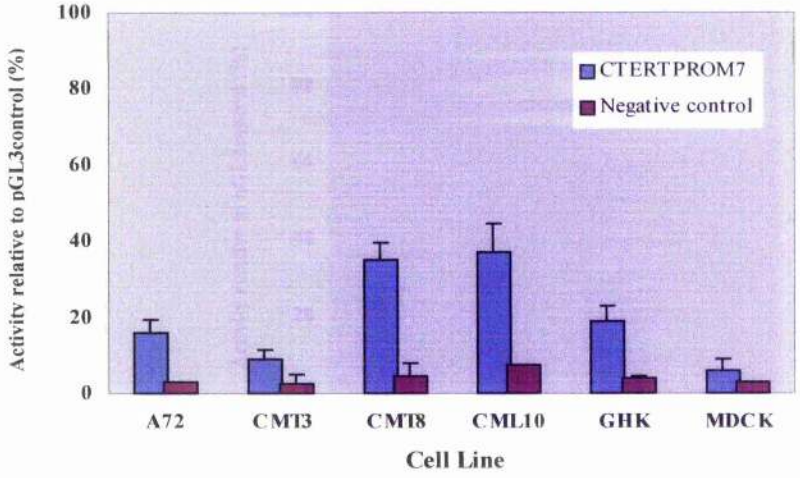
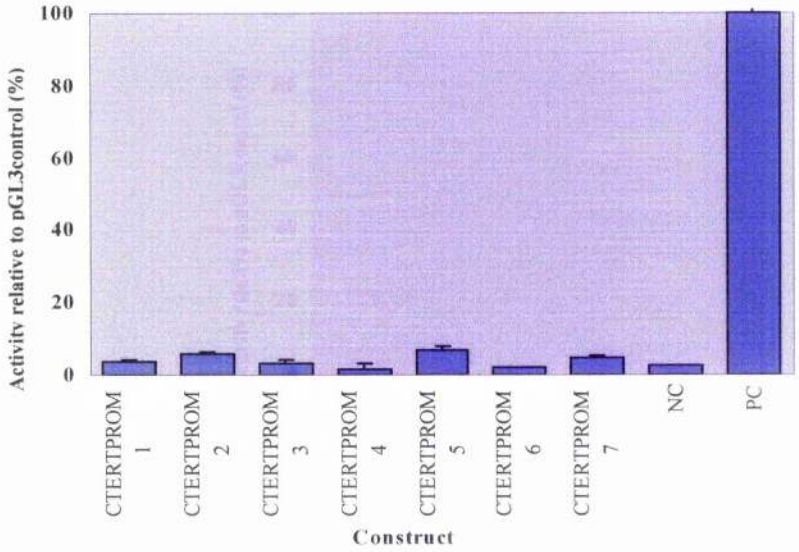


Figure 5.12: Activity of all deletion constructs in telomerase-negative K9SF cells expressed as a percentage of the activity of positive control pGL3Control (PC). Activity of negative control (pGL3Basic with no insert) also shown (NC).



5.5.3 Sequence analysis of the canTR promoter

Initial PCR amplification of the canine TR gene was performed. Using the conditions listed in 2.2.6: Amplification of DNA by the polymerase chain reaction, a 286 bp fragment was amplified from gDNA, cloned into pCR2.1®TOPO and sequenced to confirm the presence of canine TR sequence. Sequence of this product is shown in Figure 5.13.

Following initial PCR, primers were designed from trace file 271822249 to perform genome walking. The positions of these are shown in Figure 5.13. The first genome walk, using primers GSP-1R and nested primer GSP-2R, revealed the presence of a 700 bp band in the library created with *EcoR V* and a 500 bp band in the *Pvu II* library of the DNA libraries. Both fragments were excised, gel-purified, cloned into pCR2.1®TOPO and sequenced. The sequence obtained was then used to design primer GSP-3R primer for a second genome walk. This genome walk was performed with primer GSP-2R and nested primer GSP-3R. The nested PCR from the second genome walk resulted in a 600 bp product from the *EcoR V* library and a 400 bp product from the

Pvu II library (Figure 5.14). These bands were excised, gel purified, cloned into pCR2.1®TOP and sequenced. The sequences obtained from all colonies containing correct sequence were subsequently aligned and used to generate a consensus sequence of 700 bp of the promoter. The consensus sequence is shown in Figure 5.15. Following sequencing and characterisation of this promoter region, attempts were made to amplify fragments for the generation of deletion constructs and subsequent luciferase reporter assays, as performed with the canine TERT promoter. However, initial attempts at PCR were unsuccessful despite using the same modifications as previously described, and due to time constraints, further troubleshooting was not performed. As a result full characterisation of the core TR promoter could not be completed.

Figure 5.13: canine TR and promoter sequence obtained from trace file 271822249. Forward (blue) and reverse (red) primers used for initial PCR and for genome walking are shown, together with the TATA box (boxed).

Promoter:

NGGCGGATATTTCGCCTGTTGGTGGAATTCAGAAAAGAAAGAGAAAAGGAAAAGAAAAAAGAAAAAGACC

CTTTAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAGAAAAAAGAAAAGACCTAACATTTTACTT

GSP-3R GSP-2R

AAGGAACGTGGAGGAGGAGGCGGCGCAGGGTCCTCACGGCCGGAAGTAGAACCGTAGGGATGGAGAGGGA

GSP-1R

GGGGGGTGGAGAAAGGGGCGGAAGCCGGCTCTCGCGAGAGGCGAGATGACGGCTTCGGCCAATCAGCGCC

GGCGGCGGGCGCGCCCTTTATAAGCGGCGGCGGCGGGCGGCGGCGGCCG

TATA-box

TR gene:

AGGTCGCGGAGGGTGGGCCCG

DogTR1F

GGAGAGGCGGCGGCCGGCCTCCGTCTAACCCTAACTGAGCAGGGCGTGGGCGCCGCGCTTTTGTTCCCCG

CGCGCTGTTTTTCTCGCTGACTTTCAGCGGGCGGAAAAGCCTCGGCCTACCGCCGTCCACCGTCCGGGTC

TGCAGCCAACAACAAAAAATGTCAGCCGCTGGCTCGCTCGCCCCTCCCGGGAGCCTGCGGCCGCTCGCCC

GCTCGGCCCCCCGCGTCCCGCCCGGAGGCCGCGGCCGGCCCGGGGCTTCTCCGGAGGCGCCCACTGCCGT

DogTR1R

CGCGAAGAGTTGGGCTCTGTCAGCCGCGGGCCGTCTGGNGGCCGCAGGGCTGGGCTCGNGCCGCAGGGAG

AGCTACGGAGCGNGTCCCCTGCGCGCGTGCGTCTCCCTGAGCTGTGGGACTCGCACCCGGNACCGGCTCG

CACCGCNCCNCGCGGGNGCGCTGCTGGGCCCTTGGGNTCTGCGGCTGTCCTCCCCTGGCGNNGCGCCTGC

GTCTCACCGAAAGNAAAAGGGGGGCCCCCG

Figure 5.14: Fragments amplified from *EcoR* V and *Pvu* II libraries during genome walking

Genomewalker – agarose gel analysis of primary and nested PCR. Lane 1: *EcoR V* library; Lane 2: *Dra I* library; Lane 3: *Pvu II* library; Lane 4: *Ssp I* library

A: Second genome walk. Primary PCR using primer GSP2-R. Notice multiple bands in all lanes.

B: Second genome walk. Nested PCR using primer GSP3-R. Notice 600 bp band in lane 1 and 400 bp band in lane 3.

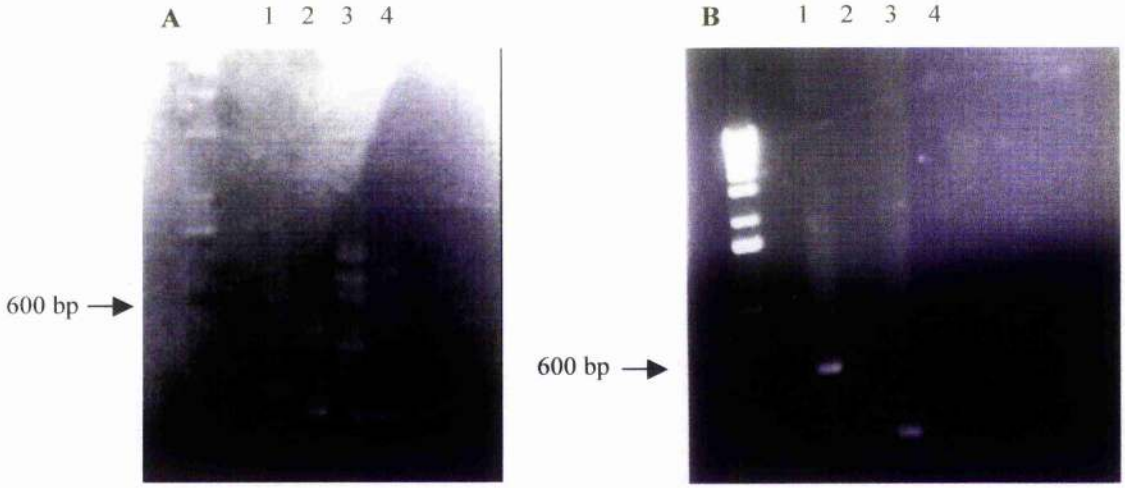


Figure 5.15: Consensus sequence of canine TR promoter showing TATA box (boxed), TR gene (underlined), and position of primers used for genome walking (purple)

```
-801  CTTATTCATG AGAGACACAG AGACCCAGGC AGAGGGAGAA GCAGGCCCCA
-751  CGCAGGGAGC CCGACGCGGA ACTCGATCCC ACGACCCCGG GTCACGTCCT
-701  GGGCCGAAGG CAGGCGCCGA ACCGCTGGGC CCCCCCGGCT GCCGTCGAGT
-651  TTCCTTAAAG GTCGCTACCA ACTGCGATGG CGTTTGTGGA AAACAGAGCC
-601  GCGCAGCTGT GCAAGTTAGT TTGTACTTTC GGAGTAACCT TAGCTCCTTC
-551  CCCTCAAAAT AAAATAAAAT AAAATAAAAT AAAATAAATA AAATAAAATA
-501  ATAAAATAAA TAAAATAAAA ATAAAATAAA TAAAAAGAAT AAAAGAAAAA
-451  GGAAAAGAAA GACAAAAAGA AAAAGAAAGA AAAGGAAAAG GAAAAGAAAA
-401  AAGAAGAAGA AAGAAAAAAG GAAAAGGAAA AGAAAAAAAA GAATAAAGGA
-351  AAAGGAAAAA AAGAAAAAGA AAAAAAAGAA AAAAGGAAAA GAAAAAGAAA
-301  GAGAAAAGGA AAAGAAAAAA GAAAAAGAAA AAGAAAAAAG AAAAAAGAAA
-251  AAAGAAAAAA GAAAAAAGAA AAAAGAAAAA AGAAAAAACC TAACATTTTA
-201  CTTAAGGAAC GTGGAGGAGG AGGCGGCGCA GGGTCCTCAC GGCCGGAAGT
                                          GSP-3R
-151  AGAACCGTAG GGATGGAGAG GGAGGGGGGT GGAGAAAGGG GCGGAAGCCG
           GSP-2R      GSP-1R
-101  GCTCTCGCGA GAGGCGAGAT GACGGCTTCG GCCAATCAGC GCCGGCGGCG
-51   GGCGCGCCCT TTATAAGCGG CGGCGGCGGG CGGCGGCGGC CGAGGTCGCG
-1    G
+1   AGGGTGGGC CCGGGAGAGG CGGCGGCCGG CCTCCGTCTA ACCCTAACTG
+51  AGCAGGGCGT GGGCGCCGCG CTTTTGTTCC CCGCGCGCTG TTTTTCTCGC
+101 TGACTTTCAG CGG
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5.6 Discussion

The principal aims of this chapter were to sequence and characterise the promoter of the canTERT gene as a potential tool for developing tumour-specific targeting of gene expression. We screened a phage library and identified a clone containing 8Kb of the promoter region upstream of the canTERT gene. From the sequence obtained from this clone we designed primers which were then used to amplify and sequence approximately 5.6 Kb of the promoter from genomic DNA in two large, overlapping subfragments. From within this region, 1.5 Kb of the promoter together with approximately 340 bp of the gene was successfully subcloned into pCR2.1®TOPO and used to generate deletion constructs that were subsequently subcloned into the pGL3basic reporter vector and transfected into telomerase-positive cell lines. Luciferase assays in telomerase positive and negative cells were then used to identify the specificity and strength of the core promoter, which was found to be contained within a region extending 314 bp upstream of the ATG start codon. In addition to sequencing and characterisation of the canine TERT promoter, using primers designed from canine DNA trace files retrieved from Genbank, genome walking was performed which allowed sequencing of a 700 bp region of the canine TR gene promoter as an initial step in its characterisation. However, attempts to amplify regions of the TR promoter to generate luciferase reporter constructs were unsuccessful, and as a result full characterisation was not performed.

5.6.1 CanTERT promoter characterisation

Sequence analysis showed that the canTERT promoter shares some regulatory motifs with the hTERT promoter: the 5'regulatory region of canTERT is GC-rich, lacks TATA and CAAT boxes and contains binding sites for multiple transcription factors. In addition, four CpG islands were identified within the 5.6 Kb of the promoter. Intriguingly, the largest of these coincides almost exactly with the core promoter, with its origin at 349 bp upstream of the ATG and extending into exon 2. This raises the question of whether methylation of the canine TERT promoter is likely to play a significant role in regulation of telomerase activity. In the human literature, the importance of promoter methylation in regulating hTERT function is as yet unclear, and the clinical relevance of promoter methylation appears to be slight, and probably tumour-type dependent (Widschwendter *et al.* 2004). Given the similarity in GC-content between the two species, this may also be the case in dogs.

Analysis of deletion constructs revealed that core activity of the promoter is contained within 314 bp upstream of the ATG. This result is consistent with the hTERT promoter where several studies have shown that core promoter lies within approximately 300 bp of the transcriptional start site (Horikawa *et al.* 2002; Takakura *et al.* 1999; Wick *et al.* 1999).

5.6.1.1 Difficulties encountered with amplifying the canTERT promoter

Considerable difficulty was encountered when we attempted to amplify the canTERT promoter for sequencing, due to the GC-rich nature of the proximal part of the gene. Ultimately, sequencing and generation of deletion constructs was performed over approximately 12 months, considerably longer than expected. An initial fragment upstream of the large CpG island that extends into the canTERT gene was amplified, cloned and sequenced with relative ease. However, the promoter fragments extending into this CpG island were considerably more difficult to isolate. Regions of genomic DNA containing a high percentage of guanidine and cytosine residues are commonly associated with a high degree of secondary structure. This is due to the fact that G-C pairs contain 3 hydrogen bonds, rather than 2 as is the case with A-T pairs. As a result, following the denaturation step of PCR amplification, which is designed to reduce secondary structure and separate the DNA strands, G-C pairing frequently recurs and secondary structure reforms, preventing access of primers to their binding sites and interfering with elongation by DNA polymerase.

Various methods exist to deal with these problem areas of the genome, many of which are designed to alter the melting behaviour of the DNA strands. DMSO, glycerol and Q-solution are all examples of additives that are used in this way. Another additive designed to alter the melting behaviour of DNA strands is betaine, present within some of the buffers provided with the kit supplied by Cambio. It may be useful to investigate this in future attempts to amplify the canTERT gene in its entirety since we did not employ it as an additive on its own. In addition, many PCR protocols involve modifying the percentage of the Mg^{2+} in the reaction mix for the same reason. Initial attempts at PCR involved lowering the annealing temperature to 55° C during the annealing step. In theory, this should lower the specificity of priming and result in primer annealing to multiple sites in addition to the desired site. However, the lower annealing temperature is likely to have resulted in reforming of secondary structure in the area of interest. Techniques including the HotStarTaq and KOD Hot Start Taq protocols are designed to improve the amplification of difficult templates by increasing the specificity of priming and elongation using hot start protocols. However, without modifying the melting behaviour of the GC-rich areas, it seems likely that reducing the temperature of the reaction during the annealing step allowed secondary structure to reform and therefore inhibited primer binding and polymerase elongation. Ultimately, the proprietary PCRx Enhancer Solution, provided by Invitrogen, was the only additive capable of dealing with our problem template. Using the Enhancer Solution, initial amplification of the largest promoter fragment (CTERTPROM1) and cloning into pCR2.1®TOPO was achieved. Once this fragment was present in a plasmid, it is likely that the amount of secondary structure present in our template was significantly reduced. We were then able to amplify smaller fragments from this

plasmid with greater success. However, future attempts to amplify or sequence other GC-rich regions of the canTERT gene are likely to be met with similar difficulties.

5.6.1.2 Potential transcription factor binding sites within the core canTERT promoter

Transcriptional analysis of the canTERT promoter demonstrated that the promoter was active only in telomerase positive cells, although the levels of transcription varied between cell lines. In contrast, no significant promoter activity could be detected in primary fibroblast cultures.

5.6.1.2.1 Comparing the canine and human TERT promoters

Within the canine core proximal promoter the most obvious feature was the presence of multiple Sp1 binding sites (Figure 5.6). Several Sp1 binding sites also exist within the core hTERT promoter and Sp1 mutations have been shown to reduce hTERT promoter activity, indicating a role in hTERT transcriptional activation (Kyo *et al.* 2000). Whilst the exact mechanism by which Sp1 enhances transcription is not known, Sp1 is a general transcription factor and is ubiquitously expressed in a wide range of cells. The question remains, however, as to how significant a contribution Sp1 makes to specific TERT activation in cancer cells. For instance, the finding that adding Sp1 and c-Myc binding sites to the hTERT promoter increases activity specifically in tumour cells suggests that regulation of the promoter in tumour cells may indeed involve the control of Sp1 (Kim *et al.* 2003b). It is likely, however, that this control is complex and involves other transcription factor binding sites in conjunction with Sp1.

Inclusion of proximal promoter sequence extending up to –1557 bp reduced transcriptional activity, indicating the presence of silencer/repressor elements within this region (-1557 bp to -314bp). However, few potential sites for known hTERT repressor binding elements were identified within the canTERT promoter. A single site for the Myeloid Zinc Finger 2 protein (MZF-2) was found at position - 605 in the canine promoter. MZF-2 binding sites have been reported in the hTERT promoter, four of which exist within a 200 bp region just upstream of the core promoter (Horikawa *et al.* 1999; Takakura *et al.* 1999) and constructs with their 5' end located within this region (-600 to -800bp) show lower expression, suggesting MZF-2-dependent repression. Interestingly, the canTERT promoter seems to follow a similar pattern, with constructs comprising 890bp and 650bp of promoter sequence showing reduced levels of activity when compared to the construct consisting of 314bp of upstream sequence. This suggests that MZF-2 may play an important negative role in the regulation of canine TERT. Furthermore, the inclusion of exon 1, intron 1 and part of exon 2 in all deletion constructs decreased promoter activity by approximately 50-75%, depending on the construct. Transcriptional repression by the structural TERT gene has been demonstrated for the

hTERT promoter (Renaud *et al.* 2003), where transcriptional activity is reduced 20- to 50-fold depending on the portion of exon 2 included and constructs comprising up to 753 bp of exon 2 result in 100-fold reduced activity . In our study, only a small portion of exon 2 was included, and it would be of interest to determine whether additional exon 2 sequences would similarly lead to further reductions in the transcriptional activity of the promoter. Other studies have also reported involvement of the human TERT gene in transcriptional regulation (Cong *et al.* 1999), and support the inhibitory nature of this region. The only known transcription binding factor identified in this region that is known to contribute to the inhibition of hTERT promoter activity is MZF-2, for which a binding site is located in exon 1 of canTERT at bp 121. It is possible that this MZF-2 binding site is important for downregulation of canTERT promoter activity, however, currently there is no evidence for a role of MZF-2 in cancers and hence the significance of MZF-2 mediated hTERT repression is unknown.

Several additional sites for known hTERT repressor binding proteins were also identified. These include a binding site for Wilms Tumor 1 tumour suppressor gene product (WT1), located within the core region of the canine promoter. In the human promoter, one WT1 site is present at position –358 bp (Poole *et al.* 2001) and mutation of the binding site has been shown to increases activity in 293T cells but not HeLa cells (Oh *et al.* 1999). The canine TERT promoter also contains a putative E box binding site at –1397 bp. Mad1/Max heterodimers have been shown to function as a negative regulator of hTERT transcription by binding to E box sequences (Xu *et al.* 2001). Conversely, c-Myc/Max heterdimers can also bind to E box sequences and activate hTERT activity (Kyo *et al.* 2000). The hTERT minimal promoter contains two E-box sequences, however only one E box sequence was identified within the canTERT promoter located upstream of the core promoter at -1397 bp.

Four putative ER/Sp1 binding were also identified within the canTERT promoter. Several oestrogen-response elements (EREs) have been found within the hTERT promoter region, some of which bind oestrogen together with its receptor (ER) and some of which bind ER and Sp1 together (Kyo *et al.* 1999; Misiti *et al.* 2000). Whilst some reports suggest the more distal ERE, located 2754 bp upstream of the start codon, as being important for TERT activation, more recent reports have suggested direct activation of hTERT expression through binding of ER to a more proximal Sp1/ER site located at -950 bp (Misiti *et al.* 2000). The significance of these binding sites in the canine promoter are currently unknown. However, the presence of a greater number of ER/Sp1 binding sites in the canine promoter, along with a greater number of PR and GR binding sites suggests that hormonal regulation may play a significant role in the canine TERT promoter. Further studies examining the activity of the canine promoter under the influence of these hormones are required to verify this, however.

Other potential transcription factor binding sites shared between the canine and human TERT promoters include c-ets-2, MAZ, NF1, MyoD, AP1, c-Myb and PR. Although the importance of many of these transcription binding sites is currently unknown their presence in both the canine and the human promoter would suggest that these may be important activators/repressors contributing to either telomerase repression in normal cells or activation in cancer cells.

Several putative binding sites within the human TERT promoter were not identified within the canine promoter, namely: AP2, AP4, CCAC, NF-E2, NFκB, NMYC or p53. In our transcriptional binding site analysis, we identified several sites that appear to be unique to the canine sequence including several glucocorticoid receptor binding sites (GR), several GR/PR sites, one SRY site, two Myc-CF1 sites, one MTF-1 site and one c-jun site (Table 5.4). With the exception of a myc-CF1 binding site (-254 bp), all other sites were located outside the core promoter region, however the role of myc-CF1 in TERT transcriptional control remains to be established. One transcription factor that may be of particular interest is c-jun. c-jun is thought to play an important role in cell proliferation working in conjunction with ras protein and recent studies have shown a dramatic increase in c-jun expression in tumour cells from patients with myeloid leukemias, Hodgkins and anaplastic large cell lymphomas (Lohr *et al.* 1997; Weiss & Bohmann 2004).

5.6.1.2.2 Other potential regulatory factors

A wide variety of potential regulatory mechanisms have been examined in the regulation of hTERT, illustrating the complexity underlying the *in vivo* activation of telomerase. Interestingly, some pathways and mechanisms seem to be implicated in oncogenesis via several routes. For instance, the human T-cell lymphotrophic virus type-1 (HTLV-1) upregulates the hTERT promoter via the nuclear factor κB (NF-κB) pathway to allow the development of an immortal phenotype in T cells during the progression to adult T cell leukaemia/lymphoma (Sinha-Datta *et al.* 2004). Other viruses also appear to exploit the hTERT promoter. Part of the Hepatitis B virus, for instance, integrates within the hTERT promoter and activates hTERT gene transcription, a factor which may be important in the development of hepatocellular carcinoma (Horikawa & Barrett 2001). Human papillomavirus (HPV) downregulates the hTERT promoter via the E2 protein to suppress cell division in host cells. This occurs in pre-malignant lesions, with E2 function subsequently being lost, allowing the E6 and E7 promoters to act, leading to the development of a malignant phenotype in host cells (Lee *et al.* 2002b). This mechanism thereby allows the virus to induce a 'dormant' state in host cells, prior to the development of cancer (Lee *et al.* 2002a).

Another potentially important mechanism for regulating the TERT promoter is through transcription factors responsive to hypoxic conditions in the external environment. Although they were not detected by the transcription factor binding search, the canine promoter appears to contain

two putative hypoxia response elements (HREs). The first of these is located within the E-box (5'-ACGTG-3'), and the second is located at bp –460 (5'-CCGTGG-3'). HREs, containing the core motif 5'-(A/C)CGT(G/C) -3' (Greco *et al.* 2002), are located within promoters or enhancers of genes involved in glycolysis, glucose transport, erythropoiesis and angiogenesis (Nishi *et al.* 2004). These elements bind the HIF-1 heterodimeric transcription factor, composed of the basic helix-loop-helix PAS protein HIF-1α and the aryl-hydrocarbon receptor nuclear translocator (ARNT – also known as HIF-1β) and thereby mediate the transcriptional response to oxygen deprivation. HIF-1β is constitutively expressed, but HIF-1α is tightly coupled to oxygen tension in the extracellular environment and consequently is the key determinant in HIF-1 activity in hypoxia. Since hypoxic stress is known to induce genetic instability by accelerating DNA breakage and gene amplification in tumour cells (Russo *et al* 95), mechanisms by which tumour cells can protect their chromosome structure offer a selective proliferative advantage in such situations. Several groups have shown that telomerase becomes activated under hypoxic conditions (Seimiya *et al.* 1999), suggesting that telomerase activation may offer just such a protective mechanism for tumour cells in humans. The mechanism by which this occurs in has now been shown to be through the upregulation of HIF-1α and its binding to the HREs present in the hTERT promoter (Yatabe *et al.* 2004). Thus it may be that hypoxia plays an important role in regulating the activity of the canine TERT gene, and this will be of considerable importance in the development of cancer-targeted therapies.

5.6.1.3 Specificity of canTERT promoter activity

In order to assess the specificity of the core promoter, we performed transient transfections in a range of telomerase-positive canine cancer cell lines, and telomerase-negative canine fibroblasts. No significant promoter activity was observed in the telomerase-negative fibroblasts, confirming that the canTERT core promoter is only active in telomerase positive cell lines. A wide range of activity was observed in the telomerase positive cell lines, with some cell lines exhibiting low transcriptional activity. Interestingly low hTERT promoter transcriptional activity has been reported in several human telomerase positive cell lines including MCF7, U737, U87, A543, 5637 (Bilsland *et al.* 2003; Koga *et al.* 2000; Plumb *et al.* 2001). Given the spectrum of canTERT promoter activity, it will be of prime importance to determine which tumour cell lines contain sufficient promoter activity to drive potential targeted therapies. It may also be necessary to consider enhancing the promoter's activity in some cell lines with weak transcriptional activity, an approach which has been pursued with the human TERT promoter.

5.6.2 Sequencing the canTR promoter

In addition to sequencing and characterising the canTERT promoter, we have also sequenced the canTR promoter. The most significant feature of the canTR promoter is that it contained fourteen repeats of the pentanucleotide sequence TAAAA (Figure 5.14), a finding not reported either in humans or in mice (Zhao *et al.* 1998). The $(TAAAA)_n$ repeat element is a common feature of repetitive elements such as *alu* sequences (Millar *et al.* 2000) and LINE elements as well as being found in the promoter of some genes, such as the sex hormone-binding globulin (SHBG) gene (Hogeveen *et al.* 2001). Interestingly, the variation in the number of repeats is highly variable between individuals, and the number of repeats regulates the expression of the gene, with increasing numbers of repeats in the SHBG promoter increasing the expression of human SHBG (Hogeveen *et al.* 2001). In addition, when six TAAAA repeats are present, SHBG expression is silenced altogether. The significance of these repeats in the canTR promoter is currently unknown, but it seems likely that the number of repeats is likely to vary between individuals. Indeed, initial attempts to subclone fragments of the canTR promoter from genomic DNA for luciferase assays suggested that allelic variation exists in the number of repeats within a single individual (data not shown). This variation may have an important influence on the levels of expression of telomerase between individuals. Indeed, it may be the $(TAAAA)_n$ repeat number will be shown to have some correlation with breed, since some breeds are notable in their predisposition to cancer. One of the original aims of this part of the project was to generate deletion constructs with varying numbers of $(TAAAA)_n$ repeats for luciferase reporter assays. However, difficulties were encountered with PCR amplification of the promoter as a single fragment, most likely related to the unusual sequence in the region of these repeats. Despite modification of the PCR conditions as described with the TERT promoter, repeated attempts at amplification were unsuccessful, and due to time constraints full troubleshooting was not performed. Therefore, we were unable to completely characterise the canine TR promoter. In the future, further analysis of other individuals as well as luciferase assays with varying numbers of $(TAAAA)_n$ repeats will be necessary to fully characterise the TR promoter. In addition, it remains to be seen whether the canine TR promoter shows similar specificity to the human TR promoter, and therefore whether it will be useful as a cancer-specific promoter.

5.6.3 Future Directions

In defining and characterising the canine TERT gene promoter we are now in a position to evaluate canTERT promoter mediated gene expression in canine cells expressing telomerase. This knowledge will help build our understanding of the events that are involved with cell immortalisation during canine oncogenesis, an important area of future research. Further work examining the importance of the different binding sites so far identified may also identify key events involved with telomerase regulation in cells in the developing animal, as well as its

regulation in stem cell populations. In addition, as has been shown in the human literature, TERT promoter-driven gene expression can potentially serve a number of purposes. Whilst several strategies have employed the hTERT promoter to drive apoptosis or the expression of suicide genes, others have used the promoter for purposes such as the improved identification of tumour cells (Umeoka *et al.* 2004; Yin *et al.* 2004). One of the more attractive possibilities for canTERT promoter-driven therapies is the specific expression of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) gene within tumour cells. TRAIL expression driven by the hTERT promoter results in a large reduction in orthotopic pancreatic tumour volume in a nude mouse model treated with an adenovirus containing the TRAIL gene driven by the hTERT promoter (Jacob *et al.* 2004). Additionally, TRAIL expression elicits a significant bystander effect through the interaction of TRAIL molecules expressed on the cell membrane with receptors on adjacent cells (Kagawa *et al.* 2001). An additional aim of this project initially was to investigate the ability of TRAIL, expressed under the control of the core canine TERT promoter, to cause specific apoptosis in canine telomerase-positive cells. Although this construct was apparently successfully generated with the help of DJ Argyle, sequencing revealed that a 170 bp region of the core promoter had been lost during cloning. Due to time constraints the reason for this was not fully elucidated and troubleshooting was not performed, and consequently we were unable to examine the ability of the core promoter to specifically direct expression of a pro-apoptotic gene within telomerase-positive cells. However, this work is now being continued in our laboratory.

Another approach which could be investigated in the treatment of canine cancer is the use of the TERT promoter to drive replication of an oncolytic virus. This has been successfully performed by a number of groups and shows considerable promise (Kawashima *et al.* 2004; Zou *et al.* 2004). One of the problems encountered in the human field is the development of resistance by some tumours to the adenoviral vectors used. This is due, in most cases, to the downregulation of the Coxsackie-adenovirus receptor (CAR), the receptor to which the adenoviral vectors bind and gain entry to target cells (Bergelson *et al.* 1997; Wickham *et al.* 1993). Modifications to the fibre protein which interacts with CAR so that binding to cells can occur via CAR or ubiquitously expressed integrins has successfully overcome this limitation (Taki *et al.* 2005). In animals, the development of adeno-associated vectors offer an alternative to the adenoviral vectors used in humans. However, it remains to be seen whether similar problems with resistance to adeno-associated viral infection will be encountered in animals. In principle, however, the theory underlying the use of these vectors as oncolytic therapy remains sound and offers a promising hope for future cancer treatments.

It may be that the canTERT promoter alone may direct specific expression of a given gene or replication of an oncolytic virus, but that the strength of expression is limited. If this proves to be the case, there are a number of strategies that may be employed to enhance its strength. Inclusion of other promoter/enhancer elements in the hTERT promoter has been shown to improve the

strength of the promoter with minimal activity in telomerase-negative cells. Initially, some groups increased the strength of the human promoter by adding Sp1 binding sites and c-Myc binding sites (Kim *et al.* 2003b). However, given the density of Sp1 binding sites in the canine TERT promoter it is perhaps unlikely the addition of further binding sites will increase strength of expression significantly. Some groups have added the SV40 promoter in constructs downstream of the hTERT promoter in addition to extra Sp1/c-Myc binding sites and used these constructs to drive strong expression of a gene of interest (Song 2004; Song 2005). This approach may bear more fruit in the canine promoter, although specificity of expression will need to be confirmed if this modification is performed.

CHAPTER VI

GENERAL DISCUSSION

6.1 Work described in this thesis

The aims of this thesis were to examine potential ways of using telomerase in the diagnosis and treatment of cancer in dogs. Following on from other work examining telomere and telomerase biology in normal canine tissue, in canine cancer cell lines and in canine cancer tissue, there is now good evidence to support the theory that telomerase activation is an important step in oncogenesis in canine cells as in human cells. There is now an enormous amount of research in man that has investigated means for using it in cancer diagnosis and therapy, and it is to be hoped that some of this knowledge can be applied to canine cancer as well as human cancer. However, this work needs to be validated in the canine species before it can be taken into clinical trials, and it is the first step of this process that this thesis tried to achieve.

Before work can begin developing telomerase-based anti-cancer therapy, an absolute prerequisite is an accurate picture of the telomerase activity of the tumour being treated. The important paper by Shay and Bachetti (Shay & Bacchetti 1997) summarised the current state of knowledge of telomerase activity across a wide spectrum of tumours. However, more detailed assessments of telomerase activity in brain tumours revealed that within this collective group, there was a wide variation in the reported percentage of tumours that had reactivated telomerase. To some extent this is not surprising, given that the number of tumour types that arise from cells in the brain is probably greater than in any other organ in the body. It would be not unexpected, therefore, to find that the different tumour subtypes acquire different hallmarks of malignancy in different ways and through different biochemical pathways. However, even bearing this in mind, the variation in percentage of glioblastomas positive for telomerase activity appeared to be very high, with anywhere from 28% to 100% of tumours in this subtype positive for telomerase activity. Leading on from this, the study reported by Falchetti et al, in comparing different ways of assessing telomerase activity in glioblastomas, suggested that amount of this variation could be due to the way in which telomerase activity was assessed, with the lowest figures obtained when the TRAP assay was performed (Falchetti *et al.* 2000). This study, and others examining telomerase activity *in situ* found that in fact the vast majority, if not all, glioblastomas, possess telomerase activity, but that variation across different regions of the tumour could lead to false negative results.

With these findings strongly supporting the value of *in situ* methods for detecting telomerase activity, we attempted to validate an immunohistochemical method for detecting TERT in canine brain tumours. Canine brain tumours represent a relatively common and debilitating disease in dogs, as in man. Surgical removal of these tumours is often challenging, if not impossible without severely compromising the patient, and adjunctive therapies in the dog are not widely accessible. In addition, the similarity between the range of tumours seen in dogs and in humans makes them an attractive model for studying human disease. Ultimately, our goal is to develop new telomerase-based therapeutic strategies for their treatment, but since no work so far has examined the

telomerase status of brain tumours in dogs, we set out to achieve this. This work raised some expected, and some unexpected findings. Firstly, in validating the NCL-hTERT antibody in the normal brain, we found that a significant proportion of normal cells stained positively for TERT in all parts of the brain examined. We also found that one part of the brain, the region of the subventricular zone near the caudate nucleus, appeared to be positive for telomerase activity. The significance of this finding needs to be confirmed since at present only one normal canine brain has been examined, and more individuals need to be surveyed. Secondly, in examining TERT staining in canine brain tumours, we found both that TERT activity appears to correlate with both malignancy and proliferation in canine brain tumours, but also that not all TERT-positive cells in canine brain tumours are tumour cells, with some endothelial cells also staining positively for TERT. This would appear to validate our decision to assess telomerase activity using an *in situ* technique. However, our study is still incomplete in that we have not compared TERT staining with any other method of assessing telomerase activity, and ideally this should be performed in the future. This will take considerably longer, since most other techniques require fresh or frozen tissue, and the rate of sample collection in dogs is much slower than in man, due to the lower numbers of dogs that present to clinics and the lower percentage of these cases that undergo definitive treatment involving tumour resection. In the future it is to be hoped that such work will be carried out using tumour banks derived from collaborative tissue collection at multiple centres, but at present these tumour banks are still in their infancy.

Once we had established that telomerase activity is associated with malignancy in canine brain tumours as well as in human brain tumours, we assessed two ways of inhibiting it *in vitro*: by using the dominant negative mutant of human TERT (DNhTERT), and by using the G-quadruplex ligand telomestatin. The initial intent of this study was to use canine brain tumour cell lines to perform this work. However, no telomerase-positive canine brain tumour cell lines had been isolated at GUVS prior to this study beginning, and no cases were obtained during the course of the study that were suitable for establishing cell lines. During the course of the study, a meningioma cell line was generously donated by T. Scase at the Animal Health Trust, Newmarket. However, this was found to be contaminated and had to be discarded. For this reason, *in vitro* work was consequently performed in other, previously established, telomerase-positive canine cancer cell lines. Perhaps the biggest problem encountered in this part of the study was the lack of good negative control cell lines for both the DNhTERT study. During the course of the DNhTERT study it became apparent that the K9SF cell line that we had previously isolated from a mature dog was sufficiently close to senescence to prove problematic. In the absence of any transfections, the growth of these cells became slower from approximately passage 8 or 9 and would cease almost entirely by approximately passage 11. Transfections in these cells were performed at passage 6, and it became apparent that the trauma of transfection dramatically slowed the growth of these cells so that they would only continue growing for a further 1 or 2 passages. Unfortunately, due to problems with contamination in the laboratory, this slow growth meant that several flasks of K9SF cells

transfected with empty pCINeo became contaminated before stable transfectants could be established. In some ways it was fortuitous that the DNhTERT construct conveyed increased growth rate to these cells, as otherwise cells constructed with this construct might also have become contaminated before establishment of stable transfectants.

Inhibition of telomerase with telomestatin was more successful. Again the issue of a suitable negative control cell line became obvious once we looked at the growth rates of canine skin fibroblasts. An alternative ideal control would be a telomerase negative canine cancer cell line that elongates telomeres using the ALT pathway. However, to date, such a cell line has not been identified. Instead, we used a commercially available primary skin fibroblast cell line that had been isolated from a young beagle. This cell line should have been suitable for our work, but in the medium used, the growth rate was too slow to provide enough samples for more detailed investigation. Despite these issues, this study showed that canine cells could prove a useful testing ground for pharmacological methods of inhibiting telomerase activity. In addition, this study has gone some way to addressing one of the key concerns of using telomerase inhibition as an anti-cancer treatment in clinical settings. Eliminating telomerase activity from cancer cells without affecting other characteristics of tumour behaviour would allow continued tumour growth until the majority of tumour cells had exhausted their telomeric reserve. In dealing with brain tumours, particularly, this lag phase may result in the death of the patient before the death of the tumour. However, our study showed that cell death occurred before the exhaustion of telomeric reserve with telomestatin treatment, probably as a result of telomere uncapping. In addition, telomestatin treatment slowed the growth rate of both D17 and CMT7 cells, although after a greater lag phase in CMT7 cells than D17 cells. Both of these findings suggest that telomestatin may be useful in a clinical setting. The implications for the treatment of brain tumours in humans are also interesting, since a greater proportion of glioblastomas in humans maintain telomere length through the ALT pathway than many other tumours, and since G-quadruplex ligands have a strong effect on these cells as well as telomerase-positive cells.

Having confirmed the potential for telomerase inhibition as a strategy for cancer therapy, in the last part of our study we began work that may ultimately lead to strategies utilising telomerase-targeted gene expression. Sequencing and characterisation of the canine TERT gene was achieved, despite some problems, and identification of the core promoter sequence was successfully performed. This is a vital first step in the generation of TERT promoter-driven gene expression. We also began the process of sequencing and characterising the canine TR promoter, although this was not completed. Similarities between the canine and human TERT gene promoters were identified, but substantial differences were also found. However, two important factors suggest that the canine TERT gene promoter may function in a similar manner to the human TERT promoter. Firstly, the core promoter region in dogs seems to be remarkably similar to that in people, and coincides almost exactly with the largest CpG island identified. Secondly, elements of the canine TERT gene seem

to be involved in regulating gene expression, as they are in people. However, a number of the transcription factor binding sites which have attracted most attention in humans are not present in the canine promoter or are present outwith the core promoter. This does suggest that the action of the canine promoter may be regulated via different mechanisms. For instance, there appears to be a greater number of hormone-related transcription factor binding sites in the canine promoter, as compared with the human promoter. A much greater difference appears to exist between the human and canine TR promoters, with the presence of the $(TAAAA)_n$ repeats being of particular interest. At this stage the distribution of TR expression in canine tissues is unknown. Given the overall similarity between the presence of telomerase activity and the correlation between telomerase activity and 'TERT' expression in dog and human tissues, it seems likely that these two species have evolved with telomerase regulation occurring along similar lines. Therefore we could expect TR expression in dogs to mimic that in humans. How the $(TAAAA)_n$ repeats contribute to any differences found is an intriguing question that deserves further investigation. Despite this, however, this last part of the study has provided a good foundation for further work designing promoter-driven gene expression targeted at telomerase-positive tumours.

6.2 Future Studies

The work carried out in this project has provided some very interesting information. However, in many ways this information could be seen as a starting point, providing the foundation for more directed studies. Some of the major themes of future work are discussed here.

6.2.1 In Situ Detection of Telomerase Activity

Our investigation of the immunohistochemical detection of TERT as an assay for telomerase activity has provided interesting data. However, it is felt that there is more work to be performed before we can be certain of the sensitivity and specificity of the NCL-hTERT antibody in canine tissues. Although we have examined the immunohistochemical detection of TERT in canine telomerase-positive and –negative cells in tissue culture, we have yet to compare TERT staining in tumour tissues with other methods of assessing telomerase activity such as TRAP assays or RT-PCR. In addition, a more comprehensive survey of TERT staining in canine brains needs to be undertaken – it is certainly possible that the single brain examined is not representative of the overall TERT staining status in canine brains. Furthermore, given the presence of so many TERT-positive cells in telomerase-negative areas, the sensitivity of the stain in canine tissue needs to be confirmed. Another strategy, which we are pursuing at the moment, is to synthesise a novel antibody directed specifically at canine TERT. Whilst the peptide for immunisation has been synthesised, immunisation and antibody purification have yet to be completed. A different way of assessing telomerase activity in situ would be to use ISH techniques, and this is worth examining to

compare with immunohistochemical staining. These techniques can be technically demanding and rely on good quality RNA, which may not be easily available from paraffin-embedded sections, however they are worth attempting.

If validation of either the NCL-hTERT antibody or a novel, canine-specific TERT antibody can be successfully completed, this could prove an enormously powerful tool for surveying telomerase activity in a wide range of tissues and pathological conditions in dogs. For instance, the possibility of telomerase activation within the brain in other conditions, such as hypoxia or trauma could be assessed. A definitive survey of which somatic cells in the body exhibit telomerase activity (eg within the gastrointestinal tract) as well as searching for other stem cell populations could be performed. With the ability to use archival material, the possibilities for investigating telomerase activity become almost boundless.

6.2.2 Telomerase Inhibition

In our study we confirmed that it is possible to inhibit telomerase activity using G-quadruplex ligands in canine cells. However, the inability to inhibit canine telomerase using the human dominant negative mutant DNhTERT has raised other intriguing possibilities for future research. Firstly, unlike the G-quadruplex ligands, the dominant-negative splice variant approach eliminates telomerase activity without interfering with telomere capping. For this reason, DNhTERT has been used extensively in the human literature to investigate the impact on telomerase-positive cell lines of telomerase inhibition. It would be valuable to perform the same work in canine cell lines. Therefore, a number of studies could be performed that could usefully help this. Firstly, sequencing and characterising the full length canine TERT transcript would allow the generation of mutant variants, and thus we could investigate whether changes to the A motif in the same region as the human α deletion splice variant also produce dominant negative function in canine cells. If we could generate such a construct, we could also investigate the possible consequence inhibiting telomerase function without changing telomere structure. For instance, does interfering with telomerase activity in canine telomerase-positive cells affect growth rate? Are there consequent downstream effects within cells that might confound future telomerase-based therapies such as compensatory upregulation of transcription factors (eg Sp1, c-myc)? These questions could be answered with similar studies to the ones described here, if such a construct could be generated. Further work examining the clinical usefulness of telomerase inhibition could also be performed using other pharmacological methods, such as oligonucleotide inhibition or the investigation of other G-quadruplex ligands. Importantly, further work characterising the behaviour of other telomerase-positive canine cell lines in response to telomestatin should be performed, to examine the growth pattern of different cell lines in response to telomestatin therapy, in particular to see

whether the lag phase in growth rate inhibition correlates with the initial telomere length of those cell lines.

6.2.3 Promoter-targeted gene expression

Perhaps the part of this project which generated the most fertile ground for further work was the work investigating and characterising the canine TERT gene and TR gene promoters, in particular the TERT gene promoter. In identifying the core promoter of the TERT gene and confirming the strength of its expression in several telomerase-positive cell lines, we now have very powerful system that can be used to drive telomerase-specific gene expression. However, in sequencing the TERT promoter and identifying putative transcription factor binding sites, we have also begun the process of characterising the ways in which canine telomerase may be regulated in cancer cells. Having obtained the core promoter, the first step should be to identify whether it can be used to specifically drive cell death in telomerase-positive canine cancer cells by using it to drive an apoptosis-inducing gene such as the TRAIL gene. As well, the core promoter could be used to assess the viability of other genes of interest, including those that offer some potential bystander effect (eg the bacterial nitroreductase gene).

The sequence of the canine TERT promoter also allows us to investigate the manner in which canine telomerase is activated within tumour cells. Given the density of Sp1 binding sites, the importance of this as an activator of gene expression should be investigate, by co-transfecting telomerase-positive cells with the pGL3-promoter constructs together with a second vector expressing Sp1. This would allow us to measure the additional upregulation of TERT gene expression by Sp1. A similar strategy could be employed with some of the transcription factors that are currently poorly understood, such as MZF-1 and WT-1. As a control, it would also be interesting to transfect telomerase-negative control cells with the same constructs to examine the effect of transcription factors in isolation or even in particular combination with each other. Another alternative method of investigating the importance of particular transcription factor binding sites that could be employed would be to mutate specific sequences to alter their function, and then to examine the effects of these mutations on promoter activity. This approach has certainly been used in humans to examine the effect of the E-boxes present within the human core promoter, and could be employed to examine the importance of some of the hormone-receptor binding sites such as ER, PR and GR binding sites. Another intriguing study would be to examine the influence of hypoxic conditions on activity of the canine TERT promoter. It should be an easy study to transfect telomerase-positive cells with the pGL3-promoter construct and incubate them under hypoxic conditions to examine the effect on the promoter.

Having explored the importance of different transcription factors on the TERT promoter, the next task should be to investigate engineering the TERT promoter construct to modify its activity under different circumstances. It may be that the first modification would be to increase its strength of expression under all circumstances, by introducing a promoter such as the SV40 promoter. In any case, once modification of the promoter is complete, it could then be tested for its ability to drive expression of an appropriate gene in telomerase-positive cells. Once this preliminary testing phase is complete, the next step would be to investigate the function of the promoter *in vivo*, potentially using a nude mouse model xenografted with canine tumour cells. This would be the first testing ground on the way to final phase I, phase II and phase III clinical trials.

These studies, which could be actively pursued with the canine TERT gene promoter, could eventually also be applied to the TR promoter. However, rather more preliminary work needs to be performed with the TR promoter, first confirming the promoter sequence, and secondly investigating the activity of the TR promoter with different numbers of $(TAAAA)_n$ repeats. In itself this work is valuable because these repeats are relatively poorly understood in humans.

GLOSSARY

ALT	Alternative Lengthening of Telomeres
BrDU	BromoDeoxyUridine
canTERT	Canine TERT
cDNA	Complementary DNA
CGH	Comparative Genomic Hybridisation
CTP	Cytosine Triphosphate
DAB	Diaminobenzine
DIG	Digoxygenin
DIG-HRP	Digoxygenin-Horseradish Peroxidase
DNA	Deoxyribonucleic Acid
DTT	Dithiothretol
ELISA	Enzyme-linked Immunosorbent Assay
FISH	Fluorescent In Situ Hybridisation
gDNA	Genomic DNA
GBM	Glioblastoma multiforme
GTP	Guanidine Triphosphate
hTERT	Human TERT
HRP	Horseradish Peroxidase
ISH	In Situ Hybridisation
LOH	Loss of Heterozygosity
mTERT	Murine TERT
NTP	Nucleotide Triphosphate
PAGE	Polyacrylamide Gel Electrophoresis
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PS	Phosphatidylserine
RNA	Ribonucleic Acid
RT-PCR	Reverse-Transcriptase PCR
TERT	Telomerase Reverse Transcriptase
TR	Telomerase RNA
TRAP	Telomerase Repeat Amplification Protocol
VEGF	Vascular Endothelial Growth Factor

APPENDIX A

IMMUNOHISTOCHEMISTRY SAMPLE DETAILS: TERT AND MIB-1 STAINING

Table 1: TERT Staining vs Malignancy for 93 Tumours

Sample	Diagnosis	Grading	TERT Staining
1	Astrocytoma	1	Negative
2	Astrocytoma	1	Negative
3	Astrocytoma	1	Negative
4	Astrocytoma	1	Negative
5	Astrocytoma	1	Negative
6	Astrocytoma: anaplastic	2	Negative
7	Astrocytoma: anaplastic	2	Negative
8	Astrocytoma: anaplastic	2	Positive
9	Astrocytoma: anaplastic	2	Positive
10	Astrocytoma: anaplastic	2	Positive
11	Astrocytoma: glioblastoma	2	Negative
12	Astrocytoma: glioblastoma	2	Negative
13	Astrocytoma: glioblastoma	2	Positive
14	Astrocytoma: glioblastoma	2	Positive
15	Choroid plexus papilloma	1	Negative
16	Choroid plexus papilloma	1	Negative
17	Choroid plexus papilloma	1	Negative
18	Choroid plexus papilloma	1	Negative
19	Choroid plexus papilloma	1	Negative
20	Choroid plexus papilloma	1	Negative
21	Choroid plexus papilloma: anaplastic	2	Positive
22	Choroid plexus papilloma: carcinoma	2	Negative
23	Ependymoma	1	Positive
24	Gliosarcoma	2	Positive
25	Medulloblastoma	2	Positive
26	Meningioma	1	Negative
27	Meningioma	1	Negative
28	Meningioma	1	Negative
29	Meningioma	1	Negative
30	Meningioma	1	Negative
31	Meningioma	1	Negative
32	Meningioma	1	Negative
33	Meningioma	1	Negative
34	Meningioma	1	Negative
35	Meningioma	1	Negative
36	Meningioma	1	Negative
37	Meningioma	1	Negative
38	Meningioma	1	Positive
39	Meningioma	1	Positive
40	Meningioma	1	Positive
41	Meningioma	1	Positive
42	Meningioma	1	Positive
43	Meningioma	1	Positive
44	Meningioma	2	Negative

45	Meningioma	2	Positive
46	Meningioma	2	Positive
47	Meningioma: anaplastic	2	Negative
48	Meningioma: anaplastic	2	Negative
49	Meningioma: anaplastic	2	Positive
50	Meningioma: anaplastic	2	Positive
51	Meningioma: anaplastic	2	Positive
52	Meningioma: anaplastic	2	Positive
53	Metastasis: adenocarcinoma	2	Negative
54	Metastasis: adenocarcinoma	2	Negative
55	Metastasis: adenocarcinoma	2	Positive
56	Metastasis: adenocarcinoma	2	Positive
57	Metastasis: adenocarcinoma	2	Positive
58	Metastasis: adenocarcinoma	2	Positive
59	Metastasis: lymphoma	2	Negative
60	Metastasis: lymphoma	2	Negative
61	Metastasis: lymphoma	2	Negative
62	Metastasis: lymphoma	2	Positive
63	Metastasis: lymphoma	2	Positive
64	Metastasis: lymphoma	2	Positive
65	Metastasis: lymphoma	2	Positive
66	Oligodendroglioma	1	Negative
67	Oligodendroglioma	1	Negative
68	Oligodendroglioma	1	Negative
69	Oligodendroglioma	1	Negative
70	Oligodendroglioma	1	Negative
71	Oligodendroglioma	1	Negative
72	Oligodendroglioma	1	Negative
73	Oligodendroglioma	1	Negative
74	Oligodendroglioma	1	Negative
75	Oligodendroglioma	1	Positive
76	Oligodendroglioma	1	Positive
77	Oligodendroglioma	1	Positive
78	Oligodendroglioma	1	Positive
79	Oligodendroglioma	2	Negative
80	Oligodendroglioma	2	Positive
81	Oligodendroglioma	2	Positive
82	Oligodendroglioma	2	Positive
83	Oligodendroglioma: anaplastic	1	Negative
84	Oligodendroglioma: anaplastic	1	Negative
85	Oligodendroglioma: anaplastic	2	Positive
86	Oligodendroglioma: anaplastic	2	Positive
87	Oligodendroglioma: anaplastic	2	Positive
88	Oligodendroglioma: anaplastic	2	Positive
89	Oligodendroglioma: anaplastic	2	Positive
90	Oligodendroglioma: anaplastic	2	Positive
91	Oligodendroglioma: anaplastic	2	Positive
92	Oligodendroglioma: anaplastic	2	Positive
93	Pituitary adenocarcinoma	2	Positive

Table 2: MIB-1 LI and TERT staining for 51 tumours

Sample	Diagnosis	Grading	MIB1 LI (%)	TERT Staining
1	Astrocytoma	1	3.2	Negative
2	Astrocytoma: anaplastic	2	14.2	Negative
3	Astrocytoma: anaplastic	2	8.9	Positive
4	Astrocytoma: glioblastoma	2	14.8	Negative
5	Astrocytoma: glioblastoma	2	26.1	Positive
6	Choroid plexus papilloma	1	0	Negative
7	Choroid plexus papilloma	1	0	Negative
8	Choroid plexus papilloma	1	0	Negative
9	Choroid plexus papilloma	1	0.9	Negative
10	Choroid plexus papilloma	1	3	Negative
11	Choroid plexus carcinoma	2	9.2	Negative
12	Gliosarcoma	2	20.2	Positive
13	Meningioma	1	0	Negative
14	Meningioma	1	0	Negative
15	Meningioma	1	0	Negative
16	Meningioma	1	0	Negative
17	Meningioma	1	0.2	Negative
18	Meningioma	1	0.4	Negative
19	Meningioma	1	0.4	Negative
20	Meningioma	1	1.2	Negative
21	Meningioma	1	1.5	Negative
22	Meningioma	1	2	Negative
23	Meningioma: anaplastic	2	13	Negative
24	Meningioma: anaplastic	2	41.4	Positive
25	Meningioma: anaplastic	2	9.8	Positive
26	Meningioma: anaplastic	2	14.2	Positive
27	Meningioma: anaplastic	2	22.4	Positive
28	Metastasis: adenocarcinoma	2	19.4	Negative
29	Metastasis: lymphoma	2	72.5	Negative
30	Metastasis: lymphoma	2	19.4	Positive
31	Metastasis: lymphoma	2	49.3	Positive
32	Oligodendroglioma	1	0	Negative
33	Oligodendroglioma	1	0	Negative
34	Oligodendroglioma	1	1.1	Negative
35	Oligodendroglioma	1	2.4	Negative
36	Oligodendroglioma	1	2.8	Negative
37	Oligodendroglioma	1	8.9	Negative
38	Oligodendroglioma	1	0.9	Negative
39	Oligodendroglioma	1	4.6	Negative
40	Oligodendroglioma	1	1	Positive
41	Oligodendroglioma	1	3.5	Positive
42	Oligodendroglioma: anaplastic	2	14.9	Negative
43	Oligodendroglioma: anaplastic	2	17.3	Positive
44	Oligodendroglioma: anaplastic	2	20.4	Positive
45	Oligodendroglioma: anaplastic	2	37.8	Positive
46	Oligodendroglioma: anaplastic	2	9.2	Positive
47	Oligodendroglioma: anaplastic	2	10.2	Positive
48	Oligodendroglioma: anaplastic	2	13.4	Positive
49	Oligodendroglioma: anaplastic	2	23.5	Positive
50	Oligodendroglioma: anaplastic	2	25	Positive
51	Pituitary adenocarcinoma	2	16.9	Positive

APPENDIX B

UNFORMATTED SEQUENCE OF CANIS FAMILIARIS TELOMERASE REVERSE TRANSCRIPTASE UPSTREAM REGULATORY REGION

>Canis familiaris telomerase reverse transcriptase promoter exon 1 intron 1 and initial part of exon2

-5390 CTGCAGAGCA CCTGCCCCCG CCCCCGGCCC CACATGGGCA

-5350 CCCACCGTCC TGGGACACCA GCTCCCCTCC CGCACCTGCC GTTCACCCAT

-5300 GGGTCTTTCC TGTGTCACAG ACCTTCCCTC CCTGAGTCCT TCAAGATTTG

-5250 TCTAGATGAC AATGTCCCAG CGCCCCTCAT CCTTGGGGGA CAGCTGTTTC

-5200 CTAGGGGCCC AGCCCCAGAC GGTCGGGGTG TCCTCGCCGG GTTGTGGCCA

-5150 TGCCGCGTGG CTGGTCCCTA TGTCCACTCT GCTGGGATGT CCATGTTGTC

-5100 CCCCAGCAAC AGGGAAGGCC AGGCGGTCTG TCCCCCCAAG CATGTCTCCC

-5050 TGTCTCCCAC GCTCGCCCTG TCCCCCCTGC CGGGCCGTGG TCTGTCCGGA

-5000 GCCCAGACCT CTGCCTGTCC TCAGACCCCG CTCTCGGGCC GCTCTGTGCT

-4950 CTGCTTCCCT CCGTCTGTCC CTCTGCTACT TCCTTCTTGC TGAACCTGTC

-4900 CTCAAGGCGC CTATGCCTCC ACATGTTGTA CGTGAGCCCC GTGAGCCCTG

-4850 TGGGCACCAC CCCTCTCGCC CCCCCACCCC GGGTGCAGGA AACACCGTGC

-4800 GCTAGGTTAC CCAATGCGCA GTTCTGCCTG TGCGGTTGGT TGTTGGACAG

-4750 CTCCCGCGTG CCCTCACTAC GGAGTGCCTG GAGGTGTTTG GGGCATGGCG

-4700 GGAATCCTGA AGCCCAGGAT GTCGGTGGCC CCTTCCTGGG GGAACTGGCG

-4650 CTTGTGCCAT TTGCGTCTGC AGGAGCCAGT GTCTCCACGG ACATGAGGCG

-4600 GGTCTTCCCA GGGCCCCATA GTCACACAGA GGAGCAGATA CAGGGCCCAG

-4550 AGACACCAGA TACAGTAACT GGTGAAAGCC AGAGCGAAGG GTCTGGATTC

-4500 AAGTCCAGAA GCTGCCAGCA GCCTCAGCAA GTGCTCGCGG GATGTGCCTC

-4450 GCTCGCACCT GTTCTGAAAG TCATTGTGGC CGCCGCCGGG AGGAGGTGGC

-4400 TGGCAGGACA GGAAGCCACA GCCAGGAGAA GACACAAGGA CACCTGGACC

-4350 CACGCCCCCC GTCTTTCTCC TGCGGAGAGG TGTCTGTGCA GGGCGGGAAT

-4300 CCAAGTGCCT CTTTGTAAAG TGCAGACGCC CTGGCTCTGC CCTCCTCACC

-4250 TCCTGCCCCA CCTGTGCCCG GGAACCAGCG GGGCCCGGTC CCTGAGCACC

-4200 CGTGTCCTTG GAGAGGCCCT CACCACCAGG ACCCAGGACA TGAGTGGGCA

-4150 CGTGCTGTTC ACAAAAGTAG AGATTAAGGA CTTAATTCTA TAAGTAAATT

-4100 CCACCTTTCA GCCTCCAAGT AGGTCAATTT ATCTTAAGAT TTTCTTAAAT

-4050 TTTCATCAAA TAAGTTGCAA GAATGCAGAA ATCCAGAGGC ACACAGGCAA

-4000 CCGGAGCTGA GCTGTGTTTG CCGAGATGCG AGGACCAAAT ACCCTCACCT

-3950 GGAGGGATTT AAGCCGTAAG CACCTTTTAT TGGTTTTCAT TAGGTGGTTG

-3900 AGGGCAAGAG AAAGGACACA GCGAGGCGGA TGCAGTCGGG ATGGATTAAT

-3850 GTGGTGGCAC CACCAGGGGA GGAGGTGAAA GGCCTCACCC ACCGGGGAGG

-3800 CGGACGCACA CTTGTTCCCC CCCGGCGCTG CCAGTCTTCC TGACCTGAGG

-3750 GCCTCTTCCT GAGGCCCCTG CCCCAGCCCG CTGCCCTCTC TCCTGACCTC

-3700 ACCCAGCACC CAAGCCAGCC GTGCACATGG GGGACCCTGG CCTGGCGCCA

-3650 GACAACCTGG GTTTGTGTCC TGGCTCTGCC CCGGGGGATC GAGGTTTCCA

-3600 GGAAGCCCCC ACCCTGAACC TGACAGGACG ATGATACTCG TCCACCTACT

-3550 TTCTGAGGGG CTCAGAGGTC ATGCCCTCAG GTGGCAAATC CCACCGACCA

-3500 GGACCATTCT TCCCACTGCT GTCTGTCCCC AGCATGAGGG CACAGAGCAG

-3450 CCACTGTTTT CTCTGCTCAC AGACACCCAC CTGGTAGAAT GAGGCTCAGG

-3400 AAATAAAGAG GGGGCTGCTT TCAGCCCCAG GTTGGGGTGA CAGGAGCAGC

-3350 TGGAACCAGG GGCCAGGAAC ATGCAGCCCG GCCTCACAAC TCCACAGACG

-3300 TGGGGCCTCA CCCCCGCCAT GGCCGTGCAT GCCCTCGCGG GACACTGTGT

-3250 AGTGTCCAGA GGGGTCGCCT CCCCATGACA GGGCGCCGTC CTTCCCCTGC

-3200 AGCGGCCTGG GCCGTGGGGC ACTGGGGTAC GTGTCTGCGG CTGAGCTGAC

-3150 CCCTAGACGG GGCTCCCACC TGAGCCACAC GCTGTTCCCT CTGCCTCTGT

-3100 CTGCCTGGAG GGGTGGTCTG GCCCAGACGG CCTCGTTTCA CACCACTGGT

-3050 TTGTGACGTG CTACAGATTT CAGTTCCCAT GGTGACCCTG TGATAAAGTA

-3000 TTGGCAAGGA CCTTGTCAAT CTCTGAGGAT AATTTTCCCC ACACACAACA

-2950 ATCCCCCTCC CAGAGGGGAT CTGTGGAGGC TTTGGGGTCC CGGGGTGTCA

-2900 CCCATGTTGC TTCCAGAAAC ACCCCATAAG ACCTAGGTCC CTAAACCTGC

-2850 CCTCCACACC CCCTCCCTTC AATCGTGCGT GGTCTGAAGG ACAGGGCTCC

-2800 TCCCATGACT CGTTGTCTAT GCTCCCTCAG CCTGAACCTG CCACAGGATC

-2750 CTCTCCTGGC CCCGGGGTCA CAGCCTCCCG GGCACCCCAT CCTGGCCGTA

-2700 TCCCTGGCAG CATGCCGACA GTACTGAAGC CCGTCCCATG CACACCCCAC

-2650 CAGAGGCTAC AGTAACAGCC AGCGAGGCCG GCACTGCTGG GAGGGCCCAG

-2600 GGCAGCAGGA GCCCTACTGG TGTCCGCCAG AACAGAAGTC AGCACCACCC

-2550 TCCTTACAGT GTGACCTGGC CGTGGCACTC CTGCTGGACC TTCATCCAGG

-2500 AACAGGACAA CTGACATAAT GAGGCACAGA ATCCTGTGTA CAAACATCCA

-2450 CAGCAGCTTT CCGTGTCACA GCCCCACAGT GCAAACATCC CCAATGTCCC

-2400 CTGCCAGGCG TGTGGTCCTC TCCACCGTGC AGCCGCAGCA CCGACTGGGT

-2350 GCAGCTTCCA CATGGGAAGC AGCAGACTGC TGGCCCCACA GGAGCATGCC

-2300 GAGCAGAAGG TCAGTGTCCG TGGGTCCTAT GTGCTGTGAC TCCATTTATC

-2250 TCCCCACAAT CACAATTTCC CTGGTCAGGG GCTCAGGTTC TGGGCTGGGG

-2200 GGGCATAAGC ATGGGGGACC CTGAGGCCAG GGGCACCACC CACAGCCAGA

-2150 ACCGTCCTGG TCATGCCGAT CCACATAGGC AGGGACAGGA CACGAGCCTC

-2100 CCACACACAC TGGGCCAGGC TTAGTGCGCT GGTTCTGGCC AAGGGTCCCC

-2050 GCGCTCCGGG AGGTCACCCG CTGGGGACAC CAGGAGAGGG TCCTGCAGGC

-2000 AGAGCCAATC AGCAGCTGTA TTCCTATCAC ACTGAGGTTG AGGGCTGAGG

-1950 GCCACCCAAG GATGCCGCAT CCTAGCCCTG GAACCTGGGG ATGTGGCCTC

-1900 GCCTGGCAGA GGGACTTTTT GGTTGGGATT TCTTGAGGGT CAAAGTTAGG

-1850 GGGATGCAGA CTGACACAGA GGCTGGCTTC AGAGCAGCTG GGAGGGTGCA

-1800 GCGTGAAGGC CTTTCCAAGG GGGCAGGGGC CAGCGGGGGA CCCCTGTTGG

-1750 CAAGGCCACA CCGTGGGCCC CACTTCTCTC TCATGCTGTT GTGAACAGCT

-1700 GTCACAGACA GTGGCTCGCA GGTCACTGAC CGCTCACATT GGGCTGTTCA

-1650 CGTGGGCAGT GGCCTGGCTG CGGACCGATG TTTGGAGTCC ACCTGTGTGT

-1600 TTGCTGGGGG TTGGGGAGGG TGCCCAGCCA GCTCTTGTAT TCTAGCTCAA

-1550 GCCAACAACA TCAATTTTTT TCTGGAACAC TTTTCACATT CATGACGCCG

-1500 TGATTTCAGA CTATCACCCG CTTCCTGAAA CAGGTGATGG AGGAGATGAA

-1450 GGGCTTCGTT CAGGCTGCGG GAACCCCCCC CGCCATCAGC ACAGTATCAG

-1400 TCCCACGTGT CCTTCTCTGC CTTGGTTTCT CCTGTTCCTC TTTTTCTAAA

-1350 AACTGATGTT TTCTGACCAG GTTTCTCCAG GTGAATCTGA GTAACTTTGT

-1300 TGGAAAAGAA CACATTTCCC CAGACAGTCG CAAAACCTCC AGTGGCCGAA

-1250 GTGAGTGGTT CTCCATCCTG GCCCTGGAGA CCACCGTGCG CCTCGGTGGA

-1200 ACTCTGTCTA GAAGGGCGAC ACGTAATCCG AGTGTTACAG GAAGGGGCAG

-1150 ACCGCCAGCA CCATGAATAT GGAAGCCCGG CCACCAAGAG GGTGCAAGGG

-1100 GCCTGTCGAA ATGCTAGGGC CATGAATAAA AGCAATTTCC TGACCACGTA

-1050 CGTTGCCAAA GGTTAACATT TGTGCAGCCG TCCAGTGTTT GCTGAGCTCA

-1000 GGAGCATTGT CCCCGGGAGC CTCCTGAGAC CCACAAGAGC CTGTTTCTCA

-950 GCAATTCAAA CTTGAACGCG TCTGCGTTTG CATGGTCTCA TTAGGGTGGC

-900 TCCTCTGGGT GTCCTGTTCA AGCGTCATGA TGAACACTGT CACAACTGTC

-850 TCCCTGGGTC TGGGGTGGCG GGTCTGCATT GTAGTGCAGA GGCTCTGCAC

-800 TGGGCGGGAA GGGTCTGTGA GGTCCCCTGA CCCGTCCAGT CCCACCTCCA

-750 GCCTGCCCTG GGGCCTGAGC ACGAGGGCAG CTGGGAGCGG CTGAGAGGAG

-700 CCTGCACAGG GTGCCACCAA GCCCTGTGGC TCCCATTTCT GCCCTCCTTT

-650 CCTGGACTGT GGTTAGGAGG GAGCTGAGTG GGGTTTTTTT GCCTGTGGTG

-600 GGGACAACAG GAGCCCGAGG ACCTGAAGGA GAAACCACAG CCTGGGGTCG

-550 AGGAGGTCAG GTCTGGGGGC AGTACTGCCG CTGCCTGCTG GCTGCTCACT

-500 GGCCGGGCCT GTCCTCTGGG GCTGCTTGTG CCCGCACCCC CCGTGGCTCA

-450 GTGGGGCAGC CCTGCTGGCC CTTCACCCCG TGTATCGTGC ACATTCTCCA

-400 CCCTGCTCCC TGCACCCCCT CCGGGCTGAA CCCCTCCCCC CTCGCCAGCC

-350 TCCAGGGAGC AGCCTCAGTG TCTGGGTCAC TCGCCCCTGT GACAGGGACA

-300 AACCCAAGGA CCCCCTGCCC AGGGGCGCAG AGCGCAGCAG GTGCCCCATG

-250 TCGGGCGGGG GCTGCCCTGG GTACCCCCGA GCCTCGGCCG ACGGCATCGC

-200 TCCCCGCCCG CGACCCTCGC CGCGCCCCCC GGGCCGGAGA GCAGAGGACG

-150 CGGCCGCGGC CCACGCGCTC CTGGGGCTCC GCACACCTCG GCGCGCACGC

-100 CCCCGGGCGC CCCGCCCCTT CCTCCCAGGA GCCCCGCCCC GCCGCGGACC

-50 CCCTCCCCCG GCCCGCCCCT TCCTCCCGCG GACCCCGCCC CTCCCCGGCC

Exon 1

+1 ATGCCGCGAG CGCCCCGGTG CCGCGCCGTG CGCGCCCTGC TGCGGGGCCG CTACCGGGAG

+61 GTGCTGCCCC TGGCCACCTT CCTGCCGCGC CTGGGGCCCC CGGGCCGGC TGCTCGTGCG

+121 GCGCGGGGAC CCGGCGGCCT TCCGCGCGCT GGTGGCGCAG TGCCTGGTGT GCGTGCCCTG

+181 GGGCGCGCGG CCGCCCCCCG CCGCCCCGTG CTTCCGCCA

Intron 1

+220 GGTGGGCCGC GGGGGACCCT GCTGGGGCCG GGGCCGGGGC TGGGGCCGGG GCCGGGGCTG

+280 GGAGGGGCAC GGCCGCGCGC TTCAAGACGC CGCCGCCCCC CCGCAG

Exon 2

+326 GTGTCCTGTC CTCAA

REFERENCES

Abdul-Ghani, R., Ohana, P., Matouk, I., Ayesh, S., Ayesh, B., Laster, M., Bibi, O., Giladi, H., Molnar-Kimber, K., Sughayer, M. A., de Groot, N., & Hochberg, A. 2000, "Use of transcriptional regulatory sequences of telomerase (hTER and hTERT) for selective killing of cancer cells", *Mol. Ther.*, vol. 2, no. 6, pp. 539-544.

Adamo, P. F., Cantile, C., & Steinberg, H. 2003, "Evaluation of progesterone and estrogen receptor expression in 15 meningiomas of dogs and cats", *Am. J. Vet. Res*, vol. 64, no. 10, pp. 1310-1318.

Aigner, S., Lingner, J., Goodrich, K. J., Grosshans, C. A., Shevchenko, A., Mann, M., & Cech, T. R. 2000, "Euplotes telomerase contains an La motif protein produced by apparent translational frameshifting", *EMBO J.*, vol. 19, no. 22, pp. 6230-6239.

Akiyama, M., Hideshima, T., Shammas, M. A., Hayashi, T., Hamasaki, M., Tai, Y. T., Richardson, P., Gryaznov, S., Munshi, N. C., & Anderson, K. C. 2003, "Effects of oligonucleotide N3'-->P5' thio-phosphoramidate (GRN163) targeting telomerase RNA in human multiple myeloma cells", *Cancer Res.*, vol. 63, no. 19, pp. 6187-6194.

Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., & Barrett, J. C. 1996, "Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts", *Proc. Natl. Acad. Sci.U.S.A*, vol. 93, no. 24, pp. 13742-13747.

Allsopp, R. C., Morin, G. B., DePinho, R., Harley, C. B., & Weissman, I. L. 2003, "Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation", *Blood*, vol. 102, no. 2, pp. 517-520.

Argyle, D. J. & Nasir, L. 2003, "Telomerase: a potential diagnostic and therapeutic tool in canine oncology", *Vet. Pathol.*, vol. 40, no. 1, pp. 1-7.

Argyle, D. J., Mckevitt, T., Gault, E., & Nasir, L. 2004, "Evaluation of telomerase-targeted therapies in canine cancer cell lines", *Vet. Comp. Oncol.*, vol. 2, no. 4, pp. 214-221.

Armbruster, B. N., Banik, S. S., Guo, C., Smith, A. C., & Counter, C. M. 2001, "N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo", *Mol. Cell Biol.*, vol. 21, no. 22, pp. 7775-7786.

Artandi, S. E., Alson, S., Tietze, M. K., Sharpless, N. E., Ye, S., Greenberg, R. A., Castrillon, D. H., Horner, J. W., Weiler, S. R., Carrasco, R. D., & DePinho, R. A. 2002, "Constitutive telomerase expression promotes mammary carcinomas in aging mice", *Proc. Natl. Acad. Sci.U.S.A*, vol. 99, no. 12, pp. 8191-8196.

Asai, A., Oshima, Y., Yamamoto, Y., Uochi, T. A., Kusaka, H., Akinaga, S., Yamashita, Y., Pongracz, K., Pruzan, R., Wunder, E., Piatyszek, M., Li, S., Chin, A. C., Harley, C. B., & Gryaznov, S. 2003, "A novel telomerase template antagonist (GRN163) as a potential anticancer agent", *Cancer Res.*, vol. 63, no. 14, pp. 3931-3939.

Athanassiadou, P., Bantis, A., Gonidi, M., Liossi, A., Aggelonidou, E., Petrakakou, E., & Giannopoulos, A. 2003, "Telomerase expression as a marker in prostate cancer: correlation to clinicopathologic predictors", *J. Exp .Clin. Cancer Res.*, vol. 22, no. 4, pp. 613-618.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G., & Struhl, K. 1994, *Current Protocols in Molecular Biology* 4th edition, John Wiley and Sons, New York.

Bachand, F. & Autexier, C. 2001, "Functional regions of human telomerase reverse transcriptase and human telomerase RNA required for telomerase activity and RNA-protein interactions", *Mol. Cell Biol.*, vol. 21, no. 5, pp. 1888-1897.

Bachand, F., Triki, I., & Autexier, C. 2001, "Human telomerase RNA-protein interactions", *Nucleic Acids Res.*, vol. 29, no. 16, pp. 3385-3393.

Bachand, F., Boisvert, F. M., Cote, J., Richard, S., & Autexier, C. 2002, "The product of the survival of motor neuron (SMN) gene is a human telomerase-associated protein", *Mol. Biol. Cell*, vol. 13, no. 9, pp. 3192-3202.

Bailey, S. M., Brenneman, M. A., & Goodwin, E. H. 2004, "Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells", *Nucleic Acids Res.*, vol. 32, no. 12, pp. 3743-3751.

Banfi, A., Bianchi, G., Notaro, R., Luzzatto, L., Cancedda, R., & Quarto, R. 2002, "Replicative aging and gene expression in long-term cultures of human bone marrow stromal cells", *Tissue Eng.*, vol. 8, no. 6, pp. 901-910.

Banik, S. S., Guo, C., Smith, A. C., Margolis, S. S., Richardson, D. A., Tirado, C. A., & Counter, C. M. 2002, "C-terminal regions of the human telomerase catalytic subunit essential for in vivo enzyme activity", *Mol. Cell Biol.*, vol. 22, no. 17, pp. 6234-6246.

Baumann, P., Podell, E., & Cech, T. R. 2002, "Human Pot1 (protection of telomeres) protein: cytolocalization, gene structure, and alternative splicing", *Mol. Cell Biol.*, vol. 22, no. 22, pp. 8079-8087.

Baykal, A., Thompson, J. A., Xu, X. C., Hahn, W. C., Deavers, M. T., Malpica, A., Gershenson, D. M., Silva, E. G., & Liu, J. 2004, "In situ human telomerase reverse transcriptase expression pattern in normal and neoplastic ovarian tissues", *Oncol. Rep.*, vol. 11, no. 2, pp. 297-302.

Beattie, T. L., Zhou, W., Robinson, M. O., & Harrington, L. 1998, "Reconstitution of human telomerase activity in vitro", *Curr. Biol.*, vol. 8, no. 3, pp. 177-180.

Beattie, T. L., Zhou, W., Robinson, M. O., & Harrington, L. 2000, "Polymerization defects within human telomerase are distinct from telomerase RNA and TEP1 binding", *Mol. Biol. Cell*, vol. 11, no. 10, pp. 3329-3340.

Beattie, T. L., Zhou, W., Robinson, M. O., & Harrington, L. 2001, "Functional multimerization of the human telomerase reverse transcriptase", *Mol. Cell Biol.*, vol. 21, no. 18, pp. 6151-6160.

Bedbrook, J. R., Jones, J., O'Dell, M., Thompson, R. D., & Flavell, R. B. 1980, "A molecular description of telometic heterochromatin in secale species", *Cell*, vol. 19, no. 2, pp. 545-560.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., & Finberg, R. W. 1997, "Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5", *Science*, vol. 275, no. 5304, pp. 1320-1323.

Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J. 2001, "Role for a bidentate ribonuclease in the initiation step of RNA interference", *Nature*, vol. 409, no. 6818, pp. 363-366.

Bhattacharyya, A. & Blackburn, E. H. 1997, "A functional telomerase RNA swap in vivo reveals the importance of nontemplate RNA domains", *Proc. Natl. Acad. Sci.U.S.A*, vol. 94, no. 7, pp. 2823-2827.

Bianchi, A., Smith, S., Chong, L., Elias, P., & de Lange, T. 1997, "TRF1 is a dimer and bends telomeric DNA", *EMBO J.*, vol. 16, no. 7, pp. 1785-1794.

Bianchi, A., Stansel, R. M., Fairall, L., Griffith, J. D., Rhodes, D., & de Lange, T. 1999, "TRF1 binds a bipartite telomeric site with extreme spatial flexibility", *EMBO J.*, vol. 18, no. 20, pp. 5735-5744.

Bickenbach, J. R., Vormwald-Dogan, V., Bachor, C., Bleuel, K., Schnapp, G., & Boukamp, P. 1998, "Telomerase is not an epidermal stem cell marker and is downregulated by calcium", *J. Invest. Dermatol.*, vol. 111, no. 6, pp. 1045-1052.

Bieche, I., Nogues, C., Paradis, V., Olivi, M., Bedossa, P., Lidereau, R., & Vidaud, M. 2000, "Quantitation of hTERT gene expression in sporadic breast tumors with a real-time reverse transcription-polymerase chain reaction assay", *Clin. Cancer Res.*, vol. 6, no. 2, pp. 452-459.

Biessmann, H. & Mason, J. M. 1992, "Genetics and molecular biology of telomeres", *Adv. Genet.*, vol. 30,pp: 185-249.

Bigner, S. H., Matthews, M. R., Rasheed, B. K., Wiltshire, R. N., Friedman, H. S., Friedman, A. H., Stenzel, T. T., Dawes, D. M., McLendon, R. E., & Bigner, D. D. 1999, "Molecular genetic aspects of oligodendrogliomas including analysis by comparative genomic hybridization", *Am. J. Pathol.*, vol. 155, no. 2, pp. 375-386.

Biller, B. J., Kitchell, B. E., & Cadile, C. D. 1998, "Evaluation of an assay for detecting telomerase activity in neoplastic tissues of dogs", *Am. J. Vet. Res*, vol. 59, no. 12, pp. 1526-1529.

Bilsland, A. E., Anderson, C. J., Fletcher-Monaghan, A. J., McGregor, F., Evans, T. R., Ganly, I., Knox, R. J., Plumb, J. A., & Keith, W. N. 2003, "Selective ablation of human cancer cells by telomerase-specific adenoviral suicide gene therapy vectors expressing bacterial nitroreductase", *Oncogene*, vol. 22, no. 3, pp. 370-380.

Birnboim, H. C. & Doly, J. 1979, "A rapid alkaline extraction procedure for screening recombinant plasmid DNA", *Nucleic Acids Res.*, vol. 7, no. 6, pp. 1513-1523.

Blackburn, E. H. & Gall, J. G. 1978, "A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena", *J. Mol. Biol.*, vol. 120, no. 1, pp. 33-53.

Blackburn, E. H. 2005, "Telomeres and telomerase: their mechanisms of action and the effects of altering their functions", *FEBS Lett.*, vol. 579, no. 4, pp. 859-862.

Blackwood, E. M. & Eisenman, R. N. 1991, "Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc", *Science*, vol. 251, no. 4998, pp. 1211-1217.

Blasco, M. A., Lee, H. W., Hande, M. P., Samper, E., Lansdorp, P. M., DePinho, R. A., & Greider, C. W. 1997, "Telomere shortening and tumor formation by mouse cells lacking telomerase RNA", *Cell*, vol. 91, no. 1, pp. 25-34.

Blasco, M. A. 2002, "Opinion: Telomerase beyond telomeres", *Nat. Rev. Cancer*, vol. 2, no. 8, pp. 627-633.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., & Wright, W. E. 1998, "Extension of life-span by introduction of telomerase into normal human cells", *Science*, vol. 279, no. 5349, pp. 349-352.

Boldrini, L., Pistolesi, S., Gisfredi, S., Ursino, S., Lupi, G., Caniglia, M., Pingitore, R., Basolo, F., Parenti, G., & Fontanini, G. 2003, "Telomerase in intracranial meningiomas", *Int. J. Mol. Med.*, vol. 12, no. 6, pp. 943-947.

Bond, J. A., Wyllie, F. S., & Wynford-Thomas, D. 1994, "Escape from senescence in human diploid fibroblasts induced directly by mutant p53", *Oncogene*, vol. 9, no. 7, pp. 1885-1889.

Bond, J. A., Haughton, M. F., Rowson, J. M., Smith, P. J., Gire, V., Wynford-Thomas, D., & Wyllie, F. S. 1999, "Control of replicative life span in human cells: barriers to clonal expansion intermediate between M1 senescence and M2 crisis", *Mol. Cell Biol.*, vol. 19, no. 4, pp. 3103-3114.

Boyd, M., Mairs, R. J., Mairs, S. C., Wilson, L., Livingstone, A., Cunningham, S. H., Brown, M. M., Quigg, M., & Keith, W. N. 2001, "Expression in UVW glioma cells of the noradrenaline transporter gene, driven by the telomerase RNA promoter, induces active uptake of [131I]MIBG and clonogenic cell kill", *Oncogene*, vol. 20, no. 53, pp. 7804-7808.

Braasch, D. A. & Corey, D. R. 2001, "Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA", *Chem. Biol.*, vol. 8, no. 1, pp. 1-7.

Braund, K. 1994, *Clinical syndromes in veterinary neurology*, 2 edn, Mosby, St Louis.

Braund, K. G. & Ribas, J. L. 1986, "Central nervous system meningiomas", *Compend. Cont. Edu.*, vol. 8, no. 4, pp. 241-248.

Bravo, R. & Macdonald-Bravo, H. 1987, "Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites", *J. Cell Biol.*, vol. 105, no. 4, pp. 1549-1554.

Brearley, M. J., Jeffery, N. D., & Phillips, S. M. 1999, "Hypofractionated Radiation Therapy of Brain Masses in Dogs: A Retrospective Analysis of Survival of 83 Cases (1991-1996)", *J. Vet. Int. Med.*, vol. 13, pp. 408-412.

Breen, M., Jouquand, S., Renier, C., Mellersh, C. S., Hitte, C., Holmes, N. G., Cheron, A., Suter, N., Vignaux, F., Bristow, A. E., Priat, C., McCann, E., Andre, C., Boundy, S., Gitsham, P., Thomas, R., Bridge, W. L., Spriggs, H. F., Ryder, E. J., Curson, A., Sampson, J., Ostrander, E. A., Binns, M. M., & Galibert, F. 2001, "Chromosome-specific single locus FISH probes allow anchorage of an 1800-marker integradated radiation-hybrid/linkage map of the dog genome to all chromosomes", *Genome Res.*, vol. 11, pp. 1784-1795.

Brenner, A. J., Stampfer, M. R., & Aldaz, C. M. 1998, "Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation", *Oncogene*, vol. 17, no. 2, pp. 199-205.

Broccoli, D., Young, J. W., & de Lange, T. 1995, "Telomerase activity in normal and malignant hematopoietic cells", *Proc. Natl. Acad. Sci. U.S.A*, vol. 92, no. 20, pp. 9082-9086.

Brown, J. P., Wei, W., & Sedivy, J. M. 1997, "Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts", *Science*, vol. 277, no. 5327, pp. 831-834.

Bruno, S. & Darzynkiewicz, Z. 1992, "Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells", *Cell Prolif.*, vol. 25, no. 1, pp. 31-40.

Bryan, T. M. & Reddel, R. R. 1997, "Telomere dynamics and telomerase activity in in vitro immortalised human cells", *Eur. J. Cancer*, vol. 33, no. 5, pp. 767-773.

Bryan, T. M., Goodrich, K. J., & Cech, T. R. 2000, "A mutant of Tetrahymena telomerase reverse transcriptase with increased processivity", *J. Biol. Chem.*, vol. 275, no. 31, pp. 24199-24207.

Buckner, J. C. 2003, "Factors influencing survival in high-grade gliomas", *Semin. Oncol.*, vol. 30, no. 6 Suppl 19, pp. 10-14.

Burger, A. M., Dai, F., Schultes, C. M., Reszka, A. P., Moore, M. J., Double, J. A., & Neidle, S. 2005, "The G-quadruplex-interactive molecule BRACO-19 inhibits tumor growth, consistent with telomere targeting and interference with telomerase function", *Cancer Res*, vol. 65, no. 4, pp. 1489-1496.

Burnett, M. 1974, *Intrinsic Mutagenesis,* Medical and Technical Publishing Co., Lancaster.

Cabuy, E. & de Ridder, L. 2001, "Telomerase activity and expression of telomerase reverse transcriptase correlated with cell proliferation in meningiomas and malignant brain tumors in vivo", *Virchows Arch.*, vol. 439, no. 2, pp. 176-184.

Cairncross, J. G., Ueki, K., Zlatescu, M. C., Lisle, D. K., Finkelstein, D. M., Hammond, R. R., Silver, J. S., Stark, P. C., Macdonald, D. R., Ino, Y., Ramsay, D. A., & Louis, D. N. 1998, "Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas", *J. Natl. Cancer Inst.*, vol. 90, no. 19, pp. 1473-1479.

Cao, Y., Li, H., Deb, S., & Liu, J. P. 2002, "TERT regulates cell survival independent of telomerase enzymatic activity", *Oncogene*, vol. 21, no. 20, pp. 3130-3138.

Caporaso, G. L., Lim, D. A., Alvarez-Buylla, A., & Chao, M. V. 2003, "Telomerase activity in the subventricular zone of adult mice", *Mol. Cell Neurosci.*, vol. 23, no. 4, pp. 693-702.

Carroll, T., Maltby, E., Brock, I., Royds, J., Timperley, W., & Jellinek, D. 1999, "Meningiomas, dicentric chromosomes, gliomas, and telomerase activity", *J. Pathol.*, vol. 188, no. 4, pp. 395-399.

Chapon, C., Cech, T. R., & Zaug, A. J. 1997, "Polyadenylation of telomerase RNA in budding yeast", *RNA.*, vol. 3, no. 11, pp. 1337-1351.

Chen, J. L., Blasco, M. A., & Greider, C. W. 2000, "Secondary structure of vertebrate telomerase RNA", *Cell*, vol. 100, no. 5, pp. 503-514.

Chen, J. L., Opperman, K. K., & Greider, C. W. 2002, "A critical stem-loop structure in the CR4-CR5 domain of mammalian telomerase RNA", *Nucleic Acids Res.*, vol. 30, no. 2, pp. 592-597.

Chen, J. L. & Greider, C. W. 2003, "Determinants in mammalian telomerase RNA that mediate enzyme processivity and cross-species incompatibility", *EMBO J.*, vol. 22, no. 2, pp. 304-314.

Cherry, J. M. & Blackburn, E. H. 1985, "The internally located telomeric sequences in the germ-line chromosomes of Tetrahymena are at the ends of transposon-like elements", *Cell*, vol. 43, no. 3 Pt 2, pp. 747-758.

Cheung, A. N., Chiu, P. M., Tsun, K. L., Khoo, U. S., Leung, B. S., & Ngan, H. Y. 2004, "Chromosome in situ hybridisation, Ki-67, and telomerase immunocytochemistry in liquid based cervical cytology", *J. Clin. Pathol.*, vol. 57, no. 7, pp. 721-727.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. 1979, "Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease", *Biochemistry*, vol. 18, no. 24, pp. 5294-5299.

Chomczynski, P. & Sacchi, N. 1987, "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction", *Anal. Biochem.*, vol. 162, no. 1, pp. 156-159.

Chong, E. Y., Pang, J. S., Ko, C. W., Poon, W. S., & Ng, H. K. 2000, "Telomere length and telomerase catalytic subunit expression in non-astrocytic gliomas", *Pathol. Res Pract*, vol. 196, no. 10, pp. 691-699.

Colgin, L. M., Wilkinson, C., Englezou, A., Kilian, A., Robinson, M. O., & Reddel, R. R. 2000, "The hTERTalpha splice variant is a dominant negative inhibitor of telomerase activity", *Neoplasia*, vol. 2, no. 5, pp. 426-432.

Collins, K., Kobayashi, R., & Greider, C. W. 1995, "Purification of Tetrahymena telomerase and cloning of genes encoding the two protein components of the enzyme", *Cell*, vol. 81, no. 5, pp. 677-686.

Collins, K. & Greider, C. W. 1995, "Utilization of ribonucleotides and RNA primers by Tetrahymena telomerase", *EMBO J.*, vol. 14, no. 21, pp. 5422-5432.

Collins, K. & Mitchell, J. R. 2002, "Telomerase in the human organism", *Oncogene*, vol. 21, no. 4, pp. 564-579.

Condon, J., Yin, S., Mayhew, B., Word, R. A., Wright, W. E., Shay, J. W., & Rainey, W. E. 2002, "Telomerase immortalization of human myometrial cells", *Biol. Reprod.*, vol. 67, no. 2, pp. 506-514.

Cong, Y. S., Wen, J., & Bacchetti, S. 1999, "The human telomerase catalytic subunit hTERT: organisation of the gene and characterization of the promoter", *Hum. Mol. Genet.*, vol. 8, no. 1, pp. 137-142.

Coons, A., Creech, H., & Jones, R. 1941, "Immunological properties of an antibody containing a fluorescent group", *Proceedings Of The Society For Experimental Biology And Medicine*, pp. 200-202.

Cooper, G. M., Brudno, M., Green, E. D., Batzoglou, S., & Sidow, A. 2003, "Quantitative estimates of sequence divergence for comparative analyses of mammalian genomes", *Genome Res*, vol. 13, no. 5, pp. 813-820.

Coppes, M. J., Campbell, C. E., & Williams, B. R. 1993, "The role of WT1 in Wilms tumorigenesis", *FASEB J.*, vol. 7, no. 10, pp. 886-895.

Corey, D. R. 2002, "Telomerase inhibition, oligonucleotides, and clinical trials", *Oncogene*, vol. 21, no. 4, pp. 631-637.

Counter, C. M., Meyerson, M., Eaton, E. N., & Weinberg, R. A. 1997, "The catalytic subunit of yeast telomerase", *Proc. Natl. Acad. Sci. U.S.A*, vol. 94, no. 17, pp. 9202-9207.

Counter, C. M., Meyerson, M., Eaton, E. N., Ellisen, L. W., Caddle, S. D., Haber, D. A., & Weinberg, R. A. 1998a, "Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase", *Oncogene*, vol. 16, no. 9, pp. 1217-1222.

Counter, C. M., Hahn, W. C., Wei, W., Caddle, S. D., Beijersbergen, R. L., Lansdorp, P. M., Sedivy, J. M., & Weinberg, R. A. 1998b, "Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization", *Proc. Natl. Acad. Sci. U.S.A*, vol. 95, no. 25, pp. 14723-14728.

Cruet-Hennequart, S., Maubant, S., Luis, J., Gauduchon, P., Staedel, C., & Dedhar, S. 2003, "alpha(v) integrins regulate cell proliferation through integrin-linked kinase (ILK) in ovarian cancer cells", *Oncogene*, vol. 22, no. 11, pp. 1688-1702.

Damm, K., Hemmann, U., Garin-Chesa, P., Hauel, N., Kauffmann, I., Priepke, H., Niestroj, C., Daiber, C., Enenkel, B., Guilliard, B., Lauritsch, I., Muller, E., Pascolo, E., Sauter, G., Pantic, M., Martens, U. M., Wenz, C., Lingner, J., Kraut, N., Rettig, W. J., & Schnapp, A. 2001, "A highly selective telomerase inhibitor limiting human cancer cell proliferation", *EMBO J.*, vol. 20, no. 24, pp. 6958-6968.

Davies, B. R., Steele, I. A., Edmondson, R. J., Zwolinski, S. A., Saretzki, G., von Zglinicki, T., & O'Hare, M. J. 2003, "Immortalisation of human ovarian surface epithelium with telomerase and temperature-sensitive SV40 large T antigen", *Exp. Cell Res.*, vol. 288, no. 2, pp. 390-402.

de Bruin, D., Lanzer, M., & Ravetch, J. V. 1994, "The polymorphic subtelomeric regions of Plasmodium falciparum chromosomes contain arrays of repetitive sequence elements", *Proc. Natl. Acad. Sci. U.S.A*, vol. 91, no. 2, pp. 619-623.

de Kok, J. B., Schalken, J. A., Aalders, T. W., Ruers, T. J., Willems, H. L., & Swinkels, D. W. 2000, "Quantitative measurement of telomerase reverse transcriptase (hTERT) mRNA in urothelial cell carcinomas", *Int. J. Cancer*, vol. 87, no. 2, pp. 217-220.

de Lange, T., Shiue, L., Myers, R. M., Cox, D. R., Naylor, S. L., Killery, A. M., & Varmus, H. E. 1990, "Structure and variability of human chromosome ends", *Mol. Cell Biol.*, vol. 10, no. 2, pp. 518-527.

de Lange, T. & Petrini, J. H. 2000, "A new connection at human telomeres: association of the Mre11 complex with TRF2", *Cold Spring Harb. Symp. Quant. Biol.*, vol. 65:265-73., pp. 265-273.

de Lange, T. 2002, "Protection of mammalian telomeres", *Oncogene*, vol. 21, no. 4, pp. 532-540.

de Wet, J. R., Wood, K. V., Helinski, D. R., & DeLuca, M. 1985, "Cloning of firefly luciferase cDNA and the expression of active luciferase in Escherichia coli", *Proc. Natl. Acad. Sci. U.S.A*, vol. 82, no. 23, pp. 7870-7873.

Del Bufalo, D., Rizzo, A., Trisciuoglio, D., Cardinali, G., Torrisi, M. R., Zangemeiser-Wittke, U., Zupi, G., & Biroccio, A. 2005, "Involvement of hTERT in apoptosis induceed by interference with Bcl-2 expression and function", *Cell Death and Differentiation* pp. 1-10.

Delany, M. E., Krupkin, A. B., & Miller, M. M. 2000, "Organization of telomere sequences in birds: evidence for arrays of extreme length and for in vivo shortening", *Cytogenet. Cell Genet.*, vol. 90, no. 1-2, pp. 139-145.

Delany, M. E. & Daniels, L. M. 2004, "The chicken telomerase reverse transcriptase (chTERT): molecular and cytogenetic characterization with a comparative analysis", *Gene*, vol. 339:61-9., pp. 61-69.

DePinho, R. A. 2000, "The age of cancer", *Nature*, vol. 408, no. 6809, pp. 248-254.

Dessain, S. K., Yu, H., Reddel, R. R., Beijersbergen, R. L., & Weinberg, R. A. 2000, "Methylation of the human telomerase gene CpG island", *Cancer Res.*, vol. 60, no. 3, pp. 537-541.

Devereux, T. R., Horikawa, I., Anna, C. H., Annab, L. A., Afshari, C. A., & Barrett, J. C. 1999, "DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene", *Cancer Res.*, vol. 59, no. 24, pp. 6087-6090.

Dimski, D. S. & Cook, J. R. 1990, "Carmustine induced partial remission of an astrocytoma in a dog", *J. Am. Anim. Hosp. Assoc.*, vol. 26, pp. 179-182.

Dome, J. S., Chung, S., Bergemann, T., Umbricht, C. B., Saji, M., Carey, L. A., Grundy, P. E., Perlman, E. J., Breslow, N. E., & Sukumar, S. 1999, "High telomerase reverse transcriptase (hTERT) messenger RNA level correlates with tumor recurrence in patients with favorable histology Wilms' tumor", *Cancer Res.*, vol. 59, no. 17, pp. 4301-4307.

du, M. S., Guillaud, P., Camus, E., Seigneurin, D., & Brugal, G. 1991, "Ki-67 labeling in postmitotic cells defines different Ki-67 pathways within the 2c compartment", *Cytometry*, vol. 12, no. 5, pp. 455-463.

Duan, W., Rangan, A., Vankayalapati, H., Kim, M. Y., Zeng, Q., Sun, D., Han, H., Fedoroff, O. Y., Nishioka, D., Rha, S. Y., Izbicka, E., Von Hoff, D. D., & Hurley, L. H. 2001, "Design and synthesis of fluoroquinophenoxazines that interact with human telomeric G-quadruplexes and their biological effects", *Mol. Cancer Ther.*, vol. 1, no. 2, pp. 103-120.

Ducrest, A. L., Amacker, M., Mathieu, Y. D., Cuthbert, A. P., Trott, D. A., Newbold, R. F., Nabholz, M., & Lingner, J. 2001, "Regulation of human telomerase activity: repression by normal chromosome 3 abolishes nuclear telomerase reverse transcriptase transcripts but does not affect c-Myc activity", *Cancer Res*, vol. 61, no. 20, pp. 7594-7602.

Dugan, S. J., Schwarz, P. D., Roberts, S. M., & Ching, S. V. 1993, "Primary optic nerve meningioma and pulmonary metastasis in a dog", *J. Am. Anim. Hosp. Assoc.*, vol. 29, pp. 11-14.

Dunn, K. A., Thomas, R., Binns, M. M., & Breen, M. 2000, "Comparative genomic hybridization (CGH) in dogs--application to the study of a canine glial tumour cell line", *Vet. J.*, vol. 160, no. 1, pp. 77-82.

Elayadi, A. N., Demieville, A., Wancewicz, E. V., Monia, B. P., & Corey, D. R. 2001, "Inhibition of telomerase by 2'-O-(2-methoxyethyl) RNA oligomers: effect of length, phosphorothioate substitution and time inside cells", *Nucleic Acids Res*, vol. 29, no. 8, pp. 1683-1689.

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., & Tuschl, T. 2001a, "Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate", *EMBO J.*, vol. 20, no. 23, pp. 6877-6888.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. 2001b, "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells", *Nature*, vol. 411, no. 6836, pp. 494-498.

Eliceiri, B. P. & Cheresh, D. A. 2001, "Adhesion events in angiogenesis", *Curr. Opin. Cell Biol.*, vol. 13, no. 5, pp. 563-568.

Engelhard, H. H., Stelea, A., & Cochran, E. J. 2002, "Oligodendroglioma: pathology and molecular biology", *Surg. Neurol.*, vol. 58, pp. 111-117.

Englert, C. 1998, "WT1--more than a transcription factor?", *Trends Biochem. Sci.*, vol. 23, no. 10, pp. 389-393.

Evans, S. M., Dayrell-Hart, B., Powlis, W., Christy, G., & VanWinkle, T. 1993, "Radiation therapy of canine brain masses.", *J. Vet. Intern. Med.*, vol. 7, no. 4, pp. 216-219.

Falchetti, M. L., Pallini, R., D'Ambrosio, E., Pierconti, F., Martini, M., Cimino-Reale, G., Verna, R., Maira, G., & Larocca, L. M. 2000, "In situ detection of telomerase catalytic subunit mRNA in glioblastoma multiforme", *Int. J Cancer*, vol. 88, no. 6, pp. 895-901.

Falchetti, M. L., Larocca, L. M., & Pallini, R. 2002, "Telomerase in brain tumors", *Child's Nervous System*, vol. 18, pp. 112-117.

Falchetti, M. L., Pierconti, F., Casalbore, P., Maggiano, N., Levi, A., Larocca, L. M., & Pallini, R. 2003, "Glioblastoma induces vascular endothelial cells to express telomerase in vitro", *Cancer Res*, vol. 63, no. 13, pp. 3750-3754.

Farwell, D. G., Shera, K. A., Koop, J. I., Bonnet, G. A., Matthews, C. P., Reuther, G. W., Coltrera, M. D., McDougall, J. K., & Klingelhutz, A. J. 2000, "Genetic and epigenetic changes in human epithelial cells immortalized by telomerase", *Am. J. Pathol.*, vol. 156, no. 5, pp. 1537-1547.

Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., & . 1995, "The RNA component of human telomerase", *Science*, vol. 269, no. 5228, pp. 1236-1241.

Fiset, S. & Chabot, B. 2001, "hnRNP A1 may interact simultaneously with telomeric DNA and the human telomerase RNA in vitro", *Nucleic Acids Res*, vol. 29, no. 11, pp. 2268-2275.

Fiveash, J. B. & Spencer, S. A. 2003, "Role of radiation therapy and radiosurgery in glioblastoma multiforme", *Cancer J.*, vol. 9, no. 3, pp. 222-229.

Ford, L. P., Suh, J. M., Wright, W. E., & Shay, J. W. 2000, "Heterogeneous nuclear ribonucleoproteins C1 and C2 associate with the RNA component of human telomerase", *Mol. Cell Biol.*, vol. 20, no. 23, pp. 9084-9091.

Ford, L. P., Shay, J. W., & Wright, W. E. 2001, "The La antigen associates with the human telomerase ribonucleoprotein and influences telomere length in vivo", *RNA.*, vol. 7, no. 8, pp. 1068-1075.

Ford, L. P., Wright, W. E., & Shay, J. W. 2002, "A model for heterogeneous nuclear ribonucleoproteins in telomere and telomerase regulation", *Oncogene*, vol. 21, no. 4, pp. 580-583.

Forsyth, N. R., Wright, W. E., & Shay, J. W. 2002, "Telomerase and differentiation in multicellular orgnanisms: Turn it off, turn it on, and turn it off again", *Differentiation*, vol. 69, pp. 188-197.

Foster, S. A., Wong, D. J., Barrett, M. T., & Galloway, D. A. 1998, "Inactivation of p16 in human mammary epithelial cells by CpG island methylation", *Mol. Cell Biol.*, vol. 18, no. 4, pp. 1793-1801.

Fournel-Fleury, C., Magnol, J. P., Chabanne, L., Ghernati, I., Marchal, T., Bonnefond, C., Bryon, P. A., & Felman, P. 1997, "Growth fractions in canine non-Hodgkin's lymphomas as determined in situ by the expression of the Ki-67 antigen", *J. Comp. Pathol.*, vol. 117, no. 1, pp. 61-72.

Fradiani, P. A., Ascenzioni, F., Lavitrano, M., & Donini, P. 2004, "Telomeres and telomerase activity in pig tissues", *Biochimie*, vol. 86, pp. 7-12.

Friedman, K. L. & Cech, T. R. 1999, "Essential functions of amino-terminal domains in the yeast telomerase catalytic subunit revealed by selection for viable mutants", *Genes Dev.*, vol. 13, no. 21, pp. 2863-2874.

Fu, W., Killen, M., Culmsee, C., Dhar, S., Pandita, T. K., & Mattson, M. P. 2000, "The catalytic subunit of telomerase is expressed in developing brain neurons and serves a cell survival-promoting function", *J. Mol. Neurosci.*, vol. 14, no. 1-2, pp. 3-15.

Fujimoto, K., Kyo, S., Takakura, M., Kanaya, T., Kitagawa, Y., Itoh, H., Takahashi, M., & Inoue, M. 2000, "Identification and characterization of negative regulatory elements of the human telomerase catalytic subunit (hTERT) gene promoter: possible role of MZF-2 in transcriptional repression of hTERT", *Nucleic Acids Res*, vol. 28, no. 13, pp. 2557-2562.

Fukushima, T., Yoshino, A., Katayama, Y., Watanabe, T., Kusama, K., & Moro, I. 2002, "Prediction of clinical course of diffusely infiltrating astrocytomas from telomerase expression and quantitated activity level", *Cancer Lett.*, vol. 187, no. 1-2, pp. 191-198.

Fukushima, M., Shimomura, N., Nakamura, K., Kammori, M., Koizumi, K., Shimizu, K., & Takubo, K. 2004, "Demonstration of human telomerase reverse transcriptase by in situ hybridization in lung carcinoma", *Oncol. Rep.*, vol. 12, no. 6, pp. 1227-1232.

Fulton, L. M. & Steinberg, H. S. 1999, "Preliminary Study of Lomustine in the Treatment of Intracranial Masses in Dogs Following Localization by Imaging Techniques", *Semin. Vet. Med. Surg. (Small Anim.)*, vol. 5, no. 4, pp. 241-245.

Gao, X. & Huang, L. 1995, "Cationic liposome-mediated gene transfer", *Gene Ther.*, vol. 2, no. 10, pp. 710-722.

Gavin, P. R., Fike, J. R., & Hoopes, P. J. 1995, "Central Nervous System Tumors", *Semin. Vet. Med. Surg. (Small Anim.)*, vol. 10, no. 3, pp. 180-189.

Gerdes, J., Lemke, H., Baisch, H., Wacker, H. H., Schwab, U., & Stein, H. 1984, "Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67", *J. Immunol.*, vol. 133, no. 4, pp. 1710-1715.

Gilson, E., Laroche, T., & Gasser, S. M. 1993, "Telomeres and the functional architecture of the nucleus", *Trends Cell Biol.*, vol. 3, no. 4, pp. 128-134.

Gomez, D., Aouali, N., Renaud, A., Douarre, C., Shin-Ya, K., Tazi, J., Martinez, S., Trentesaux, C., Morjani, H., & Riou, J. F. 2003, "Resistance to senescence induction and telomere shortening by a G-quadruplex ligand inhibitor of telomerase", *Cancer Res*, vol. 63, no. 19, pp. 6149-6153.

Gonzalez-Suarez, E., Samper, E., Flores, J. M., & Blasco, M. A. 2000, "Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis", *Nat. Genet.*, vol. 26, no. 1, pp. 114-117.

Gonzalez-Suarez, E., Samper, E., Ramirez, A., Flores, J. M., Martin-Caballero, J., Jorcano, J. L., & Blasco, M. A. 2001, "Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes", *EMBO J.*, vol. 20, no. 11, pp. 2619-2630.

Goossens, M. M., Feldman, E. C., Nelson, R. W., Theon, A. P., Koblik, P. D., Elliott, D. A., & Rutteman, G. R. 1998, "Cobalt 60 irradiation of pituitary gland tumors in three cats with acromegaly", *J. A.m Vet. Med. Assoc*, vol. 213, no. 3, pp. 374-376.

Gottschling, D. E., Aparicio, O. M., Billington, B. L., & Zakian, V. A. 1990, "Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription", *Cell*, vol. 63, no. 4, pp. 751-762.

Gowan, S. M., Heald, R., Stevens, M. F., & Kelland, L. R. 2001, "Potent inhibition of telomerase by small-molecule pentacyclic acridines capable of interacting with G-quadruplexes", *Mol. Pharmacol.*, vol. 60, no. 5, pp. 981-988.

Greco, O., Marples, B., Dachs, G. U., Williams, K. J., Patterson, A. V., & Scott, S. D. 2002, "Novel chimeric gene promoters responsive to hypoxia and ionizing radiation", *Gene Ther.*, vol. 9, no. 20, pp. 1403-1411.

Greenberg, R. A., O'Hagan, R. C., Deng, H., Xiao, Q., Hann, S. R., Adams, R. R., Lichtsteiner, S., Chin, L., Morin, G. B., & DePinho, R. A. 1999, "Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation", *Oncogene*, vol. 18, no. 5, pp. 1219-1226.

Greenwood, M. J. & Lansdorp, P. M. 2003, "Telomeres, telomerase, and hematopoietic stem cell biology", *Arch. Med. Res*, vol. 34, no. 6, pp. 489-495.

Greider, C. W. & Blackburn, E. H. 1985, "Identification of a specific telomere terminal transferase activity in Tetrahymena extracts", *Cell*, vol. 43, no. 2 Pt 1, pp. 405-413.

Greider, C. W. & Blackburn, E. H. 1989, "A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis", *Nature*, vol. 337, no. 6205, pp. 331-337.

Greider, C. W. 1991, "Telomerase is processive", *Mol. Cell Biol.*, vol. 11, no. 9, pp. 4572-4580.

Griffey, S. M., Kraegel, S. A., & Madewell, B. R. 1999, "Proliferation indices in spontaneous canine lung cancer: proliferating cell nuclear antigen (PCNA), Ki-67 (MIB1) and mitotic counts", *J. Comp. Pathol.*, vol. 120, no. 4, pp. 321-332.

Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H., & de Lange, T. 1999, "Mammalian telomeres end in a large duplex loop", *Cell*, vol. 97, no. 4, pp. 503-514.

Gronthos, S., Zannettino, A. C., Hay, S. J., Shi, S., Graves, S. E., Kortesidis, A., & Simmons, P. J. 2003, "Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow", *J. Cell Sci.*, vol. 116, no. Pt 9, pp. 1827-1835.

Gu, J., Kagawa, S., Takakura, M., Kyo, S., Inoue, M., Roth, J. A., & Fang, B. 2000, "Tumor-specific transgene expression from the human telomerase reverse transcriptase promoter enables targeting of the therapeutic effects of the Bax gene to cancers", *Cancer Res.*, vol. 60, no. 19, pp. 5359-5364.

Guiducci, C., Cerone, M. A., & Bacchetti, S. 2001, "Expression of mutant telomerase in immortal telomerase-negative human cells results in cell cycle deregulation, nuclear and chromosomal abnormalities and rapid loss of viability", *Oncogene*, vol. 20, no. 6, pp. 714-725.

Guilleret, I., Yan, P., Grange, F., Braunschweig, R., Bosman, F. T., & Benhattar, J. 2002, "Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity", *Int. J. Cancer*, vol. 101, no. 4, pp. 335-341.

Guilleret, I. & Benhattar, J. 2003, "Demethylation of the human telomerase catalytic subunit (hTERT) gene promoter reduced hTERT expression and telomerase activity and shortened telomeres", *Exp. Cell Res*, vol. 289, no. 2, pp. 326-334.

Gunes, C., Lichtsteiner, S., Vasserot, A. P., & Englert, C. 2000, "Expression of the hTERT gene is regulated at the level of transcriptional initiation and repressed by Mad1", *Cancer Res*, vol. 60, no. 8, pp. 2116-2121.

Gunther, T., Schneider-Stock, R., Hackel, C., Pross, M., Schulz, H. U., Lippert, H., & Roessner, A. 2000, "Telomerase activity and expression of hTRT and hTR in gastrointestinal stromal tumors in comparison with extragastrointestinal sarcomas", *Clin. Cancer Res*, vol. 6, no. 5, pp. 1811-1818.

Guo, Y., Liu, J., Li, Y. H., Song, T. B., Wu, J., Zheng, C. X., & Xue, C. F. 2005, "Effect of vector-expressed shRNAs on hTERT expression", *World J.Gastroenterol.*, vol. 11, no. 19, pp. 2912-2915.

Guyon, R., Lorentzen, T. D., Hitte, C., Kim, L., Cadieu, E., Parker, H., Quignon, P., Lowe, J. K., Renier, C., Gelfenbeyn, B., Vignaux, F., DeFrance, H. B., Gloux, S., Mahairas, G. G., Andre, C., Galibert, F., & Ostrandr, E. A. 2003, "A 1-Mb resolution radiation hybrid map of the canine genome", *Proc. Natl. Acad. Sci. U.S.A*, vol. 100, no. 9, pp. 5296-5301.

Hahn, W. C., Stewart, S. A., Brooks, M. W., York, S. G., Eaton, E., Kurachi, A., Beijersbergen, R. L., Knoll, J. H., Meyerson, M., & Weinberg, R. A. 1999a, "Inhibition of telomerase limits the growth of human cancer cells", *Nat. Med*, vol. 5, no. 10, pp. 1164-1170.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., & Weinberg, R. A. 1999b, "Creation of human tumour cells with defined genetic elements", *Nature*, vol. 400, no. 6743, pp. 464-468.

Hakin-Smith, V., Jellinek, D. A., Levy, D., Carroll, T., Teo, M., Timperley, W. R., McKay, M. J., Reddel, R. R., & Royds, J. A. 2003, "Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme", *Lancet*, vol. 361, no. 9360, pp. 836-838.

Hall, M. E., Nasir, L., Daunt, F., Gault, E. A., Croxall, J. P., Wanless, S., & Monaghan, P. 2004, "Telomere loss in relation to age and early environment in long-lived birds", *Proc. Biol. Sci.*, vol. 271, no. 1548, pp. 1571-1576.

Halvorsen, T. L., Leibowitz, G., & Levine, F. 1999, "Telomerase activity is sufficient to allow transformed cells to escape from crisis", *Mol. Cell Biol.*, vol. 19, no. 3, pp. 1864-1870.

Hammond, P. W. & Cech, T. R. 1997, "dGTP-dependent processivity and possible template switching of *Euplotes* telomerase", *Nucleic Acids Res*, vol. 25, no. 18, pp. 3698-3704.

Hammond, P. W., Lively, T. N., & Cech, T. R. 1997, "The anchor site of telomerase from *Euplotes aediculatus* revealed by photo-cross-linking to single- and double-stranded DNA primers", *Mol. Cell Biol.*, vol. 17, no. 1, pp. 296-308.

Hammond, P. W. & Cech, T. R. 1998, "Euplotes telomerase: evidence for limited base-pairing during primer elongation and dGTP as an effector of translocation", *Biochemistry*, vol. 37, no. 15, pp. 5162-5172.

Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., & Hannon, G. J. 2001, "Argonaute2, a link between genetic and biochemical analyses of RNAi", *Science*, vol. 293, no. 5532, pp. 1146-1150.

Hanahan, D. & Weinberg, R. A. 2000, "The hallmarks of cancer", *Cell*, vol. 100, pp. 57-70.

Hannon, G. J. 2002, "RNA interference", *Nature*, vol. 418, no. 6894, pp. 244-251.

Hansen, K. & Khanna, C. 2004, "Spontaneous and genetically engineered animal models: use in preclinical cancer drug development", *Eur. J. Cancer*, vol. 40, pp. 858-880.

Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., & Peters, G. 1996, "Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence", *Mol. Cell Biol.*, vol. 16, no. 3, pp. 859-867.

Harada, K., Kazunori, A., Kurisu, K., & Tahara, E. 2000a, "Telomerase activity and the expression of telomerase components in pituitary adenoma with malignant transformation", *Surg. Neurol.*, vol. 53, pp. 267-274.

Harada, K., Kurisu, K., Tahara, H., Tahara, E., & Ide, T. 2000b, "Telomerase activity in primary and secondary glioblastomas multiforme as a novel molecular tumor marker", *J. Neurosurg.*, vol. 93, no. 4, pp. 618-625.

Harada, K., Kurisu, K., Sadatomo, T., Tahara, H., Tahara, E., Ide, T., & Tahara, E. 2000c, "Growth inhibition of human glioma cells by transfection-induced P21 and its effects on telomerase activity", *J. Neurooncol.*, vol. 47, no. 1, pp. 39-46.

Harley, C. B., Futcher, A. B., & Greider, C. W. 1990, "Telomeres shorten during ageing of human fibroblasts", *Nature*, vol. 345, no. 6274, pp. 458-460.

Harrington, L., Zhou, W., McPhail, T., Oulton, R., Yeung, D. S., Mar, V., Bass, M. B., & Robinson, M. O. 1997, "Human telomerase contains evolutionarily conserved catalytic and structural subunits", *Genes Dev.*, vol. 11, no. 23, pp. 3109-3115.

Harrington, K. J., Linardakis, E., & Vile, R. G. 2000, "Transcriptional control: an essential component of cancer gene therapy strategies?", *Adv. Drug Deliv. Rev.*, vol. 44, no. 2-3, pp. 167-184.

Harrington, L. 2003, "Biochemical aspects of telomerase function", *Cancer Lett.*, vol. 194, no. 2, pp. 139-154.

Harrington, L. 2004, "Does the reservoir for self-renewal stem from the ends?", *Oncogene*, vol. 23, no. 43, pp. 7283-7289.

Harrison, R. J., Gowan, S. M., Kelland, L. R., & Neidle, S. 1999, "Human telomerase inhibition by substituted acridine derivatives", *Bioorg. Med. Chem. Lett.*, vol. 9, no. 17, pp. 2463-2468.

Hartson, S. D. & Matts, R. L. 1994, "Association of Hsp90 with cellular Src-family kinases in a cell-free system correlates with altered kinase structure and function", *Biochemistry*, vol. 33, no. 30, pp. 8912-8920.

Hastie, N. D., Dempster, M., Dunlop, M. G., Thompson, A. M., Green, D. K., & Allshire, R. C. 1990, "Telomere reduction in human colorectal carcinoma and with ageing", *Nature*, vol. 346, no. 6287, pp. 866-868.

Hayflick, L. & Moorhead, P. S. 1961, "The serial cultivation of human diploid cell strains", *Exp. Cell Res*, vol. 25:585-621., pp. 585-621.

Hayflick, L. 1965, "The limited in vitro lifetime of human diploid cell strains", *Exp. Cell Res*, vol. 37:614-36., pp. 614-636.

Hayflick, L. 1998, "How and why we age", *Exp. Gerontol.*, vol. 33, no. 7-8, pp. 639-653.

Heidner, G. L., Kornegay, J. N., Page, R. L., Dodge, R. K., & Thrall, D. 1991, "Analysis of survival in a retrospective study of 86 dogs with brain tumors", *J. Vet. Int. Med.*, vol. 5, no. 4, pp. 219-226.

Helman, R. G., Adams, L. G., Hall, C. L., & Read, W. K. 1980, "Metastatic neuroblastoma in a dog", *Vet. Pathol.*, vol. 17, no. 6, pp. 769-773.

Hemann, M. T., Strong, M. A., Hao, L. Y., & Greider, C. W. 2001, "The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability", *Cell*, vol. 107, no. 1, pp. 67-77.

Henderson, E. R. & Blackburn, E. H. 1989, "An overhanging 3' terminus is a conserved feature of telomeres", *Mol. Cell Biol.*, vol. 9, no. 1, pp. 345-348.

Henson, J. D., Neumann, A. A., Yeager, T. R., & Reddel, R. R. 2002, "Alternative lengthening of telomeres in mammalian cells", *Oncogene*, vol. 21, no. 4, pp. 598-610.

Henson, J. W., Gaviani, P., & Gonzalez, R. G. 2005, "MRI in treatment of adult gliomas", *Lancet Oncol.*, vol. 6, no. 3, pp. 167-175.

Herbert, B., Pitts, A. E., Baker, S. I., Hamilton, S. E., Wright, W. E., Shay, J. W., & Corey, D. R. 1999, "Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death", *Proc. Natl. Acad .Sci. U.S.A*, vol. 96, no. 25, pp. 14276-14281.

Herbert, B. S., Pongracz, K., Shay, J. W., Gryaznov, S. M., & Shea-Herbert, B. 2002, "Oligonucleotide N3'-->P5' phosphoramidates as efficient telomerase inhibitors", *Oncogene* , vol. 21, no. 4, pp. 638-642.

Herbig, U., Jobling, W. A., Chen, B. P., Chen, D. J., & Sedivy, J. M. 2004, "Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a)", *Mol. Cell*, vol. 14, no. 4, pp. 501-513.

Herman, J. G. 1999, "Hypermethylation of tumor suppressor genes in cancer", *Semin. Cancer Biol.*, vol. 9, no. 5, pp. 359-367.

Hiraga, S., Ohnishi, T., Izumoto, S., Miyahara, E., Kanemura, Y., Matsumura, H., & Arita, N. 1998, "Telomerase activity and alterations in telomere length in human brain tumors", *Cancer Res*, vol. 58, no. 10, pp. 2117-2125.

Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M. A., Shay, J. W., Ishioka, S., & Yamakido, M. 1995, "Activation of telomerase in human lymphocytes and hematopoietic progenitor cells", *J. Immunol.*, vol. 155, no. 8, pp. 3711-3715.

Hiyama, E., Hiyama, K., Yokoyama, T., & Shay, J. W. 2001, "Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues", *Neoplasia.*, vol. 3, no. 1, pp. 17-26.

Hiyama, E. & Hiyama, K. 2002, "Clinical utility of telomerase in cancer", *Oncogene*, vol. 21, no. 4, pp. 643-649.

Hiyama, E., Hiyama, K., Nishizaki, M., Reynolds, C. P., Shay, J. W., & Yokoyama, T. 2003, "Differential gene expression profiles between neuroblastomas with high telomerase activity and low telomerase activity", *J. Ped. Surg.*, vol. 38, no. 12, pp. 1730-17.

Ho, D. M., Wong, T. T., Hsu, C. Y., Ting, L. T., & Chiang, H. 1998, "MIB-1 labeling index in nonpilocytic astrocytoma of childhood: a study of 101 cases", *Cancer*, vol. 82, no. 12, pp. 2459-2466.

Ho, D. M., Hsu, C. Y., Ting, L. T., & Chiang, H. 2002, "Histopathology and MIB-1 labeling index predicted recurrence of meningiomas: a proposal of diagnostic criteria for patients with atypical meningioma", *Cancer*, vol. 94, no. 5, pp. 1538-1547.

Hogeveen, K. N., Talikka, M., & Hammond, G. L. 2001, "Human sex hormone-binding globulin promoter activity is influenced by a (TAAAA)n repeat element within an Alu sequence", *J. Biol. Chem.*, vol. 276, no. 39, pp. 36383-36390.

Holliday, R. 1996, "Endless quest", *Bioessays*, vol. 18, no. 1, pp. 3-5.

Holt, S. E., Aisner, D. L., Shay, J. W., & Wright, W. E. 1997, "Lack of cell cycle regulation of telomerase activity in human cells", *Proc. Natl. Acad. Sci. U.S.A*, vol. 94, pp. 10687-10692.

Holt, S. E., Aisner, D. L., Baur, J., Tesmer, V. M., Dy, M., Ouellette, M., Trager, J. B., Morin, G. B., Toft, D. O., Shay, J. W., Wright, W. E., & White, M. A. 1999, "Functional requirement of p23 and Hsp90 in telomerase complexes", *Genes Dev.*, vol. 13, no. 7, pp. 817-826.

Homburg, C. H., de Haas, M., dem Borne, A. E., Verhoeven, A. J., Reutelingsperger, C. P., & Roos, D. 1995, "Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro", *Blood*, vol. 85, no. 2, pp. 532-540.

Horikawa, I., Cable, P. L., Afshari, C. A., & Barrett, J. C. 1999, "Cloning and characterisation of the promoter region of human telomerase reverse transcriptase gene", *Cancer Res*, vol. 59, no. 3, pp. 826-830.

Horikawa, I. & Barrett, J. C. 2001, "cis-Activation of the human telomerase gene (hTERT) by the hepatitis B virus genome", *J. Natl. Cancer Inst.*, vol. 93, no. 15, pp. 1171-1173.

Horikawa, I., Cable, P. L., Mazur, S. J., Appella, E., Afshari, C. A., & Barrett, J. C. 2002, "Downstream E-Box-mediated Regulation of the Human Telomerase Reverse Transcriptase (hTERT) Gene Transcription: Evidence for an Endogenous Mechanism of Transcriptional Repression", *Mol. Biol. Cell*, vol. 13, no. 8, pp. 2585-2597.

Hsu, D. W., Efird, J. T., & Hedley-Whyte, E. T. 1998, "MIB-1 (Ki-67) index and transforming growth factor-alpha (TGF alpha) immunoreactivity are significant prognostic predictors for meningiomas", *Neuropathol. Appl. Neurobiol.*, vol. 24, no. 6, pp. 441-452.

Huang, T. G., Savontaus, M. J., Shinozaki, K., Sauter, B. V., & Woo, S. L. 2003, "Telomerase-dependent oncolytic adenovirus for cancer treatment", *Gene Ther.*, vol. 10, no. 15, pp. 1241-1247.

Huffman, K. E., Levene, S. D., Tesmer, V. M., Shay, J. W., & Wright, W. E. 2000, "Telomere shortening is proportional to the size of the G-rich telomeric 3'-overhang", *J. Biol. Chem.*, vol. 275, no. 26, pp. 19719-19722.

Hutvagner, G. & Zamore, P. D. 2002, "RNAi: nature abhors a double-strand", *Curr. Opin. Genet. Dev.*, vol. 12, no. 2, pp. 225-232.

Ijdo, J. W., Baldini, A., Ward, D. C., Reeders, S. T., & Wells, R. A. 1991, "Origin of human chromosome 2: an ancestral telomere-telomere fusion", *Proc. Natl. Acad. Sci. U.S.A*, vol. 88, no. 20, pp. 9051-9055.

Ikeda, S., Shibata, T., Eishi, Y., Takizawa, T., & Koike, M. 2003, "Correlation between the expression of telomerase reverse transcriptase and proliferative activity in breast cancer cells using an immunocytochemical restaining method", *Pathol. Int.*, vol. 53, no. 11, pp. 762-768.

Incles, C. M., Schultes, C. M., Kempski, H., Koehler, H., Kelland, L. R., & Neidle, S. 2004, "A G-quadruplex telomere targeting agent produces p16-associated senescence and chromosomal fusions in human prostate cancer cells", *Mol. Cancer Ther.*, vol. 3, no. 10, pp. 1201-1206.

Innis, M. A., Myambo, K. B., Gelfand, D. H., & Brow, M. A. 1988, "DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA", *Proc .Natl. Acad. Sci. U.S.A*, vol. 85, no. 24, pp. 9436-9440.

Innis, M. A. & Gelfand, D. H. 1990, *PCR protocols. A guide to methods and applications* Academic Press, San Diego, CA.

Isobe, K., Yashiro, T., Omura, S., Kaneko, M., Kaneko, S., Kamma, H., Tatsuno, I., Takekoshi, K., Kawakami, Y., & Nakai, T. 2004, "Expression of the human telomerase reverse transcriptase in pheochromocytomoa and neuroblastoma tissues", *Endocrine J.*, vol. 51, no. 1, pp. 47-52.

Jacob, D., Davis, J., Zhu, H., Zhang, L., Teraishi, F., Wu, S., Marini, F. C., III, & Fang, B. 2004, "Suppressing orthotopic pancreatic tumor growth with a fiber-modified adenovector expressing the TRAIL gene from the human telomerase reverse transcriptase promoter", *Clin. Cancer Res*, vol. 10, no. 10, pp. 3535-3541.

Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., & . 1993, "Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA", *Proc. Natl. Acad. Sci. U.S.A*, vol. 90, no. 13, pp. 6320-6324.

Jeffreys, A. J., Wilson, V., Neumann, R., & Keyte, J. 1988, "Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells", *Nucleic Acids Res*, vol. 16, no. 23, pp. 10953-10971.

Jiang, X. R., Jimenez, G., Chang, E., Frolkis, M., Kusler, B., Sage, M., Beeche, M., Bodnar, A. G., Wahl, G. M., Tlsty, T. D., & Chiu, C. P. 1999, "Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype", *Nat. Genet.*, vol. 21, no. 1, pp. 111-114.

Johnson, G. C. 1990, "Genesis and pathology of tumors of the nervous system", *Semin. Vet. Med. Surg. (Small Anim.)*, vol. 5, no. 4, pp. 210-222.

Kabanov, A. V. & Kabanov, V. A. 1995, "DNA complexes with polycations for the delivery of genetic material into cells", *Bioconjug. Chem.*, vol. 6, no. 1, pp. 7-20.

Kagawa, S., He, C., Gu, J., Koch, P., Rha, S. J., Roth, J. A., Curley, S. A., Stephens, L. C., & Fang, B. 2001, "Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene", *Cancer Res*, vol. 61, no. 8, pp. 3330-3338.

Kanaya, T., Kyo, S., Hamada, K., Takakura, M., Kitagawa, Y., Harada, H., & Inoue, M. 2000, "Adenoviral expression of p53 represses telomerase activity through down-regulation of human telomerase reverse transcriptase transcription", *Clin. Cancer Res*, vol. 6, no. 4, pp. 1239-1247.

Kanazawa, Y., Ohkawa, K., Ueda, K., Mita, E., Takehara, T., Sasaki, Y., Kasahara, A., & Hayashi, N. 1996, "Hammerhead ribozyme-mediated inhibition of telomerase activity in extracts of human hepatocellular carcinoma cells", *Biochem. Biophys. Res Commun.*, vol. 225, no. 2, pp. 570-576.

Kang, S. S., Kwon, T., Kwon, D. Y., & Do, S. I. 1999, "Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit", *J. Biol. Chem.*, vol. 274, no. 19, pp. 13085-13090.

Kang, H. J., Choi, Y. S., Hong, S. B., Kim, K. W., Woo, R. S., Won, S. J., Kim, E. J., Jeon, H. K., Jo, S. Y., Kim, T. K., Bachoo, R., Reynolds, I. J., Gwag, B. J., & Lee, H. W. 2004, "Ectopic expression of the catalytic subunit of telomerase protects against brain injury resulting from ischemia and NMDA-induced neurotoxicity", *J. Neurosci.*, vol. 24, no. 6, pp. 1280-1287.

Karlseder, J., Smogorzewska, A., & de Lange, T. 2002, "Senescence induced by altered telomere state, not telomere loss", *Science*, vol. 295, no. 5564, pp. 2446-2449.

Kawashima, T., Kagawa, S., Kobayashi, N., Shirakiya, Y., Umeoka, T., Teraishi, F., Taki, M., Kyo, S., Tanaka, N., & Fujiwara, T. 2004, "Telomerase-specific replication-selective virotherapy for human cancer", *Clin. Cancer Res*, vol. 10, no. 1 Pt 1, pp. 285-292.

Kelland, L. R. 2005, "Overcoming the immortality of tumour cells by telomere and telomerase based cancer therapeutics--current status and future prospects", *Eur. J. Cancer*, vol. 41, no. 7, pp. 971-979.

Keller, E. T. & Madewell, B. R. 1992, "Locations and types of neoplasms in immature dogs: 69 cases (1964-1989)", *J. Am. Vet. Med. Assoc.*, vol. 200, no. 10, pp. 1530-1532.

Kelsey, J. L., Moore, A. S., & Glickman, L. T. 1998, "Epidemiologic studies of risk factors for cancer in pet dogs", *Epidemiol. Rev.*, vol. 20, no. 2, pp. 204-217.

Khanna, C., Wan, X., Bose, S., Cassaday, R., Olomu, O., Mendoza, A., Yeung, C., Gorlick, R., Hewitt, S. M., & Helman, L. J. 2004, "The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis", *Nat. Med.*, vol. 10, no. 2, pp. 182-186.

Kharbanda, S., Kumar, V., Dhar, S., Pandey, P., Chen, C., Majumder, P., Yuan, Z. M., Whang, Y., Strauss, W., Pandita, T. K., Weaver, D., & Kufe, D. 2000, "Regulation of the hTERT telomerase catalytic subunit by the c-Abl tyrosine kinase", *Curr. Biol.*, vol. 10, no. 10, pp. 568-575.

Khatsenko, O., Morgan, R., Truong, L., York-Defalco, C., Sasmor, H., Conklin, B., & Geary, R. S. 2000, "Absorption of antisense oligonucleotides in rat intestine: effect of chemistry and length", *Antisense Nucleic Acid Drug Dev.*, vol. 10, no. 1, pp. 35-44.

Kickhoefer, V. A., Stephen, A. G., Harrington, L., Robinson, M. O., & Rome, L. H. 1999, "Vaults and telomerase share a common subunit, TEP1", *J. Biol. Chem.*, vol. 274, no. 46, pp. 32712-32717.

Kickhoefer, V. A., Liu, Y., Kong, L. B., Snow, B. E., Stewart, P. L., Harrington, L., & Rome, L. H. 2001, "The Telomerase/vault-associated protein TEP1 is required for vault RNA stability and its association with the vault particle", *J. Cell Biol.*, vol. 152, no. 1, pp. 157-164.

Kilian, A., Bowtell, D. D., Abud, H. E., Hime, G. R., Venter, D. J., Keese, P. K., Duncan, E. L., Reddel, R. R., & Jefferson, R. A. 1997, "Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types", *Hum. Mol. Genet.*, vol. 6, no. 12, pp. 2011-2019.

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., & Shay, J. W. 1994, "Specific association of human telomerase activity with immortal cells and cancer", *Science*, vol. 266, no. 5193, pp. 2011-2015.

Kim, N. W. & Wu, F. 1997, "Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP)", *Nucleic Acids Res.*, vol. 25, no. 13, pp. 2595-2597.

Kim, M. M., Rivera, M. A., Botchkina, I. L., Shalaby, R., Thor, A. D., & Blackburn, E. H. 2001, "A low threshold level of expression of mutant-template telomerase RNA inhibits human tumor cell proliferation", *Proc. Natl. Acad. Sci. U.S.A*, vol. 98, no. 14, pp. 7982-7987.

Kim, M. Y., Vankayalapati, H., Shin-Ya, K., Wierzba, K., & Hurley, L. H. 2002, "Telomestatin, a potent telomerase inhibitor that interacts quite specifically with the human telomeric intramolecular g-quadruplex", *J. Am. Chem. Soc.*, vol. 124, no. 10, pp. 2098-2099.

Kim, M. Y., Gleason-Guzman, M., Izbicka, E., Nishioka, D., & Hurley, L. H. 2003a, "The different biological effects of telomestatin and TMPyP4 can be attributed to their selectivity for interaction with intramolecular or intermolecular G-quadruplex structures", *Cancer Res*, vol. 63, no. 12, pp. 3247-3256.

Kim, E., Kim, J. H., Shin, H. Y., Lee, H., Yang, J. M., Kim, J., Sohn, J. H., Kim, H., & Yun, C. O. 2003b, "Ad-mTERT-delta19, a conditional replication-competent adenovirus driven by the human telomerase promoter, selectively replicates in and elicits cytopathic effect in a cancer cell-specific manner", *Hum. Gene Ther.*, vol. 14, no. 15, pp. 1415-1428.

Kinzler, K. W. & Vogelstein, B. 1996, "Lessons from hereditary colorectal cancer", *Cell*, vol. 87, no. 2, pp. 159-170.

Kipling, D. & Cooke, H. J. 1990, "Hypervariable ultra-long telomeres in mice", *Nature*, vol. 347, no. 6291, pp. 400-402.

Kipling, D. 1997, "Telomere structure and telomerase expression during mouse development and tumorigenesis", *Eur. J. Cancer*, vol. 33, no. 5, pp. 792-800.

Kipling, D. 2001, "Telomeres, replicative senescence and human ageing", *Maturitas*, vol. 38, no. 1, pp. 25-37.

Kirkness, E. F., Bafna, V., Halpern, A. L., Levy, S., Remington, K., Rusch, D. B., Delcher, A. L., Pop, M., Wang, W., Fraser, C. M., & Venter, J. C. 2003, "The dog genome: survey sequencing and comparative analysis", *Science*, vol. 301, no. 5641, pp. 1898-1903.

Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A., & Klingelhutz, A. J. 1998, "Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells", *Nature*, vol. 396, no. 6706, pp. 84-88.

Kleihues, P. & Cavenee, W. K. 1999, "Meningeal Tumours," in *Pathology and Genetics of Tumours of the Nervous System*, 1 edn, P. Kleihues & W. K. Cavenee, eds., IARC Press, Lyon, pp. 175-196.

Kleihues, P. & Cavenee, W. K. 2000, *Pathology and Genetics of Tumours of the Nervous System* IARC Press, Lyon.

Kleinschmidt-DeMasters, B. K., Hashizumi, T. L., Sze, C. I., Lillehei, K. O., Shroyer, A. L., & Shroyer, K. R. 1998a, "Telomerase expression shows differences across multiple regions of oligodendroglioma versus high grade astrocytomas but shows correlation with Mib-1 labelling", *J Clin. Pathol.*, vol. 51, no. 4, pp. 284-293.

Kleinschmidt-DeMasters, B. K., Shroyer, A. L., Hashizumi, T. L., Evans, L. C., Markham, N., Kindt, G., & Shroyer, K. R. 1998b, "Part I. Telomerase levels in human metastatic brain tumors show four-fold logarithmic variability but no correlation with tumor type or interval to patient demise", *J. Neurol. Sci.*, vol. 161, no. 2, pp. 116-123.

Klobutcher, L. A., Swanton, M. T., Donini, P., & Prescott, D. M. 1981, "All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3' terminus", *Proc. Natl. Acad. Sci. U.S.A.*, vol. 78, no. 5, pp. 3015-3019.

Koeppel, F., Riou, J. F., Laoui, A., Mailliet, P., Arimondo, P. B., Labit, D., Petitgenet, O., Helene, C., & Mergny, J. L. 2001, "Ethidium derivatives bind to G-quartets, inhibit telomerase and act as fluorescent probes for quadruplexes", *Nucleic Acids Res*, vol. 29, no. 5, pp. 1087-1096.

Koestner, A., Bilzer, T., Fatzer, R., Schulman, F. Y., Summers, B. A., & van Winkle, T. J. 1999, "World Health Organisation Histological Classification of Tumors of the nervous system of domestic animals", *Bull. World Health Organ.*, vol. 5, no. 2, pp. 1-69.

Koga, S., Hirohata, S., Kondo, Y., Komata, T., Takakura, M., Inoue, M., Kyo, S., & Kondo, S. 2000, "A novel telomerase-specific gene therapy: gene transfer of caspase-8 utilizing the human telomerase catalytic subunit gene promoter", *Hum. Gene Ther.*, vol. 11, no. 10, pp. 1397-1406.

Koga, S., Hirohata, S., Kondo, Y., Komata, T., Takakura, M., Inoue, M., Kyo, S., & Kondo, S. 2001, "FADD gene therapy using the human telomerase catalytic subunit (hTERT) gene promoter

to restrict induction of apoptosis to tumors in vitro and in vivo", *Anticancer Res.*, vol. 21, no. 3B, pp. 1937-1943.

Kolquist, K. A., Ellisen, L. W., Counter, C. M., Meyerson, M., Tan, L. K., Weinberg, R. A., Haber, D. A., & Gerald, W. L. 1998, "Expression of TERT in early premalignant lesions and a subset of cells in normal tissues", *Nat.Genet.*, vol. 19, no. 2, pp. 182-186.

Komata, T., Kondo, Y., Koga, S., Ko, S. C., Chung, L. W., & Kondo, S. 2000, "Combination therapy of malignant glioma cells with 2-5A-antisense telomerase RNA and recombinant adenovirus p53", *Gene Ther*, vol. 7, no. 24, pp. 2071-2079.

Komata, T., Kondo, Y., Kanzawa, T., Hirohata, S., Koga, S., Sumiyoshi, H., Srinivasula, S. M., Barna, B. P., Germano, I. M., Takakura, M., Inoue, M., Alnemri, E. S., Shay, J. W., Kyo, S., & Kondo, S. 2001a, "Treatment of malignant glioma cells with the transfer of constitutively active caspase-6 using the human telomerase catalytic subunit (human telomerase reverse transcriptase) gene promoter", *Cancer Res*, vol. 61, no. 15, pp. 5796-5802.

Komata, T., Koga, S., Hirohata, S., Takakura, M., Germano, I. M., Inoue, M., Kyo, S., Kondo, S., & Kondo, Y. 2001b, "A novel treatment of human malignant gliomas in vitro and in vivo: FADD gene transfer under the control of the human telomerase reverse transcriptase gene promoter", *Int. J. Oncol.*, vol. 19, no. 5, pp. 1015-1020.

Komata, T., Kanzawa, T., Kondo, Y., & Kondo, S. 2002a, "Telomerase as a therapeutic target for malignant gliomas", *Oncogene* , vol. 21, no. 4, pp. 656-663.

Komata, T., Kondo, Y., Kanzawa, T., Ito, H., Hirohata, S., Koga, S., Sumiyoshi, H., Takakura, M., Inoue, M., Barna, B. P., Germano, I. M., Kyo, S., & Kondo, S. 2002b, "Caspase-8 gene therapy using the human telomerase reverse transcriptase promoter for malignant glioma cells", *Hum. Gene Ther.*, vol. 13, no. 9, pp. 1015-1025.

Kondo, S., Tanaka, Y., Kondo, Y., Hitomi, M., Barnett, G. H., Ishizaka, Y., Liu, J., Haqqi, T., Nishiyama, A., Villeponteau, B., Cowell, J. K., & Barna, B. P. 1998a, "Antisense telomerase treatment: induction of two distinct pathways, apoptosis and differentiation", *FASEB J.*, vol. 12, no. 10, pp. 801-811.

Kondo, S., Kondo, Y., Li, G., Silverman, R. H., & Cowell, J. K. 1998b, "Targeted therapy of human malignant glioma in a mouse model by 2-5A antisense directed against telomerase RNA", *Oncogene*, vol. 16, no. 25, pp. 3323-3330.

Konstantinidou, A. E., Patsouris, E., Korkolopoulou, P., Kavantzas, N., Mahera, H., & Davaris, P. 2001, "DNA topoisomerase IIalpha expression correlates with cell proliferation but not with recurrence in intracranial meningiomas", *Histopathology*, vol. 39, no. 4, pp. 402-408.

Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., & van Oers, M. H. 1994, "Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis", *Blood*, vol. 84, no. 5, pp. 1415-1420.

Kosciolek, B. A., Kalantidis, K., Tabler, M., & Rowley, P. T. 2003, "Inhibition of telomerase activity in human cancer cells by RNA interference", *Molecular Cancer Therapeutics*, vol. 2, pp. 209-216.

Kotoula, V., Hytiroglou, P., Pyrpasopoulou, A., Saxena, R., Thung, S. N., & Papadimitriou, C. S. 2002, "Expression of human telomerase reverse transcriptase in regenerative and precancerous lesions of cirrhotic livers", *Liver*, vol. 22, no. 1, pp. 57-69.

Kotoula, V., Barbanis, S., Nikolakaki, E., Koufoyannis, D., Papadimitriou, C. S., & Karkavelas, G. 2004, "Relative expression of human telomerase catalytic subunit (hTERT) transcripts in astrocytic glioma", *Acta Neuropathol.*, vol. 107, pp. 443-451.

Kraemer, K., Fuessel, S., Schmidt, U., Kotzsch, M., Schwenzer, B., Wirth, M. P., & Meye, A. 2003, "Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells", *Clin. Cancer Res*, vol. 9, no. 10 Pt 1, pp. 3794-3800.

Krams, M., Claviez, A., Heidorn, K., Krupp, G., Parwaresch, R., Harms, D., & Rudolph, P. 2001, "Regulation of telomerase activity by alternate splicing of human telomerase reverse transcriptase RNA in a subset of neuroblastomas", *Am. J. Pathol.*, vol. 159, no. 5, pp. 1925-1932.

Krams, M., Hero, B., Berthold, F., Parwaresch, R., Harms, D., & Rudolph, P. 2003, "Full-length telomerase reverse transcriptase messenger RNA is an independent prognostic factor in neuroblastoma", *Am. J. Pathol.*, vol. 162, no. 3, pp. 1019-1026.

Kros, J. M., Hop, W. C., Godschalk, J. J., & Krishnadath, K. K. 1996, "Prognostic value of the proliferation-related antigen Ki-67 in oligodendrogliomas", *Cancer*, vol. 78, no. 5, pp. 1107-1113.

Krupp, G., Kuhne, K., Tamm, S., Klapper, W., Heidorn, K., Rott, A., & Parwaresch, R. 1997, "Molecular basis of artifacts in the detection of telomerase activity and a modified primer for a more robust 'TRAP' assay", *Nucleic Acids Res.*, vol. 25, no. 4, pp. 919-921.

Kumaki, F., Kawai, T., Hiroi, S., Shinomiya, N., Ozeki, Y., Ferrans, V., & Torikata, C. 2000, "Telomerase activity and expression of human telomerase RNA component and human telomerase reverse transcriptase in lung carcinomas", *Hum. Pathol.*, vol. 32, no. 2, pp. 188-195.

Kumamoto, H., Kinouchi, Y., & Ooya, K. 2001, "Telomerase activity and telomerase reverse transciptase (TERT) expression in ameloblastomas", *J. Oral Pathol. Med.*, vol. 30, no. 4, pp. 231-236.

Kuramoto, M., Ohsumi, K., Kishimoto, T., & Ishikawa, F. 2001, "Identification and analyses of the Xenopus TERT gene that encodes the catalytic subunit of telomerase", *Gene*, vol. 277, no. 1-2, pp. 101-110.

Kwok, S., Mack, D. H., Mullis, K. B., Poiesz, B., Ehrlich, G., Blair, D., Friedman-Kien, A., & Sninsky, J. J. 1987, "Identification of human immunodeficiency virus sequences by using in vitro enzymatic amplification and oligomer cleavage detection", *J. Virol.*, vol. 61, no. 5, pp. 1690-1694.

Kyo, S., Takakura, M., Kanaya, T., Zhuo, W., Fujimoto, K., Nishio, Y., Orimo, A., & Inoue, M. 1999, "Estrogen activates telomerase", *Cancer Res.*, vol. 59, no. 23, pp. 5917-5921.

Kyo, S., Takakura, M., Taira, T., Kanaya, T., Itoh, H., Yutsudo, M., Ariga, H., & Inoue, M. 2000, "Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT)", *Nucleic Acids Res.*, vol. 28, no. 3, pp. 669-677.

Kyo, S., Masutomi, K., Maida, Y., Kanaya, T., Yatabe, N., Nakamura, H., Tanaka, M., Takarada, M., Sugawara, I., Murakami, S., Taira, T., & Inoue, H. 2003, "Significance of immunological detection of human telomerase reverse transcriptase: re-evaluation of expression and localization of telmomerase reverse transcriptase", *Am. J. Pathol.*, vol. 163, no. 3, pp. 859-867.

Labat-Moleur, F., Steffan, A. M., Brisson, C., Perron, H., Feugeas, O., Furstenberger, P., Oberling, F., Brambilla, E., & Behr, J. P. 1996, "An electron microscopy study into the mechanism of gene transfer with lipopolyamines", *Gene Ther.*, vol. 3, no. 11, pp. 1010-1017.

Langford, L. A., Piatyszek, M. A., Xu, R., Schold, S. C. J., & Shay, J. W. 1995, "Telomerase activity in human brain tumours", *Lancet*, vol. 346, no. 8985, pp. 1267-1268.

Lantos, P. L., Louis, D. N., Rosenblum, M. K., & Kleihues, P. 2002, "Tumours of the nervous system," in *Greenfield's Neuropathology*, 7 edn, vol. 2 D. I. Graham & P. L. Lantos, eds., Arnold, London, pp. 767-980.

Lauzon, W., Sanchez, D. J., Cameron, D. W., & Badley, A. D. 2000, "Flow cytometric measurement of telomere length", *Cytometry*, vol. 42, no. 3, pp. 159-164.

Le, S., Sternglanz, R., & Greider, C. W. 2000, "Identification of two RNA-binding proteins associated with human telomerase RNA", *Mol. Biol. Cell*, vol. 11, no. 3, pp. 999-1010.

LeCouteur, R. A., Fike, J. R., Cann, C. E., & Pedroia, V. 1981, "Computed tomography of brain tumors in the caudal fossa of the dog", *Vet. Rad. Ultrasound*, vol. 22, no. 6, pp. 244-251.

LeCouteur, R. A. 1999, "Current concepts in the diagnosis and treatment of brain tumours in dogs and cats", *J. Small Anim. Pract.*, vol. 40, pp. 411-416.

Lee, C. J., Suh, E. J., Kang, H. T., Im, J. S., Um, S. J., Park, J. S., & Hwang, E. S. 2002a, "Induction of senescence-like state and suppression of telomerase activity through inhibition of HPV E6/E7 gene expression in cells immortalized by HPV16 DNA", *Exp. Cell Res.*, vol. 277, no. 2, pp. 173-182.

Lee, D., Kim, H. Z., Jeong, K. W., Shim, Y. S., Horikawa, I., Barrett, J. C., & Choe, J. 2002b, "Human papillomavirus E2 down-regulates the human telomerase reverse transcriptase promoter", *J. Biol. Chem.*, vol. 277, no. 31, pp. 27748-27756.

Lemarteleur, T., Gomez, D., Paterski, R., Mandine, E., Mailliet, P., & Riou, J. F. 2004, "Stabilization of the c-myc gene promoter quadruplex by specific ligands' inhibitors of telomerase", *Biochem. Biophys. Res Commun.*, vol. 323, no. 3, pp. 802-808.

Lendvay, T. S., Morris, D. K., Sah, J., Balasubramanian, B., & Lundblad, V. 1996, "Senescence mutants of Saccharomyces cerevisiae with a defect in telomere replication identify three additional EST genes", *Genetics*, vol. 144, no. 4, pp. 1399-1412.

Lester, N. V., Hopkins, A., Bova, F. J., Friedman, W. A., Buatti, J. M., Meeks, S. L., & Chrisman, C. L. 2001, "Radiosurgery using a stereotactic headframe system for irradiation of brain tumors in dogs", *J. Am. Vet. Med. Assoc.*, vol. 219, no. 11, pp. 1562-1567.

Levis, R., Hazelrigg, T., & Rubin, G. M. 1985, "Effects of genomic position on the expression of transduced copies of the white gene of Drosophila", *Science*, vol. 229, no. 4713, pp. 558-561.

Li, H., Zhao, L. L., Funder, J. W., & Liu, J. P. 1997, "Protein phosphatase 2A inhibits nuclear telomerase activity in human breast cancer cells", *J. Biol. Chem.*, vol. 272, no. 27, pp. 16729-16732.

Li, H., Cao, Y., Berndt, M. C., Funder, J. W., & Liu, J. P. 1999, "Molecular interactions between telomerase and the tumor suppressor protein p53 in vitro", *Oncogene*, vol. 18, no. 48, pp. 6785-6794.

Li, S., Crothers, J., Haqq, C. M., & Blackburn, E. H. 2005, "Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA", *J. Biol. Chem.*, vol. 280, no. 25, pp. 23709-23717.

Lingaas, F., Comstock, K. E., Kirkness, E. F., Sorensen, A., Aarskaug, T., Hitte, C., Nickerson, M. L., Moe, L., Schmidt, L. S., Thomas, R., Breen, M., Galibert, F., Zbar, B., & Ostrander, E. A. 2003, "A mutation in the canine BHD gene is associated with hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis in the German Shepherd dog", *Hum. Mol. Genet.*, vol. 12, no. 23, pp. 3043-3053.

Lingner, J. & Cech, T. R. 1996, "Purification of telomerase from Euplotes aediculatus: requirement of a primer 3' overhang", *Proc. Natl. Acad. Sci. U.S.A*, vol. 93, no. 20, pp. 10712-10717.

Lingner, J., Hughes, T. R., Shevchenko, A., Mann, M., Lundblad, V., & Cech, T. R. 1997, "Reverse transcriptase motifs in the catalytic subunit of telomerase", *Science*, vol. 276, no. 5312, pp. 561-567.

Liu, K., Schoonmaker, M. M., Levine, B. L., June, C. H., Hodes, R. J., & Weng, N. P. 1999, "Constitutive and regulated expression of telomerase reverse transcriptase (hTERT) in human lymphocytes", *Proc. Natl. Acad. Sci. U.S.A*, vol. 96, no. 9, pp. 5147-5152.

Liu, L., Saldanha, S. N., Pate, M. S., Andrews, L. G., & Tollefsbol, T. O. 2004, "Epigenetic regulation of human telomerase reverse transcriptase promoter activity during cellular differentiation", *Genes Chromosomes. Cancer*, vol. 41, no. 1, pp. 26-37.

Lohr, C. V., Teifke, J. P., Failing, K., & Weiss, E. 1997, "Characterization of the proliferation state in canine mammary tumors by the standardized AgNOR method with postfixation and immunohistologic detection of Ki-67 and PCNA", *Vet. Pathol.*, vol. 34, no. 3, pp. 212-221.

Londono-Vallejo, J. A., Der-Sarkissian, H., Cazes, L., Bacchetti, S., & Reddel, R. R. 2004, "Alternative lengthening of telomeres is characterized by high rates of telomeric exchange", *Cancer Res*, vol. 64, no. 7, pp. 2324-2327.

Lopez, C. C., Nielsen, L., & Edstrom, J. E. 1996, "Terminal long tandem repeats in chromosomes form Chironomus pallidivittatus", *Mol. Cell Biol.*, vol. 16, no. 7, pp. 3285-3290.

Lorenz, W. W., McCann, R. O., Longiaru, M., & Cormier, M. J. 1991, "Isolation and expression of a cDNA encoding Renilla reniformis luciferase", *Proc. Natl. Acad. Sci. U.S.A*, vol. 88, no. 10, pp. 4438-4442.

Louis, E. J., Naumova, E. S., Lee, A., Naumov, G., & Haber, J. E. 1994, "The chromosome end in yeast: its mosaic nature and influence on recombinational dynamics", *Genetics*, vol. 136, no. 3, pp. 789-802.

Louis, E. J. 1995, "The chromosome ends of Saccharomyces cerevisiae", *Yeast*, vol. 11, no. 16, pp. 1553-1573.

Lu, C., Fu, W., & Mattson, M. P. 2001, "Telomerase protects developing neurons against DNA damage-induced cell death", *Brain Res. Dev. Brain Res*, vol. 131, no. 1-2, pp. 167-171.

Ludwig, A., Saretzki, G., Holm, P. S., Tiemann, F., Lorenz, M., Emrich, T., Harley, C. B., & von Zglinicki, T. 2001, "Ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to inhibitors of topoisomerase", *Cancer Res*, vol. 61, no. 7, pp. 3053-3061.

Lukowiak, A. A., Narayanan, A., Li, Z. H., Terns, R. M., & Terns, M. P. 2001, "The snoRNA domain of vertebrate telomerase RNA functions to localize the RNA within the nucleus", *RNA.*, vol. 7, no. 12, pp. 1833-1844.

Lundblad, V. & Blackburn, E. H. 1993, "An alternative pathway for yeast telomere maintenance rescues est1- senescence", *Cell*, vol. 73, no. 2, pp. 347-360.

Lusis, E. & Gutmann, D. H. 2004, "Meningioma: an update", *Curr. Opin. Neurol.*, vol. 17, no. 6, pp. 687-692.

Ly, H., Xu, L., Rivera, M. A., Parslow, T. G., & Blackburn, E. H. 2003, "A role for a novel 'trans-pseudoknot' RNA-RNA interaction in the functional dimerization of human telomerase", *Genes Dev.*, vol. 17, no. 9, pp. 1078-1083.

Ma, H., Urquidi, V., Wong, J., Kleeman, J., & Goodison, S. 2003, "Telomerase reverse transcriptase promoter regulation during myogenic differentiation of human RD rhabdomyosarcoma cells", *Mol. Cancer Res*, vol. 1, no. 10, pp. 739-746.

Madewell, B. R. 2001, "Cellular proliferation in tumors: a review of methods, interpretation, and clinical applications", *J. Vet. Intern. Med*, vol. 15, no. 4, pp. 334-340.

Maes, L., Lippens, E., Kalala, J. P., & de Ridder, L. 2005, "The hTERT-protein and Ki-67 labelling index in recurrent and non-recurrent meningiomas", *Cell Prolif.*, vol. 38, no. 1, pp. 3-12.

Majumdar, A. S., Hughes, D. E., Lichtsteiner, S. P., Wang, Z., Lebkowski, J. S., & Vasserot, A. P. 2001, "The telomerase reverse transcriptase promoter drives efficacious tumor suicide gene therapy while preventing hepatotoxicity encountered with constitutive promoters", *Gene Ther.*, vol. 8, no. 7, pp. 568-578.

Markert, J. 2003, "Glioblastoma multiforme: introduction", *Cancer J.*, vol. 9, no. 3, p. 148.

Martin-Rivera, L., Herrera, E., Albar, J. P., & Blasco, M. A. 1998, "Expression of mouse telomerase catalytic subunit in embryos and adult tissues", *Proc. Natl. Acad. Sci. U.S.A*, vol. 95, no. 18, pp. 10471-10476.

Marusic, L., Anton, M., Tidy, A., Wang, P., Villeponteau, B., & Bacchetti, S. 1997, "Reprogramming of telomerase by expression of mutant telomerase RNA template in human cells leads to altered telomeres that correlate with reduced cell viability", *Mol. Cell Biol.*, vol. 17, no. 11, pp. 6394-6401.

Masutomi, K., Yu, E. Y., Khurts, S., Ben Porath, I., Currier, J. L., Metz, G. B., Brooks, M. W., Kaneko, S., Murakami, S., DeCaprio, J. A., Weinberg, R. A., Stewart, S. A., & Hahn, W. C. 2003, "Telomerase maintains telomere structure in normal human cells", *Cell*, vol. 114, no. 2, pp. 241-253.

Mattson, M. P., Fu, W., & Zhang, P. 2001, "Emerging roles for telomerase in regulating cell differentiation and survival: a neuroscientist's perspective", *Mech. Ageing Dev.*, vol. 122, no. 7, pp. 659-671.

McClintock, B. 1941, "The stability of broken ends of chromosomes in zea mays", *Genetics*, vol. 26, pp. 234-282.

McEachern, M. J. & Blackburn, E. H. 1995, "Runaway telomere elongation caused by telomerase RNA gene mutations", *Nature*, vol. 376, no. 6539, pp. 403-409.

McGrath, J. T. 1960, "Intracranial neoplasms," in *Neurologic examination of the dog*, 2 edn, J. T. McGrath, ed., Henry Kimpton, London, pp. 148-195.

Mergny, J. L., Lacroix, L., Teulade-Fichou, M. P., Hounsou, C., Guittat, L., Hoarau, M., Arimondo, P. B., Vigneron, J. P., Lehn, J. M., Riou, J. F., Garestier, T., & Helene, C. 2001, "Telomerase inhibitors based on quadruplex ligands selected by a fluorescence assay", *Proc. Natl. Acad. Sci. U.S.A*, vol. 98, no. 6, pp. 3062-3067.

Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q., Bacchetti, S., Haber, D. A., & Weinberg, R. A. 1997, "hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization", *Cell*, vol. 90, no. 4, pp. 785-795.

Michels, C. A., Read, E., Nat, K., & Charron, M. J. 1992, "The telomere-associated MAL3 locus of Saccharomyces is a tandem array of repeated genes", *Yeast*, vol. 8, no. 8, pp. 655-665.

Millar, D. S., Paul, C. L., Molloy, P. L., & Clark, S. J. 2000, "A distinct sequence (ATAAA)n separates methylated and unmethylated domains at the 5'-end of the GSTP1 CpG island", *J. Biol. Chem.*, vol. 275, no. 32, pp. 24893-24899.

Miller, M. C. & Collins, K. 2000, "The Tetrahymena p80/p95 complex is required for proper telomere length maintenance and micronuclear genome stability", *Mol. Cell*, vol. 6, no. 4, pp. 827-837.

Misiti, S., Nanni, S., Fontemaggi, G., Cong, Y. S., Wen, J., Hirte, H. W., Piaggio, G., Sacchi, A., Pontecorvi, A., Bacchetti, S., & Farsetti, A. 2000, "Induction of hTERT expression and telomerase activity by estrogens in human ovary epithelium cells", *Mol. Cell Biol.*, vol. 20, no. 11, pp. 3764-3771.

Mitchell, J. R. & Collins, K. 2000, "Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase", *Mol. Cell*, vol. 6, no. 2, pp. 361-371.

Miyashita, N., Shiga, K., Yonai, M., Kaneyama, K., Kobayashi, S., Kojima, T., Goto, Y., Kishi, M., Aso, H., Suzuki, T., Sakaguchi, M., & Nagai, T. 2002, "Remarkable differences in telomere lengths among cloned cattle derived from different cell types", *Biol. Reprod.*, vol. 66, no. 6, pp. 1649-1655.

Morales, C. P., Holt, S. E., Ouellette, M., Kaur, K. J., Yan, Y., Wilson, K. S., White, M. A., Wright, W. E., & Shay, J. W. 1999, "Absence of cancer-associated changes in human fibroblasts immortalized with telomerase", *Nat. Genet.*, vol. 21, no. 1, pp. 115-118.

Moriarty, T. J., Huard, S., Dupuis, S., & Autexier, C. 2002, "Functional multimerization of human telomerase requires an RNA interaction domain in the N terminus of the catalytic subunit", *Mol. Cell Biol.*, vol. 22, no. 4, pp. 1253-1265.

Morii, K., Tanaka, R., Onda, K., Tsumanuma, I., & Yoshimura, J. 1997, "Expression of telomerase RNA, telomerase activity, and telomere length in human gliomas", *Biochem. Biophys. Res. Commun.*, vol. 239, no. 3, pp. 830-834.

Morrison, W. B. 1998, "Cancer affecting the nervous system," in *Cancer in dogs and cats*, 1 edn, W. B. Morrison, ed., Williams and wilkins, Baltimore, pp. 655-665.

Moyzis, R. K., Buckingham, J. M., Cram, L. S., Dani, M., Deaven, L. L., Jones, M. D., Meyne, J., Ratliff, R. L., & Wu, J. R. 1988, "A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes", *Proc. Natl. Acad. Sci. U.S.A*, vol. 85, no. 18, pp. 6622-6626.

Mukai, S., Kondo, Y., Koga, S., Komata, T., Barna, B. P., & Kondo, S. 2000, "2-5A antisense telomerase RNA therapy for intracranial malignant gliomas", *Cancer Res*, vol. 60, no. 16, pp. 4461-4467.

Muller, H. J. 1962, in *Studies of Genetics: The Selected Papers of H. J. Muller*, Indiana University Press, Bloomington, pp. 384-408.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. 1986, "Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction", *Cold Spring Harb. Symp. Quant. Biol.*, vol. 51 Pt 1:263-73., pp. 263-273.

Mullis, K. B. & Faloona, F. A. 1987, "Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction", *Methods Enzymol.*, vol. 155:335-50., pp. 335-350.

Nagai, M., Tejima, T., Arai, T., Narita, J., Watanabe, K., Ochiai, C., Yoshimoto, Y., & Endoh, M. 1995, "Proliferative potential and malignant transformation of ganglioglioma: immunohistochemical studies by MIB-1 and p53 staining", *Noshuyo. Byori.*, vol. 12, no. 2, pp. 141-149.

Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B., & Cech, T. R. 1997, "Telomerase catalytic subunit homologs from fission yeast and human", *Science*, vol. 277, no. 5328, pp. 955-959.

Nakamura, Y., Hirose, M., Matsuo, H., Tsuyama, N., Kamisango, K., & Ide, T. 1999, "Simple, rapid, quantitative, and sensitive detection of telomere repeats in cell lysate by a hybridization protection assay", *Clin. Chem.*, vol. 45, no. 10, pp. 1718-1724.

Nakamura, M., Masutomi, K., Kyo, S., Hashimoto, M., Maida, Y., Kanaya, T., Tanaka, M., Hahn, W. C., & Inoue, H. 2005, "Efficient inhibition of human telomerase reverse transcriptase expression by RNA interference sensitizes cancer cells to ionizing radiation and chemotherapy", *Hum. Gene Ther.*, vol. 16, pp. 859-868.

Nakanishi, K., Kawai, T., Kumaki, F., Hirot, S., Mukai, M., & Ikeda, E. 2002, "Expression of human telomerase RNA component and telomerase reverse transcriptase mRNA in atypical adenomatous hyperplasia of the lung", *Hum. Pathol.*, vol. 33, no. 7, pp. 697-702.

Nakatani, K., Yoshimi, N., Mori, H., Yoshimura, S., Sakai, H., Shinoda, J., & Sakai, N. 1997, "The significant role of telomerase activity in human brain tumors", *Cancer*, vol. 80, no. 3, pp. 471-476.

Nakayama, J., Saito, M., Nakamura, H., Matsuura, A., & Ishikawa, F. 1997, "TLP1: a gene encoding a protein component of mammalian telomerase is a novel member of WD repeats family", *Cell*, vol. 88, no. 6, pp. 875-884.

Narayanan, A., Lukowiak, A., Jady, B. E., Dragon, F., Kiss, T., Terns, R. M., & Terns, M. P. 1999, "Nucleolar localization signals of box H/ACA small nucleolar RNAs", *EMBO J.*, vol. 18, no. 18, pp. 5120-5130.

Nasir, L., Devlin, P., Mckevitt, T., Rutteman, G., & Argyle, D. J. 2001, "Telomere lengths and telomerase activity in dog tissues: a potential model system to study human telomere and telomerase biology", *Neoplasia*, vol. 3, no. 4, pp. 351-359.

Nasir, L., Gault, E., Campbell, S., Veeramalai, M., Gilbert, D., McFarlane, R., Munro, A., & Argyle, D. J. 2004, "Isolation and expression of the reverse transcriptase component of the Canis familiaris telomerase ribonucleoprotein (dogTERT)", *Gene*, vol. 336, no. 1, pp. 105-113.

Neckers, L., Schulte, T. W., & Mimnaugh, E. 1999, "Geldanamycin as a potential anti-cancer agent: its molecular target and biochemical activity", *Invest. New Drugs*, vol. 17, no. 4, pp. 361-373.

Nieh, S., Chen, S. F., Fu, E., Jan, C. I., & Lee, W. H. 2005, "Detection of the human telomerase RNA component by in situ hybridization in cells from body fluids", *Acta Cytol.*, vol. 49, no. 1, pp. 31-37.

Nimmo, E. R., Cranston, G., & Allshire, R. C. 1994, "Telomere-associated chromosome breakage in fission yeast results in variegated expression of adjacent genes", *EMBO J.*, vol. 13, no. 16, pp. 3801-3811.

Nishi, H., Nakada, T., Kyo, S., Inoue, M., Shay, J. W., & Isaka, K. 2004, "Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT)", *Mol. Cell Biol.*, vol. 24, no. 13, pp. 6076-6083.

Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., & Smith, J. R. 1994, "Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen", *Exp. Cell Res.*, vol. 211, no. 1, pp. 90-98.

Norrback, K. F., Hultdin, M., Dahlenborg, K., Osterman, P., Carlsson, R., & Roos, G. 2001, "Telomerase regulation and telomere dynamics in germinal centers", *Eur. J. Haematol.*, vol. 67, no. 5-6, pp. 309-317.

Nozawa, K., Maehara, K., & Isobe, K. 2001, "Mechanism for the reduction of telomerase expression during muscle cell differentiation", *J. Biol. Chem.*, vol. 276, no. 25, pp. 22016-22023.

Nugent, C. I. & Lundblad, V. 1998, "The telomerase reverse transcriptase: components and regulation", *Genes Dev.*, vol. 12, no. 8, pp. 1073-1085.

Oh, S., Song, Y., Yim, J., & Kim, T. K. 1999, "The Wilms' tumor 1 tumor suppressor gene represses transcription of the human telomerase reverse transcriptase gene", *J. Biol. Chem.*, vol. 274, no. 52, pp. 37473-37478.

Oh, S., Song, Y. H., Yim, J., & Kim, T. K. 2000, "Identification of Mad as a repressor of the human telomerase (hTERT) gene", *Oncogene*, vol. 19, no. 11, pp. 1485-1490.

Oh, H., Taffet, G. E., Youker, K. A., Entman, M. L., Overbeek, P. A., Michael, L. H., & Schneider, M. D. 2001, "Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival", *Proc. Natl. Acad. Sci. U.S.A*, vol. 98, no. 18, pp. 10308-10313.

Ohyashiki, J. H., Hisatomi, H., Nagao, K., Honda, S., Takaku, T., Zhang, Y., Sashida, G., & Ohyashiki, K. 2005, "Quantitative relationship between functionally active telomerase and major telomerase components (hTERT and hTR) in acute leukaemia cells", *Br. J. Cancer*, vol. ..

Oka, Y. & Thomas, C. A., Jr. 1987, "The cohering telomeres of Oxytricha", *Nucleic Acids Res*, vol. 15, no. 21, pp. 8877-8898.

Olby, N. 2005. Personal Communication

Olovnikov, A. M. 1973, "A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon", *J. Theor. Biol.*, vol. 41, no. 1, pp. 181-190.

Ouellette, M. M., Liao, M., Herbert, B. S., Johnson, M., Holt, S. E., Liss, H. S., Shay, J. W., & Wright, W. E. 2000, "Subsenescent telomere lengths in fibroblasts immortalized by limiting amounts of telomerase", *J. Biol. Chem.*, vol. 275, no. 14, pp. 10072-10076.

Pallini, R., Pierconti, F., Falchetti, M. L., D'Arcangelo, D., Fernandez, E., Maira, G., D'Ambrosio, E., & Larocca, L. M. 2001, "Evidence for telomerase involvement in the angiogenesis of astrocytic tumors: expression of human telomerase reverse transcriptase messenger RNA by vascular endothelial cells", *J. Neurosurg.*, vol. 94, no. 6, pp. 961-971.

Palmqvist, R., Zhang, A., Xu, D., Golovleva, I., Norrback, K. F., Gruber, A., Oberg, A., Stenling, R., & Roos, G. 2005, "hTERT gene copy number is not associated with hTERT RNA expression or telomerase activity in colorectal cancer", *Int. J. Cancer*, vol. ..

Park, J. P., Dossu, J. R., & Rhodes, C. H. 1996, "Telomere associations in desmoplastic infantile ganglioglioma", *Cancer Genet. Cytogenet.*, vol. 92, no. 1, pp. 4-7.

Parkinson, G. N., Lee, M. P., & Neidle, S. 2002, "Crystal structure of parallel quadruplexes from human telomeric DNA", *Nature*, vol. 417, no. 6891, pp. 876-880.

Parney, I. F. & Chang, S. M. 2003, "Current chemotherapy for glioblastoma", *Cancer J.*, vol. 9, no. 3, pp. 149-156.

Parsch, D., Fellenberg, J., Brummendorf, T. H., Eschlbeck, A. M., & Richter, W. 2004, "Telomere length and telomerase activity during expansion and differentiation of human mesenchymal stem cells and chondrocytes", *J. Mol. Med.*, vol. 82, no. 1, pp. 49-55.

Pascolo, E., Wenz, C., Lingner, J., Hauel, N., Priepke, H., Kauffmann, I., Garin-Chesa, P., Rettig, W. J., Damm, K., & Schnapp, A. 2002, "Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate", *J. Biol. Chem.*, vol. 277, no. 18, pp. 15566-15572.

Peng, Y., Mian, I. S., & Lue, N. F. 2001, "Analysis of telomerase processivity: mechanistic similarity to HIV-1 reverse transcriptase and role in telomere maintenance", *Mol. Cell*, vol. 7, no. 6, pp. 1201-1211.

Pennarun, G., Granotier, C., Gauthier, L. R., Gomez, D., Hoffschir, F., Mandine, E., Riou, J. F., Mergny, J. L., Mailliet, P., & Boussin, F. D. 2005, "Apoptosis related to telomere instability and cell cycle alterations in human glioma cells treated by new highly selective G-quadruplex ligands", *Oncogene*, vol. 24, no. 18, pp. 2917-2928.

Perrem, K., Colgin, L. M., Neumann, A. A., Yeager, T. R., & Reddel, R. R. 2001, "Coexistence of alternative lengthening of telomeres and telomerase in hTERT-transfected GM847 cells", *Mol. Cell Biol.*, vol. 21, no. 12, pp. 3862-3875.

Phan, A. T. & Mergny, J. L. 2002, "Human telomeric DNA: G-quadruplex, i-motif and Watson-Crick double helix", *Nucleic Acids Res*, vol. 30, no. 21, pp. 4618-4625.

Pitts, A. E. & Corey, D. R. 1998, "Inhibition of human telomerase by 2'-O-methyl-RNA", *Proc. Natl. Acad. Sci. U.S.A*, vol. 95, no. 20, pp. 11549-11554.

Platt, S. R., Garosi, L. S., Adams, V., Murphy, S., & Abramson, C. J. 2004 (Abstract), "Canine intracranial glioma outcome following corticosteroids, hypofractionated radiotherapy or multimodality therapy: 30 cases", BSAVA Congress, Birmingham, p. 544.

Platt, S. R., Scase, T., Adams, V., Adamo, P. F., & Long, S. 2005 (Abstract), "Vascular endothelial growth factor expression in canine intracranial meningiomas", ACVIM Congress, Baltimore.

Platz, S. J., Breuer, W., Pfleghaar, S., Minkus, G., & Hermanns, W. 1999, "Prognostic value of histopathological grading in canine extramedullary plasmacytomas", *Vet. Pathol.*, vol. 36, no. 1, pp. 23-27.

Plumb, J. A., Bilsland, A., Kakani, R., Zhao, J., Glasspool, R. M., Knox, R. J., Evans, T. R., & Keith, W. N. 2001, "Telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase sensitize human cancer cells to the pro-drug CB1954", *Oncogene*, vol. 20, no. 53, pp. 7797-7803.

Pollack, I. F., Hamilton, R. L., Burnham, J., Holmes, E. J., Finkelstein, S. D., Sposto, R., Yates, A. J., Boyett, J. M., & Finlay, J. L. 2002, "Impact of proliferation index on outcome in childhood malignant gliomas: results in a multi-institutional cohort", *Neurosurg.*, vol. 50, no. 6, pp. 1238-1244.

Poole, J. C., Andrews, L. G., & Tollefsbol, T. O. 2001, "Activity, function and gene regulation of the catalytic subunit of telomerase (hTERT)", *Gene*, vol. 269, pp. 1-12.

Poremba, C., Hero, B., Heine, B., Scheel, C., Schaefer, K. L., Christiansen, H., Berthold, F., Kneif, S., Stein, H., Juergens, H., Boecker, W., & Dockhorn-Dworniczak, B. 2000a, "Telomerase is a strong indicator for assessing the proneness to progression in neuroblastomas", *Med. Ped. Oncol.*, vol. 35, pp. 651-655.

Poremba, C., Scheel, C., Hero, B., Christiansen, H., Schaefer, K. L., Nakayama, J., Berthold, F., Juergens, H., Boecker, W., & Dockhorn-Dworniczak, B. 2000b, "Telomerase activity and telomerase subunits gene expression patterns in neuroblastoma: a molecular and immunohistochemical study establishing prognostic tools for fresh-frozen and paraffin-embedded tissues", *J. Clin. Oncol.*, vol. 18, no. 13, pp. 2582-2592.

Powell, M. D., Ghosh, M., Jacques, P. S., Howard, K. J., Le Grice, S. F., & Levin, J. G. 1997, "Alanine-scanning mutations in the "primer grip" of p66 HIV-1 reverse transcriptase result in selective loss of RNA priming activity", *J. Biol. Chem.*, vol. 272, no. 20, pp. 13262-13269.

Prescott, J. & Blackburn, E. H. 1997, "Functionally interacting telomerase RNAs in the yeast telomerase complex", *Genes Dev.*, vol. 11, no. 21, pp. 2790-2800.

Prober, J. M., Trainor, G. L., Dam, R. J., Hobbs, F. W., Robertson, C. W., Zagursky, R. J., Cocuzza, A. J., Jensen, M. A., & Baumeister, K. 1987, "A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides", *Science*, vol. 238, no. 4825, pp. 336-341.

Prowse, K. R., Avilion, A. A., & Greider, C. W. 1993, "Identification of a nonprocessive telomerase activity from mouse cells", *Proc. Natl. Acad. Sci. U.S.A*, vol. 90, no. 4, pp. 1493-1497.

Prowse, K. R. & Greider, C. W. 1995, "Developmental and tissue-specific regulation of mouse telomerase and telomere length", *Proc. Natl. Acad. Sci. U.S.A*, vol. 92, no. 11, pp. 4818-4822.

Pruzan, R., Pongracz, K., Gietzen, K., Wallweber, G., & Gryaznov, S. 2002, "Allosteric inhibitors of telomerase: oligonucleotide N3'-->P5' phosphoramidates", *Nucleic. Acids. Res*, vol. 30, no. 2, pp. 559-568.

Ray, S., Karamysheva, Z., Wang, L., Shippen, D. E., & Price, C. M. 2002, "Interactions between telomerase and primase physically link the telomere and chromosome replication machinery", *Mol. Cell Biol.*, vol. 22, no. 16, pp. 5859-5868.

Read, M., Harrison, R. J., Romagnoli, B., Tanious, F. A., Gowan, S. H., Reszka, A. P., Wilson, W. D., Kelland, L. R., & Neidle, S. 2001, "Structure-based design of selective and potent G quadruplex-mediated telomerase inhibitors", *Proc. Natl. Acad. Sci. U.S.A*, vol. 98, no. 9, pp. 4844-4849.

Reddel, R. R. 2003, "Alternative lengthening of telomeres, telomerase, and cancer", *Cancer Lett.*, vol. 194, no. 2, pp. 155-162.

Reddel, R. R. & Bryan, T. M. 2003, "Alternative lengthening of telomeres: dangerous road less travelled", *Lancet*, vol. 361, no. 9372, pp. 1840-1841.

Renaud, S., Bosman, F. T., & Berger, M. S. 2003, "Implication of the exon region in the regulation of the human telomerase reverse transcriptase gene promoter", *Biochem. Biophys. Res Commun.*, vol. 300, pp. 47-54.

Richards, E. J., Chao, S., Vongs, A., & Yang, J. 1992, "Characterization of Arabidopsis thaliana telomeres isolated in yeast", *Nucleic Acids Res*, vol. 20, no. 15, pp. 4039-4046.

Riou, J. F., Guittat, L., Mailliet, P., Laoui, A., Renou, E., Petitgenet, O., Megnin-Chanet, F., Helene, C., & Mergny, J. L. 2002, "Cell senescence and telomere shortening induced by a new series of specific G-quadruplex DNA ligands", *Proc. Natl. Acad. Sci. U.S.A*, vol. 99, no. 5, pp. 2672-2677.

Robson, T. & Hirst, D. G. 2003, "Transcriptional targeting in cancer gene therapy", *J. Biomed. Biotech.*, vol. 2, pp. 110-137.

Rogan, E. M., Bryan, T. M., Hukku, B., Maclean, K., Chang, A. C., Moy, E. L., Englezou, A., Warneford, S. G., Dalla-Pozza, L., & Reddel, R. R. 1995, "Alterations in p53 and p16INK4 expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts", *Mol. Cell Biol.*, vol. 15, no. 9, pp. 4745-4753.

Rossi, E., Floridia, G., Casali, M., Danesino, C., Chiumello, G., Bernardi, F., Magnani, I., Papi, L., Mura, M., & Zuffardi, O. 1993, "Types, stability, and phenotypic consequences of chromosome rearrangements leading to interstitial telomeric sequences", *J. Med. Genet.*, vol. 30, no. 11, pp. 926-931.

Rouyer, F., de la, C. A., Andersson, M., & Weissenbach, J. 1990, "An interspersed repeated sequence specific for human subtelomeric regions", *EMBO J.*, vol. 9, no. 2, pp. 505-514.

Rubin, G. M. 1978, "Isolation of a telomeric DNA sequence from Drosophila melanogaster", *Cold Spring Harb. Symp. Quant. Biol.*, vol. 42 Pt 2:1041-6., pp. 1041-1046.

Rudolph, K. L., Chang, S., Lee, H. W., Blasco, M., Gottlieb, G. J., Greider, C., & DePinho, R. A. 1999, "Longevity, stress response, and cancer in aging telomerase-deficient mice", *Cell*, vol. 96, no. 5, pp. 701-712.

Rudolph, K. L., Millard, M., Bosenberg, M. W., & DePinho, R. A. 2001, "Telomere dysfunction and evolution of intestinal carcinoma in mice and humans", *Nat. Genet.*, vol. 28, no. 2, pp. 155-159.

Rushing, E. J., Brown, D. F., Hladik, C. L., Risser, R. C., Mickey, B. E., & White, C. L. 1998, "Correlation of bcl-2, p53, and MIB-1 expression with ependymoma grade and subtype", *Mod. Pathol.*, vol. 11, no. 5, pp. 464-470.

Sachsinger, J., Gonzalez-Suarez, E., Samper, E., Heicappell, R., Muller, M., & Blasco, M. A. 2001, "Telomerase inhibition in RenCa, a murine tumor cell line with short telomeres, by overexpression of a dominant negative mTERT mutant, reveals fundamental differences in telomerase regulation between human and murine cells", *Cancer Res*, vol. 61, no. 14, pp. 5580-5586.

Saffer, J. D., Jackson, S. P., & Annarella, M. B. 1991, "Developmental expression of Sp1 in the mouse", *Mol. Cell Biol.*, vol. 11, no. 4, pp. 2189-2199.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. 1988, "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase", *Science*, vol. 239, no. 4839, pp. 487-491.

Saji, M., Xydas, S., Westra, W. H., Liang, C. K., Clark, D. P., Udelsman, R., Umbricht, C. B., Sukumar, S., & Zeiger, M. A. 1999, "Human telomerase reverse transcriptase (hTERT) gene expression in thyroid neoplasms", *Clin. Cancer Res*, vol. 5, no. 6, pp. 1483-1489.

Sallinen, P., Miettinen, H., Sallinen, S. L., Haapasalo, H., Helin, H., & Kononen, J. 1997, "Increased expression of telomerase RNA component is associated with increased cell proliferation in human astrocytomas", *Am. J. Pathol.*, vol. 150, no. 4, pp. 1159-1164.

Sallinen, P. K., Sallinen, S. L., Helen, P. T., Rantala, I. S., Rautiainen, E., Helin, H. J., Kalimo, H., & Haapasalo, H. K. 2000, "Grading of diffusely infiltrating astrocytomas by quantitative histopathology, cell proliferation and image cytometric DNA analysis. Comparison of 133 tumours in the context of the WHO 1979 and WHO 1993 grading schemes", *Neuropathol. Appl. Neurobiol.*, vol. 26, no. 4, pp. 319-331.

Samper, E., Fernandez, P., Eguia, R., Martin-Rivera, L., Bernad, A., Blasco, M. A., & Aracil, M. 2002, "Long-term repopulating ability of telomerase-deficient murine hematopoietic stem cells", *Blood*, vol. 99, no. 8, pp. 2767-2775.

Sanger, F., Nicklen, S., & Coulson, A. R. 1977, "DNA sequencing with chain-terminating inhibitors", *Proc. Natl. Acad. Sci. U.S.A*, vol. 74, no. 12, pp. 5463-5467.

Santarius, T., Kirsch, M., Rossi, M. L., & Black, P. M. 1997, "Molecular aspects of neuro-oncology", *Clin. Neurol. Neurosurg.*, vol. 99, no. 3, pp. 184-195.

Saretzki, G., Ludwig, A., von Zglinicki, T., & Runnebaum, I. B. 2001, "Ribozyme-mediated telomerase inhibition induces immediate cell loss but not telomere shortening in ovarian cancer cells", *Cancer Gene Ther.*, vol. 8, no. 10, pp. 827-834.

Saretzki, G., Petersen, S., Petersen, I., Kolble, K., & von Zglinicki, T. 2002, "hTERT gene dosage correlates with telomerase activity in human lung cancer cell lines", *Cancer Lett.*, vol. 176, no. 1, pp. 81-91.

Sarli, G., Benazzi, C., Preziosi, R., & Marcato, P. S. 1994, "Proliferative activity assessed by anti-PCNA and Ki67 monoclonal antibodies in canine testicular tumours", *J. Comp. Pathol.*, vol. 110, no. 4, pp. 357-368.

Sawada, T., Yamada, O., Yoshimura, N., Hatori, K., Fuchinoue, S., & Teraoka, S. 2002, "Xenoantigen, an alphaGal epitope-expression construct driven by the hTERT-promoter, specifically kills human pancreatic cancer cell line", *Cancer Cell Int.*, vol. 2, no. 1, p. 14.

Scharf, S. 1990, "Cloning with PCR," in *PCR protocols. A guide to methods and applications*, M. A. Innis & D. H. Gelfand, eds., Academic Press, San Diego, CA, pp. 84-91.

Schulman, F. Y., Ribas, J. L., Carpenter, J. L., Sisson, A. F., & LeCouteur, R. A. 1992, "Intracranial meningioma with pulmonary metastasis in three dogs", *Vet. Pathol.*, vol. 29, no. 3, pp. 196-202.

Schulte, T. W. & Neckers, L. M. 1998, "The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin", *Cancer Chemother. Pharmacol.*, vol. 42, no. 4, pp. 273-279.

Sciamanna, I., Landriscina, M., Pittoggi, C., Quirino, M., Mearelli, C., Beraldi, R., Mattei, E., Serafino, A., Cassano, A., Sinibaldi-Vallebona, P., Garaci, C., & Spadaforda, C. 2005, "Inhibition of endogenous reverse transcriptase antagonizes human tumor growth", *Oncogene*, vol. 24, pp. 3923-3931.

Scott, R. J., Hall, P. A., Haldane, J. S., van Noorden, S., Price, Y., Lane, D. P., & Wright, N. A. 1991, "A comparison of immunohistochemical markers of cell proliferation with experimentally determined growth fraction", *J. Pathol.*, vol. 165, no. 2, pp. 173-178.

Seenisamy, J., Bashyam, S., Gokhale, V., Vankayalapati, H., Sun, D., Siddiqui-Jain, A., Streiner, N., Shin-Ya, K., White, E., Wilson, W. D., & Hurley, L. H. 2005, "Design and synthesis of an expanded porphyrin that has selectivity for the c-MYC G-quadruplex structure", *J. Am. Chem. Soc.*, vol. 127, no. 9, pp. 2944-2959.

Seimiya, H., Sawada, H., Muramatsu, Y., Shimizu, M., Ohko, K., Yamane, K., & Tsuruo, T. 2000, "Involvement of 14-3-3 proteins in nuclear localization of telomerase", *EMBO J.*, vol. 19, no. 11, pp. 2652-2661.

Sen, D. & Gilbert, W. 1988, "Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis", *Nature*, vol. 334, no. 6180, pp. 364-366.

Senger, D., Cairncross, J. G., & Forsyth, P. A. 2003, "Long-term survivors of glioblastoma: statistical aberration or important unrecognized molecular subtype?", *Cancer J.*, vol. 9, no. 3, pp. 214-221.

Sepp-Lorenzino, L., Ma, Z., Lebwohl, D. E., Vinitsky, A., & Rosen, N. 1995, "Herbimycin A induces the 20 S proteasome- and ubiquitin-dependent degradation of receptor tyrosine kinases", *J. Biol. Chem.*, vol. 270, no. 28, pp. 16580-16587.

Seto, A. G., Zaug, A. J., Sobel, S. G., Wolin, S. L., & Cech, T. R. 1999, "Saccharomyces cerevisiae telomerase is an Sm small nuclear ribonucleoprotein particle", *Nature*, vol. 401, no. 6749, pp. 177-180.

Shammas, M. A., Shmookler Reis, R. J., Li, C., Koley, H., Hurley, L. H., Anderson, K. C., & Munshi, N. C. 2004, "Telomerase inhibition and cell growth arrest after telomestatin treatment in multiple myeloma", *Clin. Cancer Res*, vol. 10, no. 2, pp. 770-776.

Sharma, H. W., Sokoloski, J. A., Perez, J. R., Maltese, J. Y., Sartorelli, A. C., Stein, C. A., Nichols, G., Khaled, Z., Telang, N. T., & Narayanan, R. 1995, "Differentiation of immortal cells inhibits telomerase activity", *Proc. Natl. Acad. Sci. U.S.A*, vol. 92, pp. 12343-12346.

Sharma, M., Ralte, A., Arora, R., Santosh, V., Shankar, S. K., & Sarkar, C. 2004, "Subependymal giant cell astrocytoma: a clinicopathological study of 23 cases with special emphasis on proliferative markers and expression of p53 and retinoblastoma gene proteins", *Pathology*, vol. 36, no. 2, pp. 139-144.

Sharp, P. A. 2001, "RNA interference--2001", *Genes Dev.*, vol. 15, no. 5, pp. 485-490.

Shay, J. W., Wright, W. E., Brasiskyte, D., & Van der Haegen, B. A. 1993, "E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts", *Oncogene*, vol. 8, no. 6, pp. 1407-1413.

Shay, J. W. & Bacchetti, S. 1997, "A survey of telomerase activity in human cancer", *Eur. J. Cancer*, vol. 33, no. 5, pp. 787-791.

Shay, J. W. & Wright, W. E. 2000, "Hayflick, his limit, and cellular ageing", *Nat. Rev. Mol. Cell Biol.*, vol. 1, no. 1, pp. 72-76.

Shelton, D. N., Chang, E., Whittier, P. S., Choi, D., & Funk, W. D. 1999, "Microarray analysis of replicative senescence", *Curr. Biol.*, vol. 9, no. 17, pp. 939-945.

Shih, I. M., Zhou, W., Goodman, S. N., Lengauer, C., Kinzler, K. W., & Vogelstein, B. 2001, "Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis", *Cancer Res.*, vol. 61, no. 3, pp. 818-822.

Shin-Ya, K., Wierzba, K., Matsuo, K., Ohtani, T., Yamada, Y., Furihata, K., Hayakawa, Y., & Seto, H. 2001, "Telomestatin, a novel telomerase inhibitor from Streptomyces anulatus", *J. Am. Chem. Soc.*, vol. 123, no. 6, pp. 1262-1263.

Shin, K. H., Kang, M. K., Dicterow, E., & Park, N. H. 2003, "Hypermethylation of the hTERT promoter inhibits the expression of telomerase activity in normal oral fibroblasts and senescent normal oral keratinocytes", *Br. J. Cancer*, vol. %20;89, no. 8, pp. 1473-1478.

Shroyer, K. R. 2002, "Immunohistochemical markers of cell immortality", *Hum. Pathol.*, vol. 33, no. 7, pp. 683-685.

Siebert, P. D., Chenchik, A., Kellogg, D. E., Lukyanov, K. A., & Lukyanov, S. A. 1995, "An improved PCR method for walking in uncloned genomic DNA", *Nucleic Acids Res.*, vol. 23, no. 6, pp. 1087-1088.

Simmler, M. C., Rouyer, F., Vergnaud, G., Nystrom-Lahti, M., Ngo, K. Y., de la, C. A., & Weissenbach, J. 1985, "Pseudoautosomal DNA sequences in the pairing region of the human sex chromosomes", *Nature*, vol. 317, no. 6039, pp. 692-697.

Simoes, J. P., Schoning, P., & Butine, M. 1994, "Prognosis of canine mast cell tumors: a comparison of three methods", *Vet. Pathol.*, vol. 31, no. 6, pp. 637-647.

Simon, M., Park, T. W., Leuenroth, S., Hans, V. H., Loning, T., & Schramm, J. 2000, "Telomerase activity and expression of the telomerase catalytic subunit, hTERT, in meningioma progression", *J Neurosurg.*, vol. 92, no. 5, pp. 832-840.

Singer, M. S. & Gottschling, D. E. 1994, "TLC1: template RNA component of Saccharomyces cerevisiae telomerase", *Science*, vol. 266, no. 5184, pp. 404-409.

Sinha-Datta, U., Horikawa, I., Michishita, E., Datta, A., Sigler-Nicot, J., Brown, M., Kazanji, M., Barrett, J. C., & Nicot, C. 2004, "Transcriptional activation of hTERT through the NF-KB pathway in HTLV-1-transformed cells", *Blood*, vol. 104, no. 8, pp. 2523-2531.

Slijepcevic, P. & Hande, M. P. 1999, "Chinese hamster telomeres are comparable in size to mouse telomeres", *Cytogenet. Cell Genet.*, vol. 85, no. 3-4, pp. 196-199.

Smith, D. L., Soria, J. C., Morat, L., Yang, Q., Sabatier, L., Liu, D. D., Nemr, R. A., Rashid, A., & Vauthey, J. N. 2004, "Human telomerase reverse transcriptase (hTERT) and Ki-67 are better predictors of survival than established clinical indicators in patients undergoing curative hepatic resection for colorectal metastases", *Ann. Surg. Oncol.*, vol. 11, no. 1, pp. 45-51.

Smogorzewska, A., van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M. R., Schnapp, G., & de Lange, T. 2000, "Control of human telomere length by TRF1 and TRF2", *Mol. Cell Biol.*, vol. 20, no. 5, pp. 1659-1668.

Song, J. S., Kim, H. P., Yoon, W. S., Lee, K. W., Kim, M. H., Kim, K. T., Kim, H. S., & Kim, Y. T. 2003, "Adenovirus-mediated suicide gene therapy using the human telomerase catalytic

subunit (hTERT) gene promoter induced apoptosis of ovarian cancer cell line", *Biosci. Biotechnol. Biochem.*, vol. 67, no. 11, pp. 2344-2350.

Song, J. S. 2004, "Activity of the human telomerase catalytic subunit (hTERT) gene promoter could be increased by the SV40 enhancer", *Biosci. Biotechnol. Biochem.*, vol. 68, no. 8, pp. 1634-1639.

Song, J. S. 2005, "Adenovirus-mediated suicide SCLC gene therapy using the increased activity of the hTERT promoter by the MMRE and SV40 enhancer", *Biosci. Biotechnol. Biochem.*, vol. 69, no. 1, pp. 56-62.

Soria, J. C., Moon, C., Wang, L., Hittelman, W. N., Jang, S. J., Sun, S. Y., Lee, J. J., Liu, D., Kurie, J. M., Morice, R. C., Lee, J. S., Hong, W. K., & Mao, L. 2001, "Effects of N-(4-hydroxyphenyl)retinamide on hTERT expression in the bronchial epithelium of cigarette smokers", *J. Natl. Cancer Inst.*, vol. 93, no. 16, pp. 1257-1263.

Southern, E. 1975, "Long range periodicities in mouse satellite DNA", *J. Mol. Biol.*, vol. 94, pp. 51-69.

Southern, E. 1979, "Gel electrophoresis of restriction fragments", *Methods in Enzymology*, vol. 68, pp. 152-176.

Stampfer, M. R., Garbe, J., Levine, G., Lichtsteiner, S., Vasserot, A. P., & Yaswen, P. 2001, "Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor beta growth inhibition in p16INK4A(-) human mammary epithelial cells", *Proc. Natl. Acad. Sci. U.S.A*, vol. 98, no. 8, pp. 4498-4503.

Stancato, L. F., Chow, Y. H., Hutchison, K. A., Perdew, G. H., Jove, R., & Pratt, W. B. 1993, "Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system", *J. Biol. Chem.*, vol. 268, no. 29, pp. 21711-21716.

Starling, J. A., Maule, J., Hastie, N. D., & Allshire, R. C. 1990, "Extensive telomere repeat arrays in mouse are hypervariable", *Nucleic Acids Res.*, vol. 18, no. 23, pp. 6881-6888.

Stepanova, L., Leng, X., Parker, S. B., & Harper, J. W. 1996, "Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4", *Genes Dev.*, vol. 10, no. 12, pp. 1491-1502.

Sternberger, L., Hardy, P. J., Cuculis, J., & Meyer, H. 1970, "The unlabelled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes", *J. Histochem. Cytochem.*, vol. 18, no. 5, pp. 315-333.

Stewart, S. A., Hahn, W. C., O'Connor, B. F., Banner, E. N., Lundberg, A. S., Modha, P., Mizuno, H., Brooks, M. W., Fleming, M., Zimonjic, D. B., Popescu, N. C., & Weinberg, R. A. 2002, "Telomerase contributes to tumorigenesis by a telomere length-independent mechanism", *Proc. Natl. Acad. Sci. U.S.A*, vol. 99, no. 20, pp. 12606-12611.

Strahl, C. & Blackburn, E. H. 1996, "Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines", *Mol. Cell Biol.*, vol. 16, no. 1, pp. 53-65.

Streutker, C. J., Thorner, P., Fabricius, N., Weitzman, S., & Zielenska, M. 2001, "Telomerase activity as a prognostic factor in neuroblastomas", *Ped. Dev. Pathol.*, vol. 4, pp. 62-67.

Summers, B. A., Cummings, J. F., & de Lahunta, A. 1995, "Tumors of the central nervous system," in *Veterinary Neuropathology*, 1 edn, B. A. Summers, J. F. Cummings, & A. de Lahunta, eds., Mosby, St Louis, pp. 351-401.

Sun, D., Thompson, B., Cathers, B. E., Salazar, M., Kerwin, S. M., Trent, J. O., Jenkins, T. C., Neidle, S., & Hurley, L. H. 1997, "Inhibition of human telomerase by a G-quadruplex-interactive compound", *J. Med. Chem.*, vol. 40, no. 14, pp. 2113-2116.

Sundquist, W. I. & Klug, A. 1989, "Telomeric DNA dimerizes by formation of guanine tetrads between hairpin loops", *Nature*, vol. 342, no. 6251, pp. 825-829.

Swiggers, S. J., Nibbeling, H. A., Zeilemaker, A., Kuijpers, M. A., Mattern, K. A., & Zijlmans, J. M. 2004, "Telomerase activity level, but not hTERT mRNA and hTR level, regulates telomere length in telomerase-reconstituted primary fibroblasts", *Exp. Cell Res.*, vol. 297, no. 2, pp. 434-443.

Szatmari, I., Tokes, S., Dunn, C. B., Bardos, T. J., & Aradi, J. 2000, "Modified telomeric repeat amplification protocol: a quantitative radioactive assay for telomerase without using electrophoresis", *Anal. Biochem.*, vol. 282, no. 1, pp. 80-88.

Tahara, H., Yasui, W., Tahara, E., Fujimoto, J., Ito, K., Tamai, K., Nakayama, J., Ishikawa, F., Tahara, E., & Ide, T. 1999, "Immuno-histochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections", *Oncogene*, vol. 18, no. 8, pp. 1561-1567.

Takahashi, J. A., Ueba, T., Hashimoto, N., Nakashima, Y., & Katsuki, N. 2004, "The combination of mitotic and Ki-67 indices as a useful method for predicting short-term recurrence of meningiomas", *Surg. Neurol.*, vol. 61, no. 2, pp. 149-155.

Takakura, M., Kyo, S., Kanaya, T., Tanaka, M., & Inoue, M. 1998, "Expression of human telomerase subunits and correlation with telomerase activity in cervical cancer", *Cancer Res*, vol. 58, no. 7, pp. 1558-1561.

Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., & Inoue, M. 1999, "Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells", *Cancer Res.*, vol. 59, no. 3, pp. 551-557.

Takeda, T., Inaba, H., Yamazaki, M., Kyo, S., Miyamoto, T., Suzuki, S., Ehara, T., Kakizawa, T., Hara, M., DeGroot, L. J., & Hashizume, K. 2003, "Tumor-specific gene therapy for undifferentiated thyroid carcinoma utilizing the telomerase reverse transcriptase promoter", *J. Clin. Endocrinol. Metab*, vol. 88, no. 8, pp. 3531-3538.

Takeuchi, H., Kanzawa, T., Kondo, Y., Komata, T., Hirohata, S., Kyo, S., & Kondo, S. 2004, "Combination of caspase transfer using the human telomerase reverse transcriptase promoter and conventional therapies for malignant glioma cells", *Int. J. Oncol.*, vol. 25, no. 1, pp. 57-63.

Taki, M., Kagawa, S., Nishizaki, M., Mizuguchi, H., Hayakawa, T., Kyo, S., Nagai, K., Urata, Y., Tanaka, N., & Fujiwara, T. 2005, "Enhanced oncolysis by a tropism-modified telomerase-specific replication-selective adenoviral agent OBP-405 ('Telomelysin-RGD')", *Oncogene*, vol. 24, no. 19, pp. 3130-3140.

Tanaka, M., Kyo, S., Takakura, M., Kanaya, T., Sagawa, T., Yamashita, K., Okada, Y., Hiyama, E., & Inoue, M. 1998, "Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a menstrual phase-dependent manner correlated with cell proliferation", *Am. J. Pathol.*, vol. 153, no. 6, pp. 1985-1991.

Tauchi, T., Shin-Ya, K., Sashida, G., Sumi, M., Nakajima, A., Shimamoto, T., Ohyashiki, J. H., & Ohyashiki, K. 2003, "Activity of a novel G-quadruplex-interactive telomerase inhibitor, telomestatin (SOT-095), against human leukemia cells: involvement of ATM-dependent DNA damage response pathways", *Oncogene*, vol. 22, no. 34, pp. 5338-5347.

Taylor, H. A. & Delany, M. E. 2000, "Ontogeny of telomerase in chicken: impact of downregulation on pre- and postnatal telomere length in vivo", *Dev. Growth Differ.*, vol. 42, no. 6, pp. 613-621.

Tchirkov, A., Rolhion, C., Kemeny, J. L., Irthum, B., Puget, S., Khalil, T., Chinot, O., Kwiatkowski, F., Perissel, B., Vago, P., & Verrelle, P. 2003, "Clinical implications of quantitative real-time RT-PCR analysis of hTERT gene expression in human gliomas", *Br. J. Cancer*, vol. 88, no. 4, pp. 516-520.

Theimer, C. A., Blois, C. A., & Feigon, J. 2005, "Structure of the human telomerase RNA pseudoknot reveals conserved tertiary interactions essential for function", *Mol. Cell*, vol. 17, no. 5, pp. 671-682.

Theon, A. P., Lecouteur, R. A., Carr, E. A., & Griffey, S. M. 2000, "Influence of tumor cell proliferation and sex-hormone receptors on effectiveness of radiation therapy for dogs with incompletely resected meningiomas", *J. Am. Vet. Med. Assoc.*, vol. 216, no. 5, pp. 701-705.

Thomas, E., al Baker, E., Dropcova, S., Denyer, S., Ostad, N., Lloyd, A., Kill, I. R., & Faragher, R. G. 1997, "Different kinetics of senescence in human fibroblasts and peritoneal mesothelial cells", *Exp. Cell Res*, vol. 236, no. 1, pp. 355-358.

Thomson, S. A. M., Kennerly, E., Olby, N., Mickelson, J. R., Hoffman, D. E., Dickinson, P. J., Gibson, G., & Breen, M. 2005, "Microarray analysis of differentially expressed genes of primary tumors in the canine central nervous system", *Vet. Pathol.*, vol. 42, pp. 550-558.

Toschi, L. & Bravo, R. 1988, "Changes in cyclin/proliferating cell nuclear antigen distribution during DNA repair synthesis", *J. Cell Biol.*, vol. 107, no. 5, pp. 1623-1628.

Turrel, J. M., Fike, J. R., LeCouteur, R. A., & Higgins, R. J. 1986, "Computed tomographic characteristics of primary brain tumors in 50 dogs.", *J. Am. Vet. Med. Assoc.*, vol. 188, no. 8, pp. 851-856.

Uchida, K., Nakayama, H., Endo, Y., Kai, C., Tatewaki, S., Yamaguchi, R., Doi, K., & Tateyama, S. 2003, "Ganglioglioma in the thalamus of a puppy", *J. Vet. Med. Sci.*, vol. 65, no. 1, pp. 113-115.

Uhlenbeck, O. & Gumport, R. 1982, "T4 DNA Ligase," in *The Enzymes*, P. Boyer, ed., Academic Press, San Diego, CA.

Ulaner, G. A., Hu, J. F., Vu, T. H., Giudice, L. C., & Hoffman, A. R. 1998, "Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts", *Cancer Res.*, vol. 58, no. 18, pp. 4168-4172.

Ulaner, G. A., Hu, J. F., Vu, T. H., Oruganti, H., Giudice, L. C., & Hoffman, A. R. 2000, "Regulation of telomerase by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium", *Int. J. Cancer*, vol. 85, no. 3, pp. 330-335.

Umeoka, T., Kawashima, T., Kagawa, S., Teraishi, F., Taki, M., Nishizaki, M., Kyo, S., Nagai, K., Urata, Y., Tanaka, N., & Fujiwara, T. 2004, "Visualization of intrathoracically disseminated solid tumors in mice with optical imaging by telomerase-specific amplification of a transferred green fluorescent protein gene", *Cancer Res.*, vol. 64, no. 17, pp. 6259-6265.

van den Bent, M. J. 2004, "Advances in the biology and treatment of oligodendrogliomas", *Curr. Opin. Neurol.*, vol. 17, no. 6, pp. 675-680.

van Engeland, M., Nieland, L. J. W., Ramaekers, F. C. S., Schutte, B., & Reutelingsperger, C. P. M. 1998, "Annexin V-Affinity Assay: A review on an apoptosis detection system based on phosphatidylserine exposure", *Cytometry*, vol. 31, pp. 1-9.

Varley, H., Pickett, H. A., Foxon, J. L., Reddel, R. R., & Royle, N. J. 2002, "Molecular characterization of inter-telomere and intra-telomere mutations in human ALT cells", *Nat. Genet.*, vol. 30, no. 3, pp. 301-305.

Vaziri, H. & Benchimol, S. 1998, "Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span", *Curr. Biol.*, vol. 8, no. 5, pp. 279-282.

Veldscholte, J., Berrevoets, C. A., Brinkmann, A. O., Grootegoed, J. A., & Mulder, E. 1992, "Anti-androgens and the mutated androgen receptor of LNCaP cells: differential effects on binding affinity, heat-shock protein interaction, and transcription activation", *Biochemistry*, vol. 31, no. 8, pp. 2393-2399.

Venkatesan, R. N. & Price, C. 1998, "Telomerase expression in chickens: constitutive activity in somatic tissues and down-regulation in culture", *Proc. Natl. Acad. Sci. U.S.A*, vol. 95, no. 25, pp. 14763-14768.

Verhoven, B., Schlegel, R. A., & Williamson, P. 1995, "Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes", *J. Exp. Med.*, vol. 182, no. 5, pp. 1597-1601.

Vermes, I., Haanen, C., Steffens-Nakken, H., & Reutelingsperger, C. 1995, "A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V", *J. Immunol. Methods*, vol. 184, no. 1, pp. 39-51.

Villa, R., Folini, M., Porta, C. D., Valentini, A., Pennati, M., Daidone, M. G., & Zaffaroni, N. 2003, "Inhibition of telomerase activity by geldanamycin and 17-allylamino, 17-demethoxygeldanamycin in human melanoma cells", *Carcinogenesis*, vol. 24, no. 5, pp. 851-859.

Vite, C. H., McGowan, J. C., Niogi, S. N., Passini, M. A., Drobatz, K. J., Haskins, M. E., & Wolfe, J. H. 2005, "Effective gene therapy for an inherited CNS disease in a large animal model", *Ann. Neurol.*, vol. 57, no. 3, pp. 355-364.

von Zglinicki, T., Saretzki, G., Docke, W., & Lotze, C. 1995, "Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence?", *Exp. Cell Res*, vol. 220, no. 1, pp. 186-193.

Waguri, N., Suda, T., Nomoto, M., Kawai, H., Mita, Y., Kuroiwa, T., Igarashi, M., Kobayashi, M., Fukuhara, Y., & Aoyagi, Y. 2003, "Sensitive and specific detection of circulating cancer cells in patients with hepatocellular carcinoma; detection of human telomerase reverse transcriptase messenger RNA after immunomagnetic separation", *Clin. Cancer Res*, vol. 9, no. 8, pp. 3004-3011.

Wang, J., Xie, L. Y., Allan, S., Beach, D., & Hannon, G. J. 1998, "Myc activates telomerase", *Genes Dev.*, vol. 12, no. 12, pp. 1769-1774.

Wang, E. S., Wu, K., Chin, A. C., Chen-Kiang, S., Pongracz, K., Gryaznov, S., & Moore, M. A. 2004, "Telomerase inhibition with an oligonucleotide telomerase template antagonist: in vitro and in vivo studies in multiple myeloma and lymphoma", *Blood*, vol. 103, no. 1, pp. 258-266.

Weil, R. J., Wu, Y. Y., Vortmeyer, A. O., Moon, Y. W., Delgado, R. M., Fuller, B. G., Lonser, R. R., Remaley, A. T., & Zhuang, Z. 1999, "Telomerase activity in microdissected human gliomas", *Mod. Pathol.*, vol. 12, no. 1, pp. 41-46.

Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Tesmer, V. M., Holt, S. E., Bodnar, A. G., Lichtsteiner, S., Kim, N. W., Trager, J. B., Taylor, R. D., Carlos, R., Andrews, W. H., Wright, W. E., Shay, J. W., Harley, C. B., & Morin, G. B. 1997, "Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT", *Nat. Genet.*, vol. 17, no. 4, pp. 498-502.

Weiss C & Bohmann D 2004, "Deregulated repression of c-Jun provides a potential link to its role intumorigenesis", *Cell Cycle*, vol. 3, no. 2, pp. 111-113.

Wellinger, R. J., Ethier, K., Labrecque, P., & Zakian, V. A. 1996, "Evidence for a new step in telomere maintenance", *Cell*, vol. 85, no. 3, pp. 423-433.

Wellinger, R. J. & Sen, D. 1997, "The DNA structures at the ends of eukaryotic chromosomes", *Eur. J. Cancer*, vol. 33, no. 5, pp. 735-749.

Weng, N. P., Levine, B. L., June, C. H., & Hodes, R. J. 1996, "Regulated expression of telomerase activity in human T lymphocyte development and activation", *J. Exp. Med.*, vol. 183, no. 6, pp. 2471-2479.

Wenz, C., Enenkel, B., Amacker, M., Kelleher, C., Damm, K., & Lingner, J. 2001, "Human telomerase contains two cooperating telomerase RNA molecules", *EMBO J.*, vol. 20, no. 13, pp. 3526-3534.

Wheelhouse, R. T., Sun, D., Han, H., Han, F. X., & Hurley, L. H. 1998, "Cationic porphyrins as telomerase inhibitors: the interaction of tetra-(N-methyl-4-pyridyl)porphine with quadruplex DNA", *J. Am. Chem. Soc.*, vol. 120, pp. 3261-3262.

Wick, M., Zubov, D., & Hagen, G. 1999, "Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT)", *Gene*, vol. 232, no. 1, pp. 97-106.

Wickham, T. J., Mathias, P., Cheresh, D. A., & Nemerow, G. R. 1993, "Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment", *Cell*, vol. 73, no. 2, pp. 309-319.

Widschwendter, A., Muller, H. M., Hubalek, M. M., Wiedemair, A., Fiegl, H., Goebel, G., Mueller-Holzner, E., Marth, C., & Widschwendter, M. 2004, "Methylation status and expression of human telomerase reverse transcriptase in ovarian and cervical cancer", *Gynecol. Oncol.*, vol. 93, no. 2, pp. 407-416.

Wikstrand, C. J., Fung, K., Trojanowski, J. Q., McLendon, R. E., & Bigner, D. D. 1998, "Antibodies and molecular immunology," in *Russell and Rubinstein's Pathology of Tumors of the Nervous System*, 6 edn, D. D. Bigner, R. E. McLendon, & J. M. Bruner, eds., Arnold, London, pp. 251-304.

Wilkie, A. O., Higgs, D. R., Rack, K. A., Buckle, V. J., Spurr, N. K., Fischel-Ghodsian, N., Ceccherini, I., Brown, W. R., & Harris, P. C. 1991, "Stable length polymorphism of up to 260 kb at the tip of the short arm of human chromosome 16", *Cell*, vol. 64, no. 3, pp. 595-606.

Williamson, J. R., Raghuraman, M. K., & Cech, T. R. 1989, "Monovalent cation-induced structure of telomeric DNA: the G-quartet model", *Cell*, vol. 59, no. 5, pp. 871-880.

Wirth, T., Zender, L., Schulte, B., Mundt, B., Plentz, R., Rudolph, K. L., Manns, M., Kubicka, S., & Kuhnel, F. 2003, "A telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer", *Cancer Res*, vol. 63, no. 12, pp. 3181-3188.

Withrow, S. J. & MacEwan, E. G. 2001, *Small Animal Clinical Oncology*, 3 edn, W.B. Saunders, Philadelphia, PA.

Wong, J. M., Kusdra, L., & Collins, K. 2002, "Subnuclear shuttling of human telomerase induced by transformation and DNA damage", *Nat. Cell Biol.*, vol. 4, no. 9, pp. 731-736.

Wright, W. E. & Hayflick, L. 1975, "Nuclear control of cellular aging demonstrated by hybridization of anucleate and whole cultured normal human fibroblasts", *Exp. Cell Res.*, vol. 96, no. 1, pp. 113-121.

Wright, W. E. & Shay, J. W. 1992, "Telomere positional effects and the regulation of cellular senescence", *Trends Genet.*, vol. 8, no. 6, pp. 193-197.

Wright, W. E., Shay, J. W., & Piatyszek, M. A. 1995, "Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity", *Nucleic Acids Res.*, vol. 23, no. 18, pp. 3794-3795.

Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W., & Shay, J. W. 1996, "Telomerase activity in human germline and embryonic tissues and cells", *Dev. Genet.*, vol. 18, no. 2, pp. 173-179.

Wright, W. E., Tesmer, V. M., Huffman, K. E., Levene, S. D., & Shay, J. W. 1997, "Normal human chromosomes have long G-rich telomeric overhangs at one end", *Genes Dev.*, vol. 11, no. 21, pp. 2801-2809.

Wu, K. J., Grandori, C., Amacker, M., Simon-Vermot, N., Polack, A., Lingner, J., & Dalla-Favera, R. 1999, "Direct activation of TERT transcription by c-MYC", *Nat. Genet.*, vol. 21, no. 2, pp. 220-224.

Wu, Y. Y., Hruszkewycz, A. M., Delgado, R. M., Yang, A., Vortmeyer, A. O., Moon, Y. W., Weil, R. J., Zhuang, Z., & Remaley, A. T. 2000, "Limitations on the quantitative determination of telomerase activity by the electrophoretic and ELISA based TRAP assays", *Clin. Chim. Acta*, vol. 293, no. 1-2, pp. 199-212.

Wynford-Thomas, D. 1997, "Proliferative lifespan checkpoints: cell-type specificity and influence on tumour biology", *Eur. J. Cancer*, vol. 33, no. 5, pp. 716-726.

Wynford-Thomas, D. 1999, "Cellular senescence and cancer", *J. Pathol.*, vol. 187, no. 1, pp. 100-111.

Xia, J., Peng, Y., Mian, I. S., & Lue, N. F. 2000, "Identification of functionally important domains in the N-terminal region of telomerase reverse transcriptase", *Mol. Cell Biol.*, vol. 20, no. 14, pp. 5196-5207.

Xu, D., Popov, N., Hou, M., Wang, Q., Bjorkholm, M., Gruber, A., Menkel, A. R., & Henriksson, M. 2001, "Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells", *Proc. Natl. Acad. Sci. U.S.A*, vol. 98, no. 7, pp. 3826-3831.

Yan, P., Coindre, J. M., Benhattar, J., Bosman, F. T., & Guillou, L. 1999, "Telomerase activity and human telomerase reverse transcriptase mRNA expression in soft tissue tumors: correlation with grade, histology, and proliferative activity", *Cancer Res.*, vol. 59, no. 13, pp. 3166-3170.

Yashima, K., Maitra, A., Rogers, B. B., Timmons, C. F., Rathi, A., Pinar, H., Wright, W. E., Shay, J. W., & Gazdar, A. F. 1998, "Expression of the RNA component of telomerase during human development and differentiation", *Cell Growth Differ.*, vol. 9, no. 9, pp. 805-813.

Yatabe, N., Kyo, S., Maida, Y., Nishi, H., Nakamura, M., Kanaya, T., Tanaka, M., Isaka, K., Ogawa, S., & Inoue, M. 2004, "HIF-1-mediated activation of telomerase in cervical cancer cells", *Oncogene*, vol. 23, no. 20, pp. 3708-3715.

Yazawa, M., Okuda, M., Setoguchi, A., Iwabuchi, S., Nishimura, R., Sasaki, N., Masuda, K., Ohno, K., & Tsujimoto, H. 2001, "Telomere length and telomerase activity in canine mammary gland tumors", *Am. J. Vet. Res.*, vol. 62, no. 10, pp. 1539-1543.

Yi, X., Shay, J. W., & Wright, W. E. 2001, "Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells", *Nucleic Acids Res*, vol. 29, no. 23, pp. 4818-4825.

Yin, J., Fu, P., Wang, X., Jin, G., & Xiu, R. 2004, "Tumor-specific expression detected with the use of an expression vector driven by human telomerase reverse transcriptase gene promoter", *J. Lab. Clin. Med.*, vol. 144, no. 6, pp. 302-306.

Yokoyama, Y., Takahashi, Y., Shinohara, A., Lian, Z., Wan, X., Niwa, K., & Tamaya, T. 1998, "Attenuation of telomerase activity by a hammerhead ribozyme targeting the template region of telomerase RNA in endometrial carcinoma cells", *Cancer Res*, vol. 58, no. 23, pp. 5406-5410.

Zacchetti, A., van Garderen, E., Teske, E., Nederbragt, H., Dierendonck, J. H., & Rutteman, G. R. 2003, "Validation of the use of proliferation markers in canine neoplastic and non-neoplastic tissues: comparison of KI-67 and proliferating cell nuclear antigen (PCNA) expression versus in vivo bromodeoxyuridine labelling by immunohistochemistry", *APMIS*, vol. 111, no. 3, pp. 430-438.

Zaffaroni, N., Della, P. C., Villa, R., Botti, C., Buglioni, S., Mottolese, M., & Grazia, D. M. 2002, "Transcription and alternative splicing of telomerase reverse transcriptase in benign and malignant breast tumours and in adjacent mammary glandular tissues: implications for telomerase activity", *J. Pathol.*, vol. 198, no. 1, pp. 37-46.

Zahler, A. M. & Prescott, D. M. 1989, "DNA primase and the replication of the telomeres in Oxytricha nova", *Nucleic Acids Res*, vol. 17, no. 15, pp. 6299-6317.

Zakian, V. A. 1995, "Telomeres: beginning to understand the end", *Science*, vol. 270, no. 5242, pp. 1601-1607.

Zhang, X., Mar, V., Zhou, W., Harrington, L., & Robinson, M. O. 1999, "Telomere shortening and apoptosis in telomerase-inhibited human tumor cells", *Genes Dev.*, vol. 13, no. 18, pp. 2388-2399.

Zhang, R., Wang, X., Guo, L., & Xie, H. 2002, "Growth inhibition of BEL-7404 human hepatoma cells by expression of mutant telomerase reverse transcriptase", *Int. J. Cancer*, vol. 97, no. 2, pp. 173-179.

Zhao, J. Q., Hoare, S. F., McFarlane, R., Muir, S., Parkinson, E. K., Black, D. M., & Keith, W. N. 1998, "Cloning and characterization of human and mouse telomerase RNA gene promoter sequences", *Oncogene*, vol. 16, no. 10, pp. 1345-1350.

Zhou, X. Z. & Lu, K. P. 2001, "The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor", *Cell*, vol. 107, no. 3, pp. 347-359.

Zhu, J., Zhang, L., Hanisch, U. K., Felgner, P. L., & Reszka, R. 1996, "A continuous intracerebral gene delivery system for in vivo liposome-mediated gene therapy", *Gene Ther.*, vol. 3, no. 6, pp. 472-476.

Zhu, J., Wang, H., Bishop, J. M., & Blackburn, E. H. 1999, "Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening", *Proc. Natl. Acad. Sci. U.S.A.*, vol. 96, no. 7, pp. 3723-3728.

Zhu, H., Fu, W., & Mattson, M. P. 2000, "The catalytic subunit of telomerase protects neurons against amyloid beta-peptide-induced apoptosis", *J. Neurochem.*, vol. 75, no. 1, pp. 117-124.

Zhu, Y., Tomlinson, R. L., Lukowiak, A. A., Terns, R. M., & Terns, M. P. 2004, "Telomerase RNA accumulates in Cajal bodies in human cancer cells", *Mol. Biol. Cell*, vol. 15, no. 1, pp. 81-90.

Zimmermann, S., Voss, M., Kaiser, S., Kapp, U., Waller, C. F., & Martens, U. M. 2003, "Lack of telomerase activity in human mesenchymal stem cells", *Leukemia*, vol. 17, no. 6, pp. 1146-1149.

Zingg, J. M. & Jones, P. A. 1997, "Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis", *Carcinogenesis*, vol. 18, no. 5, pp. 869-882.

Zou, W., Luo, C., Zhang, Z., Liu, J., Gu, J., Pei, Z., Qian, C., & Liu, X. 2004, "A novel oncolytic adenovirus targeting to telomerase activity in tumor cells with potent", *Oncogene*, vol. 23, no. 2, pp. 457-464.

Zou, L., Zhang, P., Luo, C., & Tu, Z. 2005, "shRNA-targeted hTERT suppresses cell proliferation of bladder cancer by inhibiting telomerase activity", *Cancer Chemother. Pharmacol.*