# Development of telomerase targeted gene therapy for treatment of cancer.

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***For Poonam……***

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# Abbreviations

| | |
|---|---|
| **AAV** | Adeno associated virus |
| **AdV** | Adenovirus |
| **ALT** | Alternative lengthening of telomeres |
| **BCA** | Bichinchoninic Acid |
| **bp** | Base pairs |
| **BPV** | Bovine papillomavirus |
| **BSA** | Bovine serum albumin |
| **°C** | Degree centigrade |
| **CAR** | Coxsackie and adenovirus receptor |
| **CAV** | Canine adenovirus |
| **cm** | Centimeters |
| **CMV** | Cytomegalovirus |
| **CPE** | Cytopathic effect |
| **CRAd** | Conditionally replicative adenovirus |
| **DAPI** | 4'-6-Diamidino-2-phenylindole |
| **DMEM** | Dulbecco's modified Eagle's medium |
| **DNA** | Deoxyribonucleic acid |
| **E.coli** | Escherichia coli |
| **ECL** | Enhanced chemiluminescence |
| **F** | Farad |
| **FCS** | Fetal calf serum |
| **g** | G-Force |
| **GFP** | Green fluorescent protein |
| **hAdV** | Human adenovirus |

| | |
|---|---|
| **HIV** | Human immuno deficiency virus |
| **HSV-1** | Human herpes virus type 1 |
| **HSV-tk** | Herpes simplex virus thymidine kinase |
| **IgG** | Immunoglobulin G |
| **kDa** | Kilo Dalton |
| **l** | Litre |
| **M** | Mol |
| **µg** | Microgram |
| **µl** | Microlitre |
| **min** | Minutes |
| **ml** | Millilitre |
| **mm** | Millimetres |
| **mRNA** | Messenger ribonucleic acid |
| **OD** | Optical density |
| **PARP** | Poly (ADP-ribose) polymerase |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **Pfu** | Plaque forming units |
| **PSA** | Prostate specific antigen |
| **RFP** | Red fluorescent protein |
| **RNA** | Ribonucleic acid |
| **rpm** | Revolutions per minute |
| **rTdT** | Recombinant terminal deoxynucleotidyl transferase |
| **SCID-X1** | X-linked severe combined immune deficiency |
| **sec** | Seconds |
| **siRNA** | Small interfering ribonucleic acid |
| **SV40** | Simian virus 40 |

| | |
|---|---|
| **TERT** | Telomerase reverse transcriptase |
| **tk** | Thymidine kinase |
| **TNF** | Tumour necrosis factor |
| **TR** | Telomerase RNA template |
| **TRAIL** | Tumour necrosis factor related apoptosis inducing ligand |
| **TRAIL-R** | Tumour necrosis factor related apoptosis inducing ligand receptor |
| **TSTA** | Two step transcriptional amplification |
| **UV** | Ultraviolet |
| **v/v** | Volume per unit volume |
| **V** | Volt |
| **w/v** | Weight per unit volume |

**Single letter amino acid code**

| | |
|---|---|
| **Alanine** | **Ala (A)** |
| **Arginine** | **Arg (R)** |
| **Asparagine** | **Asn (N)** |
| **Aspartic acid** | **Asp (D)** |
| **Cysteine** | **Cys (C)** |
| **Glutamic acid** | **Glu (E)** |
| **Glutamine** | **Gln (Q)** |
| **Glycine** | **Gly (G)** |
| **Histidine** | **His (H)** |
| **Isoleucine** | **Ile (I)** |
| **Leucine** | **Leu (L)** |
| **Lysine** | **Lys (K)** |
| **Methionine** | **Met (M)** |
| **Phenylalanine** | **Phe (F)** |
| **Proline** | **Pro (P)** |
| **Serine** | **Ser (S)** |
| **Threonine** | **Thr (T)** |
| **Tryptophan** | **Trp (W)** |
| **Tyrosine** | **Tyr (Y)** |
| **Valine** | **Val (V)** |

# Abstract

Gene therapy is an area that is currently developing for treatment of various congenital and acquired diseases. The basic concept is that a transgene is expressed in a target cell resulting in alleviation of disease. In general there are certain obstacles limiting gene therapy regardless of the target disease. These include restriction of transgene expression to target cells, inadequate transgene expression and ineffective *in vivo* transgene delivery.

Cancer is one of the leading diseases to cause death in the human and canine population. For this reason gene therapy has been studied as a potential novel treatment for cancer.

Two step transcriptional amplification system is a system applied to gene therapy in order to improve efficacy. In this system a relatively weak tissue or disease specific promoter initiates the transcription of a transcriptional activator protein. This protein when expressed in target cells binds to binding sites upstream of a secondary promoter which then drives the transcription of the transgene of interest at a high level.

By use of a two step amplification mechanism and a novel transcriptional activator fusion protein VP16E2, we increased transgene expression compared to the commonly used Gal4VP16 transcriptional activator fusion protein. By incorporating the human telomerase promoter hTERT as a primary promoter and the minimal 6xE23'BpTATA promoter as a secondary promoter, transgene expression is maintained in telomerase positive cells representing cancer cells, but is restricted in telomerase negative cell lines. By inserting the tumour necrosis factor apoptosis inducing ligand (TRAIL) gene as a transgene in this system we have shown that apoptosis is elicited in telomerase positive cells although not in telomerase negative cells.

The similarities in incidence and nature of human and canine cancer forms and the need for a better translational model for human disease has put canines in focus for cancer studies and drug development. Conditionally replicative oncolytic viruses developed in humans are species specific and therefore can not be studied satisfactorily in rodent preclinical models. We have made a mutant adenovirus based on the canine adenovirus type 1. We have put the viral replication under control of our two step amplification mechanism making the viral replication dependent on telomerase activity of the infected cell. This virus is able to kill telomerase positive canine cells but not

telomerase negative canine cells *in vitro*. This virus is a potential tool for studying a conditionally replicative oncolytic virus in vivo in naturally occurring canine cancer cases. This is a good translational model for human cancer treatment and thereby benefits both species.

# Chapter 1.

## Introduction and background information.

Gene therapy is a relatively new science which has been developed as an approach to treat acquired and congenital disorders for which other treatment options are inadequate. Gene therapy is simplistically defined as the transfer of genetic material into cells of an organism for the purpose of curing or alleviating disease. This definition covers a wide range of methods depending on the application. The first approved gene therapy trial was carried out in 1990 and since then numerous trials have been carried out for treatment of various diseases (Trent & Alexander 2004). The outcome of the gene therapy trials to date has been disappointing due to challenges in targeting of transgene expression to target cells, insufficient transgene expression and inadequate in vivo transgene delivery. One success in gene therapy is the correction of the genetic defect in X-linked severe combined immune deficiency (SCID-X1) children after in vitro retroviral transduction of T cells.

There are major considerations to be taken into account when developing a gene therapy system. Targeting the cell populations of interest is an important safety issue since transgene expression in "off target" cells can have adverse effects. A cell population can be targeted by considering anatomical compartmental restrictions of transgene delivery or looking at target cell phenotypic characteristics which could be used for targeting gene expression to the cells. The blood retina barrier and the anatomy of the eye as an example offer an opportunity for local gene delivery to the eye with minimal risk of contamination to "off target" sites. The altered cellular gene expression patterns in specific tissues or diseases offer an opportunity to design gene expression systems that will function only in these cells by use of disease or tissue specific promoters controlling the gene expression.

The magnitude of transgene expression in cells is a limitation and is often not adequate to elicit a therapeutic effect. This is particularly pronounced in conjunction with tissue or disease specific promoters since these are relatively weak. Therefore two step transcriptional amplification (TSTA) systems have been developed in order to increase the magnitude of gene expression elicited by the tissue or disease specific promoters. The concept of these systems is to be able to preserve the cellular sensitivity

of the promoters to the target cells albeit at the same time allowing for a high level of transgene expression without compromising the specificity of the system.

In vivo transgene delivery is a major challenge. This can in some circumstances be traversed by harvesting of cells and transducing them in vitro before returning them to the host as an allograft. Several gene delivery mechanisms for in vivo delivery are being developed of either viral or non viral origin. When considering a gene delivery mechanism one should take into account what sort of gene expression is required for a therapeutic outcome. Congenital disorders will often require life long transgene expression in order to antagonise clinical symptoms. This can be achieved by using gene vectors which allow for genomic integration and hence stable expression of the transgene. This can be accomplished by using integrating viruses such as retroviruses or adeno associated virus however these types of vectors harbour a great risk for insertional mutagenesis (Hacein-Bey-Abina et al. 2003). For acquired conditions such as cancer, fractures or tissue repair only transient transgene expression is required until the condition is eliminated and therefore a broader range of both viral and non viral non integrating transgene delivery systems can be considered.

Despite efforts in engineering highly developed systems to target gene expression to specific cells, to amplify the transgene transcription and to deliver the transgenes to cells the efficacy of current systems is low and there is a demand for improvement of current systems and a need for better in vivo animal models.

## 1.1 Gene therapy.

Gene therapy can be categorised as replacement gene therapy, immunotherapy, suicide gene therapy, effect gene therapy, protective gene therapy, silencing gene therapy and oncolytic gene therapy; each category aimed at specific diseases for which they are suitable.

Originally gene therapy was thought of as a method to cure or alleviate fatal diseases, however recent studies also focus on non fatal diseases such as arthritis, fracture repair, retinal degeneration and skin diseases for which no successful treatment option is applicable and for which the life quality of the patient could be radically improved by gene therapy (Alton 2007, Alton et al. 2007, Cotrim & Baum 2008, Mochizuki et al. 2008).

### 1.1.1 Replacement gene therapy

Replacement gene therapy is, as the name indicates, expression of a functional exogenous gene for which the cells are deficient. This type of gene therapy has therapeutic value for congenital or acquired disorders in which a gene product is faulty or absent either systemically or in a certain location or cell type. Examples of diseases in which replacement gene therapy is a potential treatment option with the corrective transgene are diseases such as cystic fibrosis with the cystic fibrosis transmembrane regulator gene (Selkirk 2004), Duchene muscular dystrophy with the dystrophin transgene (Alton 2007, Selkirk 2004), lysosomal storage disease with lysosomal enzymes as transgenes (Alton 2007), haemophilia with factor VIII or IX as transgenes (Alton et al. 2007), Parkinson's disease with dopamine expressing transgenes (Mochizuki et al. 2008) and cancer with the tumour suppressor gene p53 as a transgene (El-Aneed 2004b, Selkirk 2004). Some major obstacles exist for replacement gene therapy especially for congenital genetic disorders. Since these diseases are genetic disorders, a permanent lifelong expression of the transgene is often required to reverse clinical symptoms. On the other hand, since the gene used as a transgene is often not expressed in the diseased individual there is a significant risk of the individual producing antibodies against the transgene and thereby nullifying the therapeutic effect (Zaldumbide & Hoeben 2007). Another problem is the restriction and regulation of the gene expression to certain cell types without exceeding normal concentrations resulting in adverse effects from overproduction. Under normal physiological conditions the cells are able to regulate the gene expression by various mechanisms however such mechanism will often not apply to exogenous expressed genes.

The only successful gene therapy trial resulting in correction of a genetic disorder in humans to date is the reconstitution of a long lasting immune response in SCID-X1 affected children (Cavazzana-Calvo et al. 2000, Hacein-Bey-Abina et al. 2003, Trent & Alexander 2004, Alton 2007, Cavazzana-Calvo & Fischer 2007, Sokolic et al. 2008). In this trial bone marrow was harvested from the host and CD34+ lymphocytes were isolated and infected repetitively with a retroviral vector encoding the γc cytokine receptor gene which is absent in this disease. The treated cells were redelivered to the host as an allograft resulting in circulation of T-cells expressing the γc cytokine receptor gene and correction of clinical symptoms (Cavazzana-Calvo et al. 2000). To date successful reconstitution of long term immune response has been

achieved in 17 out of 20 treated patients (Sokolic et al. 2008). A major drawback for this treatment was development of leukaemia in 5 of the 17 successfully treated patients which lead to death in one patient (Sokolic et al. 2008). The development of leukaemia was due to insertional mutagenesis by integration of the viral vector in the LMO2 proto-oncogene leading to increased expression of this gene resulting in carcinogenesis (Hacein-Bey-Abina et al. 2003, Bester et al. 2006). Despite the success in correcting the SCID-X1 genetic disorder the severe side effects in the form of leukaemia observed with this trial has encouraged development of safer gene therapy treatment regimes.

### 1.1.2 Immunotherapy

Immunotherapy is an area of gene therapy in which the immune system is activated to recognise the transgene. This can be done indirectly by inducing transgene expression in normal tissue and thereby evoking an immune response by stimulating antigen presenting cells or directly by in vitro transfection of antigen presenting cells with the antigen of interest (El-Aneed 2004b). Immuno gene therapy is currently being developed for treatment of different types of cancers since this procedure could offer a possibility of targeting distant tumour metastasis and residual cancer cells after surgery (Bergman et al. 2003, Barough 2006, Domcheck et al. 2007, Yamano et al. 2007, Wolchok et al. 2007, Bird et al. 2008, Hunder et al. 2008).

### 1.1.3 Suicide gene therapy

Suicide gene therapy is a form of gene therapy in which the exogenous gene is an enzyme which when expressed in a cell can cleave a pro-drug to its active cytotoxic form and thereby kill the host cell. This treatment form is being developed for treatment of various types of cancer (Nathwani 2004, Selkirk 2004, Lawler 2006 Alton et al. 2007). Suicide gene therapy in cancer has many advantages compared to conventional drug treatments offering higher efficacy and lower toxicity. The pro-drug commonly does not have an effect on normal cells and since the pro-drug will only be enzymatically activated in the target cells, a high concentration of the toxic drug will be present at the target site (Portsmouth et al. 2007). An important condition is that the suicide gene is a gene that is not normally expressed by the host cells. Another advantage with the system is the bystander effect which is often observed with suicide

gene therapy. This is an effect in which non transfected cells surrounding a transfected cell will be affected by the activated pro-drug. This bystander effect can be a result of free diffusion of the activated pro-drug through the cell membranes to surrounding cells however some of the activated pro-drugs have a charge that does not allow them to freely diffuse across the cell membrane. These substances often exert a bystander effect via gap-junctions formed between closely situated cells which allow for the transfer of activated pro-drug between cells (Luminiczky & Safrany 2006, Portsmouth et al. 2007).

The list of suicide gene and pro-drug combinations is long and many of these have already been tested in clinical trials with variable outcome. The thymidine kinase enzyme derived from herpes simplex virus type 1 which can convert ganciclovir to its phosphorylated cytotoxic counterpart is the most studied suicide gene therapy system (Lawler et al. 2006, Portsmouth et al. 2007). The herpes simplex virus 1 thymidine kinase (HSV-tk) ganciclovir suicide gene therapy system has been studied intensively in vitro and in vivo in preclinical animal models and has also been tested in phase I, II and III clinical trials for cancer therapy but has failed to show significant efficacy (Portsmouth et al. 2007).

### 1.1.4 Effect gene therapy.

Effect gene therapy is not an official categorization however a definition in which I have chosen to group gene therapy forms which could not be grouped under any of the other classifications. Effect gene therapy is a type of gene therapy where the transgene exerts a function which would normally not be seen in the target cell. This is a very broad definition and includes a broad range of diseases and transgenes. The concept is not very different from replacement gene therapy except that the transgene is not replacing a normal cell product. Likewise the outcome of the transgene expression is broad. Examples of diseases which are proposed to be treated with this form of gene therapy are Alzheimer's disease where nerve growth factor is used as a transgene (Selkirk 2004), atherosclerosis and peripheral vascular disease with vascular endothelial growth factor as a transgene (Selkirk 2004), fractures with bone morphogenetic protein as a transgene (Carofino & Lieberman 2008) and cancer with the tumour necrosis factor apoptosis inducing ligand (TRAIL) as a transgene (Kim et al. 2004a, Ma et al. 2005).

### 1.1.5 Protective gene therapy.

Protective gene therapy is conducted in order to protect certain cell types from cytotoxic therapy that may be applied to adjacent cells. A good example is gene therapy for radiation therapy induced toxicity on normal cells. These can be protected by administering super-oxide dismutase expressing plasmids in the area surrounding the irradiation target (Min et al. 2005, Cotrim & Baum 2008). Another example is the transfer of drug resistance genes into haematopoietic stem cells in order to protect these from cytotoxic drugs targeting replicating cells. This sort of therapy is often performed in vitro after which the stem cells are reintroduced to the bone marrow (Blaese et al. 1995, Gunji et al. 2000). A variety of chemoprotective genes are currently being developed for use in cancer therapy (Sorrentino 2002, Seth 2005).

### 1.1.6 Silencing gene therapy

Silencing gene therapy is a method that has evolved recently after the description of RNA interference in Caenorhabditis elegans in 1998 (Fire et al. 1998). This technique allows for silencing of gene expression at a post transcriptional level by targeting the RNA transcript (Fire et al. 1998, Lage 2005). RNA interference is only one of many ways to silence gene expression. Other methods include DNAzymes, ribozymes and antisense oligodeoxynucleotides each having different advantages and disadvantages for gene silencing (Lage 2005, Dass et al. 2008). Silencing gene therapy is currently being developed for various types of cancer in which various genes associated with apoptosis regulation and cell signalling can be silenced (Masiero et al. 2007). Other diseases in which silencing gene therapy has been taken forward are Huntington's disease targeting the Huntington gene (Mochizuki et al. 2008).

### 1.1.7 Limitations in gene therapy

All gene therapy approaches face similar obstacles with regard to efficacy and patient safety when applied to an in vivo situation. In general certain elements need to be fulfilled for a gene therapy protocol to be successful. These are efficient delivery and uptake of the transgene in cells, specific expression of the transgene in target cells

avoiding adverse effects in non target cells and adequate levels of transgene expression allowing for a therapeutic effect.

### 1.2 Gene delivery mechanisms

A condition that needs to be considered in gene therapy when selecting a possible delivery mechanism is the final mode of drug application. The mode of application depends on the disease treated and the effect aimed for. Topical delivery is useful for conditions that affect, for example the skin (Hengge 2005). Systemic application is useful for diseases that potentially affect multiple organ systems and where a systemic effect could be beneficial, which can be seen in diseases such as cancer. Local application can be performed either by injection directly into an organ of interest or by intramuscular, intracutaneous or subcutaneous injections. Local application can also be performed via an intravascular route in which the blood flow is restricted to a certain area. Direct application into a surgical site is also a potential mode of application. Other modes of delivery are delivery into some of the fluid filled body cavities such as intra peritoneal and intra cerebrospinal which is beneficial for diseases localised in those areas. These modes of application are all based on in vivo application; however in vitro gene delivery is also a potential mode of delivery. This method is based on harvest of cells or tissue and then performing *in vitro* gene delivery before transplanting the cells or tissue back into the host (Ghali et al. 2008).

There are two major groups of gene delivery mechanisms in gene therapy; viral and non-viral. Each group compromises different advantages and disadvantages but as a general rule the viral gene therapy delivery mechanisms are considered more efficient although are regarded as less safe (Kaneda & Tabata 2006).

#### 1.2.1 Non-viral gene delivery mechanisms

Non-viral gene delivery mechanisms are generally safer than viral methods though not as effective as viral gene delivery mechanisms.

### 1.2.1.1 Naked plasmid DNA

The simplest of transgene delivery forms is delivery of naked plasmid DNA. This method has been widely used and data show that a great proportion of human clinical gene therapy trials have been done using plasmid DNA and that this proportion is rising (Edelstein et al. 2004, Edelstein et al. 2007). This increase is probably a consequence of the low risk associated with in vivo transfection of cells with plasmid DNA.

Delivery of naked plasmid DNA can be done by simple injection locally or systemically. An advantage with using naked plasmid DNA for gene therapy in vivo is that it is relatively poorly immunogenic and does normally not induce an immune response and therefore is a minimal risk to bio-safety (Trent & Alexander 2004). Another advantage is that plasmid DNA is easy to work with and relatively easy to produce (Trent & Alexander 2004, Kawakami et al. 2008). The naked plasmid DNA is however subject to fast DNA degradation by blood nucleases and clearance from the liver when delivered systemically and in this context the half life of naked plasmid DNA delivered intravenously is estimated to be 5 minutes (Henshaw & Yuan 2008). Once the DNA has traversed the cell membrane the half life in the cell cytoplasm is approximately 90 minutes (Henshaw & Yuan 2008). The physical state of DNA influences the cellular DNA uptake and in this context supercoiled DNA shows a higher level of uptake compared to linear and circular DNA (Henshaw & Yuan 2008). In order to improve the cellular uptake of naked plasmid DNA various physical delivery mechanisms have been developed (Ferber 2001).

### 1.2.1.2 Electroporation.

Electroporation is a simple way to enhance cellular uptake of plasmid DNA in vivo. This method is based on the concept that cells in an electric field will form temporary hydrophilic pores in the cell membrane which will allow for uptake of the DNA molecules by passive diffusion (Escoffre et al. 2007, Henshaw & Yuan 2008). The plasmid DNA can be applied either locally or systemically and the electric field is then applied to the location of interest. The electric field can be created by use of either parallel-plate electrodes or needle electrodes of which the first is limited to surface application (Henshaw & Yuan 2008). The electric field also improves the spread of plasmid DNA by electrophoresis of the negatively charged DNA molecules in the tissue

(Escoffre et al. 2007, Henshaw & Yuan 2008). Electroporation has been shown to increase DNA uptake 80 times compared to naked DNA (Ferber 2001). A limitation of the method is that it is mostly applicable to superficial structures such as skin and that only relatively weak currents can be used to avoid cellular damage (Yamashita et al. 2002, Henshaw & Yuan 2008). Electroporation is not only used for gene therapy but is also used for chemotherapy to improve uptake of chemotherapy drugs into tumour cells (Tijink et al. 2006).

#### 1.2.1.3 Sonoporation.

Ultrasound is another physical method used to increase the uptake of plasmid DNA in cells. This method, also named sonoporation works in a similar fashion as electroporation however using sound waves instead of electric current to permeabilise the cell membrane (De Carvelho et al. 2007). A combination of electroporation and sonoporation called electrosonoporation has also shown to enhance the uptake of naked plasmid DNA into cells. This method allows for a higher level of DNA uptake and fewer side effects compared to either electroporation or sonoporation alone (Yamashita et al. 2002, Yamashita et al. 2004).

#### 1.2.1.4 Hydrodynamics.

Another physical method which has been used to increase the uptake of naked DNA in vivo is hydrodynamics. This method also known as hydroporation, uses hydrostatic pressure to force the DNA into the cells. This can be done by either rapid systemic injection of large volumes of plasmid DNA in a physiologic solution or by clamping the vein in the location of interest and thereby increasing the pressure by stagnation of blood. The increased pressure generates pores in the plasma membrane by which the DNA can be taken up (Ferber 2001, Neal et al. 2007, Suda & Liu 2007). In rodent cancer models hydrodynamic vein delivery has shown to be a successful method to deliver naked plasmid DNA to hepatocytes and this method also had an antitumour effect (Yonenaga et al. 2007). This method is however not applicable to humans since the volumes used in the mouse studies are equivalent to 8-10 % of the animals body weight which would be equivalent to infusing around 8 litres of fluid intravenously into an adult human, a procedure which could lead to heart failure (Ferber 2001, Suda & Liu

2007, Yonenaga et al. 2007). Percutaneous ethanol injection is a widely used treatment for hepatocellular carcinoma and therefore one should consider if the effect seen in the hydrodynamic vein delivery studies is due to the effect of the plasmid DNA or from the fluid pressure (Schwartz et al. 2007). Surgical clamping of a vein and subsequent plasmid DNA injection might be a more suitable way of performing hydroporation for gene delivery in larger animals and humans (Feber 2001).

#### 1.2.1.5 Gene gun.

Another physical method of improving cellular uptake of naked plasmid DNA is by tissue bombardment with DNA coated gold particles using a device called a gene gun (Kawakami et al. 2008). The gene gun accelerates the DNA coated gold particles to a high velocity at which they can penetrate the cell membrane. This method can be applied in vivo on superficial structures such as skin or on internal organs under surgery or it can be applied in vitro for transplanted cells (Cambell et al. 2007, Chang et al. 2008).

#### 1.2.1.6 Chemical methods.

Cellular plasmid DNA uptake can also be enhanced by chemical methods. Cationic polymers which can be of either natural or synthetic origin, or cationic lipids also know as liposomes, can be used to form a complex with the negatively charged plasmid DNA (Haider et al. 2005, Kaneda & Tabata 2006). This will result in an overall positive surface charge of the complex which allows the complex to interact and be taken up through the negatively charged cell membrane (El-Aneed 2004a). Modification of these complexes can be done in order to make them more stable or to allow for cell specific uptake (Haider et al. 2005, Kawakami et al. 2008).

### 1.2.2 Viral gene delivery mechanisms

Viruses have evolved to infect host cells by evading the naturally occurring host defence barriers. They can therefore be considered optimised vehicles for in vivo and ex vivo gene delivery to cells. The ability of viruses to cause disease and interact with the host genome is though a concern for the safety of viral gene therapy.

Many viruses are pathogenic and even though they do not all induce direct cell death to the infected cell they often have other adverse effects in the host (Verma & Weitzman 2005). In order to use viruses as vectors the viral components responsible for the pathogenic effect are removed and replaced with the transgene of interest. There are many different viruses that have been studied for their potential use as a gene delivery vectors. Each type offers different advantages and disadvantages and many conditions need to be considered before choosing a viral vector. The size of the transgene is of importance since viruses have different capacities for carrying foreign DNA. The target cell type should be considered since some viruses transduce specific cell types more efficiently than others such as herpes simplex virus which has been shown to transduce cells in the central nervous system efficiently (Lawler et al. 2006). The mode and effect of the required transgene expression should also be considered. If it is the aim to correct a genetic disorder then a permanent expression of the corrective transgene is often required in order to achieve a clinical effect. Viruses that integrate into the host DNA will result in continuous expression of the transgene whilst non integrative viruses that stay episomal often have a transient effect that will wane after cell division and time.

The most commonly used viral vectors for gene therapy are based on retroviruses, adenoviruses, vaccinia viruses, poxviruses, adeno-associated viruses and herpes simplex viruses (Edelstein et al. 2004, Edelstein et al. 2007).

#### 1.2.2.1 Retroviruses.

Retroviruses were previously the most commonly used vectors for human clinical gene therapy trials (Edelstein et al. 2004). Newer figures however show a decrease in the overall use of retroviral vectors for gene therapy (Edelstein et al. 2007). Retroviruses are single stranded enveloped diploid RNA viruses (Verma & Weitzman 2005). The viruses code for an integrase protein which facilitates the integration of proviral DNA, generated by reverse transcription, into the host genome and thereby establishing stable viral gene expression by use of host transcriptional machinery (Verma & Weitzman 2005).

The prototypic retroviruses such as oncoretroviruses are characterised by their ability to only infect replicating cells. The murine leukaemia virus (MLV) was successfully used as a viral vector for gene therapy treatment of SCID-X1 in children (Cavazzana-Calvo et al. 2000) as described in chapter 1.1.1. As described the

development of leukaemia in several patients was a major drawback for this trial. It has been shown that the MLV virus does not insert randomly in the host genome but selects certain regions above others. The insertion of the virus is therefore not completely random and the risk of integration induced oncogenesis is therefore increased which is why major focus has been on using other retrovirus vectors (Pfeifer 2004, Sokolic et al. 2008).

Lentiviruses belong to a subgroup of retroviruses that have been well studied for use in gene therapy and also for easy generation of transgenic animals (Pfeifer 2004). Conventional retroviruses can only infect dividing cells and an advantage with lentiviruses is their ability to infect both dividing and non dividing cells. Lentiviruses are in general more complex than other retroviruses in that they code for additional genes in their genome which facilitate their more complex lifecycle and their ability to infect non dividing cells and evade host immune responses (Quinonex & Sutton 2002). The human immuno deficiency virus type 1 (HIV-1) is the most well studied lentivirus for use in gene therapy but other viruses such as feline immuno deficiency virus (FIV) and simian immuno deficiency virus (SIV) have been studied for use as vectors due to the risk associated with the pathogenesis of the HIV-1 virus (Pfeifer 2004, Saenz & Poeschla 2004, Verma & Weitzman 2005). In order to make the viral vectors safe, most of the viral genome is removed except for a few essential sequences and the packaging sequences (Pfeifer 2004). The modified lentiviral based vectors have a packaging capacity of approximately 8.5 kb foreign DNA and the vector inserts randomly in the host genome (Pfeifer 2004). A great safety issue when using retrovirus based vectors is the risk of insertion of the virus at a site where it could activate an oncogene and thereby induce a carcinogenic phenotype. The modified lentivirus vectors have however been shown to insert randomly into the host genome and the risk of oncogene activation upon insertion is estimated to be 1/100000 (Pfeifer 2004). HIV-1 virus has a predilection for CD4 expressing cells and therefore further modifications have been done on HIV-1 to increase its tropism to a wider range of cells. Other modifications include construction of self inactivating lentiviruses in which viral enhancers and promoters have been removed (Quinonex & Sutton 2002, Pfeifer 2004).

### 1.1.2.2 Herpesviruses.

Herpesviruses have been studied for their use as viral vectors in gene therapy but their use is minor compared to retroviruses (Edelstein et al. 2007). Herpes viruses are a group of linear double stranded enveloped DNA viruses which have an advantage over other viruses in that the virus vector has a large gene carrying capacity of up to 30kb, allowing the insertion of large complex genes (Verma & Weitzman 2005). The human herpes virus type 1 has been studied for use in gene therapy. The virus has a tropism for neuronal tissue as reflected by the pathogenesis of the virus and therefore it is commonly studied for gene therapy in neuronal diseases (Hoffmann & Wildner 2007). The virus can infect both dividing and non dividing cells and the virus does not integrate in the host genome which allows for only transient transgene expression when used as a transgene vector (Verma & Weitzman 2005, Witlox et al. 2007). Wild type herpes viruses induce cell lysis in the host cell upon replication and therefore it is important that the virus is replication incompetent when used as a viral vector (Verma & Weitzman 2005). A disadvantage of using herpesviruses for gene therapy is their strong immunogenicity and potential cytotoxic effect (Edelstein et al. 2004, Verma & Weitzman 2005, Witlox et al. 2007). The human herpes virus type 1 has not only been studied for its use as a gene delivery vector but has also been studied for use as an oncolytic virus for cancers of neuronal origin in particular, due to its ability to induce cell lysis upon viral replication (Kuruppu et al. 2007, Liu et al. 2007).

### 1.1.2.3 Adeno-associated viruses.

Adeno-associated viruses (AAV) are single stranded DNA parvoviruses (Verma & Weitzman 2005, Witlox et al. 2005). This virus type is increasingly being used for human gene therapy trials (Edelstein et al. 2004, Edelstein et al. 2007). AAV has been in particular focus for gene therapy because it is a non-pathogenic virus which can infect a wide range of cells and only replicate productively in association with a helper virus, such as adenovirus (Verma & Weitzman 2005). AAV has also been shown to integrate at specific locations in the host genome making it a useful tool for long term gene therapy for genetic disorders. It has also been shown that it is possible to direct AAV integration to a site specific position using homologous recombination although most of the virus will integrate randomly. In general only a small proportion of virus integrates into the host genome whilst most persist as episomal DNA (Schultz &

Chamberlain 2008). The AAV is approximately 4.7kb however the viral packaging signals are located in the inverted terminal repeats sequences flanking the viral genome and the whole viral genome can therefore be replaced by a maximum of 5 kb foreign DNA (El-Aneed 2004a, Schultz & Chamberlain 2008). The AAV is in great focus for gene therapy but the risk and consequences of random insertion of the transgene in the host genome and the high immunogenicity of the virus are disadvantageous. Furthermore the virus is difficult to culture due to its dependence on other viruses for replication (Verma & Weitzman 2005, Witlox et al. 2007, Schultz & Chamberlain 2008).

### 1.2.3 Adenoviruses for gene therapy.

Adenoviruses are some of the most commonly used vectors in human gene therapy trials (Edelstein et al. 2007). As this virus is of particular interest for this project a more detailed description of this virus will follow.

Adenoviruses were discovered and isolated in the early 1950's (Ginsberg 1999). The Adenoviridae family comprises the genera mastadenovirus, aviadenovirus, atadenovirus, siadenovirus and a possible $5^{th}$ genus which is uncertain. The aviadenoviruses are restricted to infections in birds and the mastadenoviruses are restricted to infection in mammals. The atadenovirus and siadenovirus have less restricted host spectrum (Davison et al. 2003). Adenoviruses are widely prevalent and often associated with only mild disease which reflects the viruses' ability to infect a host and delay the host's early and innate immune response allowing for an initial large production of viral progeny which can infect other individuals before the host immune response eradicates the virus (Russell 2000).

#### 1.2.3.1 Adenovirus characteristics

Adenoviruses replicate in the cell nucleus (Goncalves 2006) which makes these viruses suitable for use as gene therapy vectors since transgenes are delivered to the cell nucleus.

Adenoviruses are double stranded linear DNA viruses, encapsidated by an icosahedral protein capsid. The size of the viral DNA varies from 26 to 45 kbp and the genome is characterised by an inverted terminal repeat and the binding of a terminal protein to the 5' ends of the linear DNA (Davison et al. 2003). The mastadenovirus

genus is the major focus group for gene therapy vectors as this group comprises the human adenoviruses. There are many well characterised mammalian adenoviruses but most studies have been done on the human adenovirus type 2 and 5 (Shenk 1996). The following description of adenoviruses will therefore be mostly based on studies done in human adenovirus type 2 and 5, however many characteristics are shared between these and other mammalian adenoviruses (Davison et al. 2003). The atadenovirus, aviadenovirus and siadenovirus differ from the mastadenovirus group in that some of the early gene regions are not present in these genera and the following description will therefore not be applicable for these genera (Davison et al. 2003).

The adenovirus icosahedral capsid is characterised by 20 facets formed by a trimeric hexon protein and 12 vertices formed by the penton base protein as illustrated in figure 1.1. From each vertice a knobbed trimeric fibre projects externally. The penton base and the fibre-knob are important for the interaction and entry of the virus to the host cell (Shenk 1996, Seiradake et al. 2006). Upon infection the viral fiber knob binds to the coxsackie adenovirus receptor (CAR) on the cell surface. The fiber-knob interaction with the receptor is followed by binding of an arginine-glycine-asparagine (RGD) motif in the penton base to integrins in the cell membrane (Shenk 1996). This leads to clathrin mediated endocytosis of the viral particle (Russell 2000). Some adenoviruses lack the RGD motif and enter the cell using a different mechanism and some viruses have also been shown to infect cells independently of the CAR (Soudais et al. 2000, Stoff-Khalili 2005, Seiradake et al. 2006).

Once inside the cell the viral capsid is partly degraded by virus encoded proteases and the virus is trafficked to the cell nucleus by interaction between the virus and cellular structures (Russell 2000). Experimentally virus has been detected in the cytoplasm and the cell nucleus 1 and 2 hours respectively after infection (Russell 2000). Once the virus enters the cell nucleus viral transcription is initiated by use of the RNA polymerase type II (Goncalves & Vries 2006).

### 1.2.3.2 Adenovirus genome

The adenovirus genome contains several overlapping reading frames for genes that can be divided into early and late transcription units according to their expression relative to the virus' replicative life cycle. The early genes are transcribed prior to the virus DNA replication whilst the late gene transcription is initiated after the initiation of DNA replication (Russell 2000). Splicing is a common method by which each of the

viral genes gives rise to multiple mRNAs by use of different splice sites (Shenk 1996). A schematic presentation of the adenovirus genome is shown in figure 1.2.

The early genes comprise the E1, E2, E3 and E4 gene regions (Shenk 1996). The E1 gene can be divided into the E1a and E1b. The main function of the E1a gene is to alter the cellular metabolism to favour viral replication (Russell 2000). The E1b gene is responsible for prolonging the survival of the infected cell by reducing the transcription of the p53 gene which is induced as a cellular defence by the expression of the E1a gene (Russell 2000). The E1b gene also plays a role in transcription of late viral genes. The viral E2 gene can also be devided into two regions E2a and E2b. This gene is responsible for the machinery for viral DNA replication and also plays a role in transcription of late viral genes. At an early stage it was shown that the E3 gene is non essential for viral replication but plays a role for the pathogenesis of the virus (Ginsberg 1999). The basic function of the E3 gene is to protect the virus from the host cell defence mechanisms by means of suppressing the expression of the major histocompatibility complex class 1 molecule on the cell surface and by inhibition of pro-apoptotic pathways (Russell 2000, Horwitz 2004). An important E3 gene product is the adenovirus death protein since this protein promotes cytolysis of the host cell and release of viral progeny after completion of viral replication (Horwitz 2004). Removal of the E3 gene reduces the ability of the virus to evade the host immune response and also reduces its cytopathic effect (Muruve 2004).

The genes encoded in the E4 region are responsible for arresting the host protein synthesis and also initiation of viral DNA replication (Goncalves & Vries 2006).

After the early adenovirus genes have been expressed the viral DNA replication is initiated, starting in the inverted repeat sequence (Shenk 1996). The newly replicated DNA can act as a template for further DNA replication or be a template for late viral gene transcription (Goncalves & Vries 2006). The late adenoviral genes comprise the L1, L2, L3, L4 and L5 genes. These encode the structural proteins of the virus (Russell 2000). Once expressed these proteins are transported back into the nucleus where the viral progeny is assembled in an immature form. This immature state is related to some of the structural proteins being formed as precursor proteins which need to be cleaved by viral encoded proteases in order to function (Shenk 1996, Russell 2000). Following viral maturation by protein cleavage the nuclear membrane is permeabilised and the viral progeny are released from the cell by cell lysis (Rao et al. 1996, Tollefson et al. 1996).

**Figure 1.1**

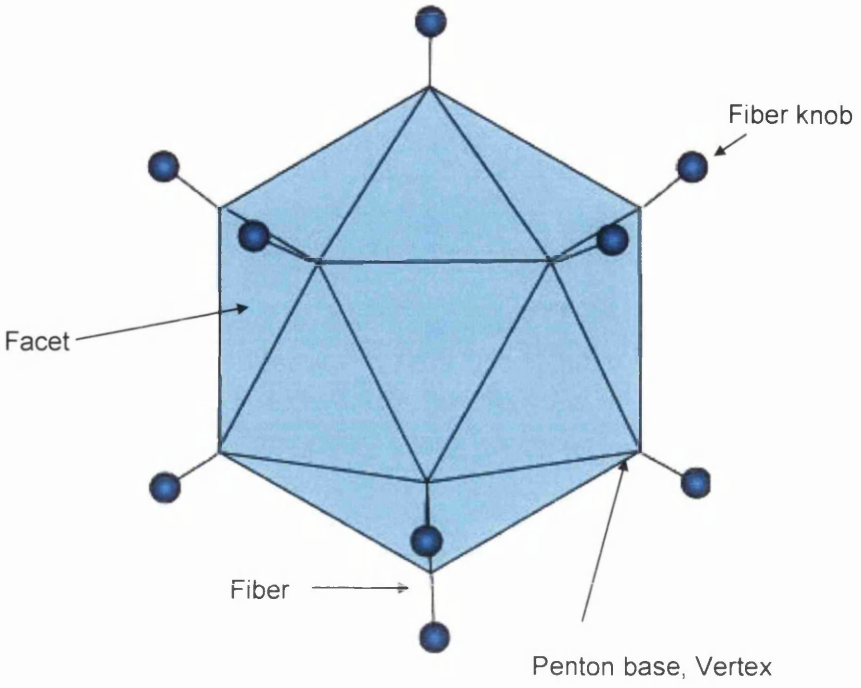



**Figure 1.1 Adenovirus model.**

Figure showing simplistic model of the three dimensional structure of the adenovirus capsid (Figure by Jamie McClement).

**Figure 1.2**

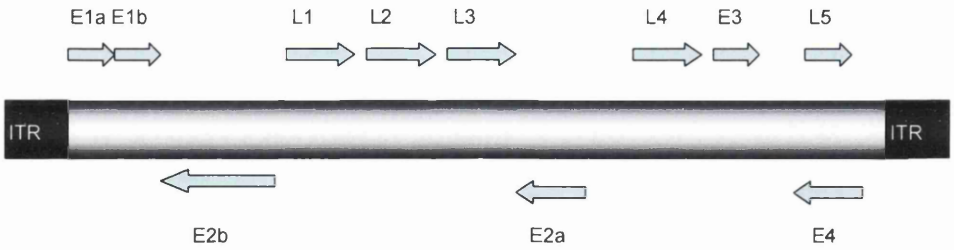



**Figure 1.2 Model of the adenovirus genome.**

Model showing schematic presentation of the adenovirus genome and location of early E1a, E1b, E2a, E2b, E3, E4 and late L1, L2, L3, L4, L5 genes. ITR : inverted terminal repeat..

### 1.2.3.3 Adenovirus vectors

The adenovirus has been widely used as a vector in gene therapy. This is mainly based on the ability of the virus to infect a broad range of different dividing and non-dividing cell types and the low morbidity of the virus in vivo (Cao et al. 2004, El-Aneed 2004a). The clear disadvantages of adenovirus vectors in gene therapy are the high antigenicity of the virus and the high prevalence of the virus in the human population. As a result of this antibodies against adenovirus can be detected in 90% of the human population which antagonises the use of adenovirus especially for long term gene expression (Russell 2000, Muruve 2004).

The most commonly used adenoviruses for gene therapy vectors are the human type 5 and less commonly type 2 adenoviruses (Cao et al. 2004, Cotrim & Baum 2008). The wild type adenovirus has a gene carrying capacity of approximately 2 kbp foreign DNA (Russell 2000). Adenovirus replication results in lysis of the infected cell and therefore for use as a gene therapy vector the adenovirus must be made replication incompetent (Verma & Weitzman 2007). This can be achieved by removing some of the genes essential for viral replication which in turn results in a higher gene carrying capacity of the virus. This capacity can also be further increased by deletion of some of the viral non essential genes (Alba et al. 2005). The development of adenoviral vectors has gone from being very simple single gene deletion viruses to optimised hybrid systems.

### 1.2.3.4 First and second generation adenoviral vectors

The first generation virus vectors were depleted in the E1 gene region increasing the gene carrying capacity to approximately 4kbp (Cao et al. 2004). The removal of the E1 genes result in a replication incompetent virus and the virus is therefore propagated by use of complementary cells expressing the E1 genes in trans (Alba et al. 2005, Zhang & Godbey 2006). Further increase in the gene carrying capacity was achieved by removing the non essential E3 gene region increasing the carrying capacity by further 4kb (Cao et al. 2004). The second generation adenoviral vectors would in addition to having E1 and E3 deleted also have the E2 or E4 genes deleted which could increase the gene carrying capacity to around 10-13 kbp (Russell 2000, Cao et al. 2004, Flotte 2007).

The use of adenovirus vectors for gene therapy is limited by the virus vector capsid proteins which elicit a strong inflammatory response including induction of inflammatory cytokines and chemokines, when virus vectors are administered systemically to humans in vivo at a high dose (Cao et al. 2004, Muruve 2004). This effect which is dose dependent leads to a risk of cytotoxic shock in the host. Lower doses and also doses administered locally are better tolerated. The acute shock reaction is part of the host's innate immune response which is activated prior to viral gene expression. The E3 and E1 gene products have been shown to mediate the host immune response however these genes are commonly lacking in the first and second generation adenoviral vectors leading to increased inflammation (Muruve 2004). The innate immune response recruits macrophages, natural killer cells and neutrophils and it has been observed that this can lead to rapid clearance of first generation adenovirus vectors from the liver with only 10-20% left 24 hours after systemic injection (Muruve 2004, Alba et al. 2005).

Host immune response is a major obstacle for use of adenoviral vectors. As previously mentioned most people carry antibodies against adenovirus. These antibodies are mostly directed against the virus capsid proteins and are effective in eliminating infective particles in the host. To combat the fast clearance of the virus, methods have been developed to shield the virus from the host immune response and thereby increase the virus vector half life. This has been done by administering immune suppressant drugs or by binding the adenovirus capsid proteins to polyethylene glycol compounds which are immunologically inert and thereby protect the virus from being recognised by the host (Russell 2000, Cao et al. 2004).

#### 1.2.3.5 Helper dependent adenoviruses

Helper dependent adenoviruses, also known as third generation adenoviruses, are viral vectors in which only the inverted repeat sequences and the viral packaging signal are present while the remainder of the viral genome is excised (Alba et al. 2005, Zhang & Godbey 2006). These viral vectors do not express any viral proteins and therefore they must be generated in the presence of a helper adenovirus expressing all the viral genes in trans (Alba et al. 2005). This introduces a risk of contaminating the viral vector stock with replication competent helper virus and introducing a high safety threat for in vivo use. Several methods have been developed to overcome this obstacle

and produce large amounts of virus vectors with minimal contamination (Cao et al. 2004, Alba et al. 2005, Goncalves & Vries 2006).

Firstly, using helper viruses with defective packaging signals was originally thought to be the solution, however the high risk of recombination between the viruses and thereby introduction of a functional packaging signal lead to a high level of contamination from the helper virus (Alba et al. 2005). Specific removal of the helper virus packaging signal by use of the cyclization recombination protein (Cre) in conjunction with the locus of X-over P1 (loxP) site also known as the Cre/Lox system is a way to overcome this problem (Cao et al. 2004, Alba et al. 2005, Goncalves & Vries 2006). This system allows for the specific excision of unwanted DNA sequences. In this system the viral packaging signal in the helper adenovirus is flanked by lox P sites. To propagate the viral vector both the helper virus and the helper dependent virus are infected or transfected into Cre expressing cells. The Cre protein then binds to the LoxP site resulting in site specific cleavage and religation and thereby excision of the packaging signal from the helper virus. This method reduced the helper virus contamination to a range between 0.1-10% (Alba et al. 2005). The residual contamination is most likely to be a result of inadequate activity of the Cre protein and therefore other recombinases have been investigated for use (Goncalves & Vries 2006). Thirdly, another improvement was insertion of the packaging signal in the helper virus in reverse which could limit recombination events and reduced the contamination levels of helper virus to 0.02-0.1 % (Alba et al. 2005).

The helper dependent adenoviruses allow for the insertion of large cDNA sequences, regulatory sequences and promoters required for tissue specific gene expression at a regulated physiological level (Goncalves & Vries 2006). For the viral particle to be packaged efficiently the insert DNA must be similar in size to the wild type virus which is around 36kb; this can be achieved by using stuffer DNA if the inserted gene is too small (Cao et al. 2004, Alba et al. 2005). Due to the lack of viral protein expression the helper dependent adenoviral vectors are advantageous to use since they are less immunogenic and result in a reduced adaptive cellular immune response when delivered systemically compared to first and second generation vectors (Cao et al. 2004, Alba et al. 2005). The innate immune response which is triggered independently of adenoviral gene expression is still a concern for the stability and toxicity of these vectors when administered in vivo (Alba et al. 2005, Flotte 2007).

### 1.2.3.6 Animal adenoviruses

Animal adenoviruses are being exploited as alternatives to human adenoviruses for use in human gene therapy, for use in gene therapy in large animal models and to create hybrid viruses between human and animal viruses to improve infectious potential and evade the host pre-existing immune response (Kremer et al. 2000, Hemminki et al. 2003, Lucas et al. 2003, Kremer 2004, Perreau et al. 2007, Zheng et al. 2007) Another risk that can be eliminated by using animal adenoviruses for human gene therapy is replication of adenovirus vectors in the presence of wild type adenoviruses in the host with accompanying risk of recombination (Rasmussen et al. 1999).

Studies have shown that the bovine adenovirus type 3 can infect human cells without cytotoxicity, as it does not replicate and does not recombine with co-infected human adenoviruses (Rasmussen et al. 1999). This study also showed that the E1 and E3 regions in the virus could be excised allowing for the insertion of exogenous DNA opening a window for the use of this virus as an alternative vector for human adenoviruses in gene therapy.

### 1.2.3.7 Canine adenoviruses

The canine adenoviruses type 1 (CAV-1) and type 2 (CAV-2) have been studied for use as gene therapy vectors both in humans and canines (Rasmussen et al. 1999, Kremer et al. 2000, Hay 2003, Hemminki et al. 2003, Kremer 2004, Stoff-Khalili et al. 2005). These two viruses share many similarities in sequence but differ significantly in pathogenicity (Linne 1992). The two viruses can routinely be distinguished by PCR (Morrison et al. 1997, Decaro et al. 2007). The canine adenoviruses share many features with the human adenovirus however diverge on several aspects. Genetically it has been shown that the CAV-1 and CAV-2 are depleted in the CG nucleotide showing a basic evolutionary difference from the human adenoviruses (Davison et al. 2003).

### 1.2.3.8 CAV-1

The CAV-1 is a highly morbid virus in the canine population and is not associated with disease in any other species. The virus has a predilection for infecting vascular endothelial cells and hepatocytes most commonly causing the disease known as canine infectious hepatitis although the virus can be found in various organs

throughout the body upon infection (Cullan & MacLachlan 2001). Infection with CAV-1 can be fatal especially in young dogs however it can also result in asymptomatic or mild disease. The clinical signs associated with clinical disease are fever, inappetance, abdominal pain, vomiting, diarrhea and dyspnea (Cullan & MacLachlan 2001, Decaro et al. 2007). Prolonged infection with CAV-1 can result in immune-mediated glomerulonephritis which is a result of soluble immune complexes being deposited in the kidneys, followed by complement stimulation and recruitment of neutrophils (Confer & Panciera 2001, Cullan & MacLachlan 2001). Uveitis, also known as blue-eye, is another secondary effect from prolonged CAV-1 infection as a result of a type III hypersensitivity reaction. Disease as a result of CAV-1 infection is uncommon in the developed world due to vaccination practice often using freeze dried attenuated CAV-2 virus (Cullan & MacLachlan 2001, Decaro et al. 2007). The CAV-2 virus is used for vaccination because this virus is associated with only mild disease and provides cross-reactivity for the CAV-1 virus and vaccination is therefore less hazardous. Previous vaccination protocols with attenuated CAV-1 virus showed profound side effects (Decaro et al. 2007).

The complete DNA sequence of the CAV-1 virus field strain RI261 has been published and the virus genome contained the same gene arrangements and features as the human adenoviruses type 2, 5, 12 and 40 and the CAV-2 virus to which it was compared (Morrison et al. 1997). The E3 region of CAV-1 containing only 2 open reading frames, differs significantly from the human adenovirus E3 region in which 5 and 8 open reading frames are identified for the hAd 40 and hAd 2 respectively. The complete CAV-1 genome is 30536 base pairs and is therefore smaller than the human adenoviruses 2, 5, 12 and 40 which are 35937, 35935, 34125 and 34214 base pairs respectively. Similar to the human viruses the genome is flanked by 161 base pair long inverted repeat sequences (Morrison et al. 1997, Davisons et al. 2003).

#### 1.2.3.9 CAV-2

Infection with CAV-2 is often involved in the multifactorial infectious tracheobronchitis seen in dogs also commonly known as kennel cough (Lopez 2001). This disease as the name indicates has been implicated with dogs housed in close proximity such as in boarding kennels. The clinical symptoms of the disease are

generally mild causing rhinitis, pharyngitis, tonsillitis, conjunctivitis and in more severe cases pneumonia (Lopez 2001).

The complete genome of CAV-2 has been sequenced showing that this virus shares many sequence similarities with CAV-1 which was already demonstrated early by hybridisation analysis that estimated 75% nucleotide identity between the two strains (Linné 1992, Morrison et al. 1997, Davison et al. 2003). The full length CAV-2 genome is 31323 base pairs in size and therefore in a similar size range to the CAV-1 virus (Davisons et al. 2003). One of the major differences found between the CAV-1 and CAV-2 virus is the E3 coding region. A comparison in this region between the two strains has shown that the CAV-2 encodes for a 40.7 kDa polypeptide whilst the CAV-1 only encoded a 22 kDa polypeptide. Further differences between the E3 regions were found suggesting that the E3 region is functionally different in these two strains (Linne 1992). It is hypothesised that the E3 region can account for the major difference in pathogenity observed between the two CAV strains (Linné 1992). The CAV-2 virus lacks the RGF motif which is used for interaction of the virus with integrins on the cell surface in order to enter the cell by endocytosis (Davison et al. 2003). This suggests that the virus enters the cell by a different mechanism (Soudais et al. 2000).

Both the CAV-1 and CAV-2 have been studied for use as gene therapy vectors. Most research has been focused on the use of the CAV-2 due to the mild disease associated with this virus (Rasmussen et al. 1999).

### 1.2.3.10 CAV vectors for gene therapy.

The CAV virus structure has been studied in regard to broadening the tropism of already existing human adenovirus gene therapy systems for use in cancer therapy in particular. The commonly used human adenoviral vectors depend primarily on CAR expression on the cell surface in order to gain entry to the cell however this receptor is down regulated in some cancer types (Stoff-Khalili et al. 2005). It was shown that hybrid hAd 5 virus vectors containing chimeric fiber knobs derived from the CAV-1 or CAV-2 demonstrated a major infectivity enhancement and consequently transgene expression in CAR deficient cells and that this effect was especially pronounced when the CAV-1 fiber knob protein was used (Stoff-Khalili et al. 2005).

Other studies have examined the use of CAV-2 as an alternative vector for delivery of transgenes to human cells and by using this approach circumventing some of

the problems with regard to pre-existing immune responses against adenoviruses in humans (Kremer 2004). The CAV-2 virus's ability to transduce different human and non human cell types has been evaluated. The CAV-2 shows a slightly different tropism than the hAd 5 when used as a vector. It has been shown that any cell that can be transduced with CAV-2 can be transduced by hAd 5 but that the reverse situation is not so (Kremer 2004). Experiments show that the transduction of certain cell lines and transgene expression is lower with the CAV-2 than the hAd5 derived vectors however this disadvantage may be outbalanced by the potentially poorer immune response against CAV-2 in humans (Kremer et al. 2000, Keriel et al. 2006). This offers a potential for long term transgene therapy in humans with a CAV-2 based vector since the virus will not be eradicated as quickly by the humoral immune response.

The CAV-2 also represents an opportunity for specific targeting of certain cell types and in this context it has been shown that the CAV-2 vectors have a preference for transducing neuronal tissue and it could therefore be useful in treatment of neuronal disorders (Kremer et al. 2000, Kremer 2004). The CAV-2 vector could also be used for research purposes as a neuronal tracer (Kremer 2004).

The CAV-1 has not been studied as extensively as the CAV-2 for use in gene therapy. This could be due to the pathogenesis of this virus which can lead to fatal disease especially in neonates and immunosuppressed dogs (Cullan & MacLachlan 2001, Decaro et al. 2007). The ability of this virus to infect a broad range of cells in vivo could suggest that this virus would be an improvement especially for cell types which are difficult to transduce.

#### 1.2.3.11 Adenoviral immune response

The pre-existing host immune response against adenoviruses is the major concern in adenovirus based gene therapy. Long term gene expression from adenoviral vectors is limited by the immune response, likewise systemically administered virus particles can be rapidly cleared from the blood stream. The mode of application could have a great impact on the efficacy of the treatment. Local gene therapy such as intratumoural injection is not initially affected by circulating neutralizing antibodies however the innate immune response is still a concern (Hay 2003). A clinical trial using an adenovirus gene therapy vector which had a severe outcome was the fatal systemic inflammatory response in an 18 year old boy who received an intravenous

injection in the hepatic vein with an E1 and E4 deleted human adenovirus type 5 vector expressing human ornithine transcarbamylase (Raper et al. 2003). The patient in this trial suffered clinically from ornithine transcarbamylase deficiency due to a gene mutation. This is a disease that though severe can be managed by dietary restrictions. However in some severe cases the patients succumb to hyperammonemic coma (Raper et al. 2003). The result from this trial shows one of the problems with the immunogenity of adenoviruses and the trial has had a great impact on the planning of future clinical trials not least because the disease for which the patient was treated was not fatal itself.

### 1.3 Targeting gene therapy to specific cells.

It is important in gene therapy to restrict transgene expression to target cells. Expression of a transgene in non target cells can have adverse effects on the patient. One way to restrict transgene expression to certain cell types has been by harvesting target cells and infecting them in vitro and returning them to the patient. This method is however restricted to a few diseases and cell types. Another way to restrict transgene expression to target cells which has shown great potential is the use of tissue or disease specific promoters. Using tissue or disease specific promoters to drive the transcription of a transgene can restrict the expression of this gene to target cells were the promoters are active.

There are many promoters that have been applied to gene therapy for cancer in particular in order to restrict gene expression to target cells. These include the survivin promoter which has shown to be active in cancer cells (Zhu et al. 2004, Uchide et al. 2005), the prostate specific antigen promoter which has been shown to be active in prostate cells and in particular prostate cancer cells (Diamandis 1998, Zhang et al. 2002), the cholecystokinin type A receptor promoter which is up-regulated in pancreatic cancer (Li et al. 2006) and the carcino embryonic antigen promoter which is active in cancer cells in particular colon cancer cells (Ueda 2003).

#### 1.3.1 Telomerase targeted gene therapy.

The promoters associated with transcription of the telomerase enzyme complex have been studied thoroughly for their use to specifically target cancer cells. Since these

promoters are of particular interest for this thesis a more detailed description of the telomerase associated genes and their promoters will follow.

### 1.3.1.1 Telomerase and the end replication problem.

The telomerase enzyme complex is active in cells in order to overcome the DNA end replication problem at cell division (Meyerson 2000, Chan & Blackburn 2003). The inability of the DNA polymerase to replicate the 3' end of the lagging strand results in loss of 50-200 nucleotides under each cell cycle as seen in figure 1.3 (Blackburn 1991, Kim et al. 1994, Chan & Blackburn 2003). This would be detrimental to the chromosomes and therefore ends of the chromosomes are capped by telomeres. Telomeres consist of multiple repeats of the non coding TTAGGG nucleotide sequence (Blackburn 1991). This sequence is conserved amongst most eukaryotes however the number of repeats present varies between species (Kelland 2005). The 3' end of the telomeres contain a G-rich overhang which together with the repeat sequence interacts with a protein complex called shelterin which consists of several telomere binding proteins. The function of the shelterin complex is to protect the chromosome ends and control telomere lengthening (Blasco 2007). The G-rich overhang is particularly important since this structure can fold back onto the double stranded DNA and thereby form a structure known as the T-loop. This conformation protects the 3' end of the chromosome from DNA repair mechanisms. In this manner the telomeres prevent the chromosomal ends against being treated as double stranded DNA breaks (Blasco 2007). Under each cell cycle telomeric DNA is lost resulting in continuously shortening of the telomeres. Normal somatic cells are not able to compensate for telomeric loss resulting in critical short telomeres after multiple cell divisions. Once the telomeres have reached a critical short length the cell is no longer able to replicate and the cell goes into a state of senescence. The telomeres can therefore be referred to as a cellular mitotic clock (Meyerson 2000, Harley 2008). The average telomere length in adult human somatic cells is 7-12 kb. In comparison the length in the dog is 12-23kb and the length in the mouse and rat are 40-100 and 20-100 kb respectively (Kelland 2005).

Stem cells, activated lymphocytes, keratinocytes and male germ cells have a greater replication potential than other cells and in order to circumvent the cellular senescence set by the attrition of telomeres, they maintain the length of their telomeres by activation of the telomerase enzyme (Meyerson 2000, Keith et al. 2002). Many

cancer cells which are continuously replicating will likewise activate the telomerase enzyme in order to expand their replicative potential and therefore the telomerase associated promoters are of particular interest in cancer directed gene therapy (Blackburn 2005, Harley 2008, Shay & Keith 2008).

**Figure 1.3**

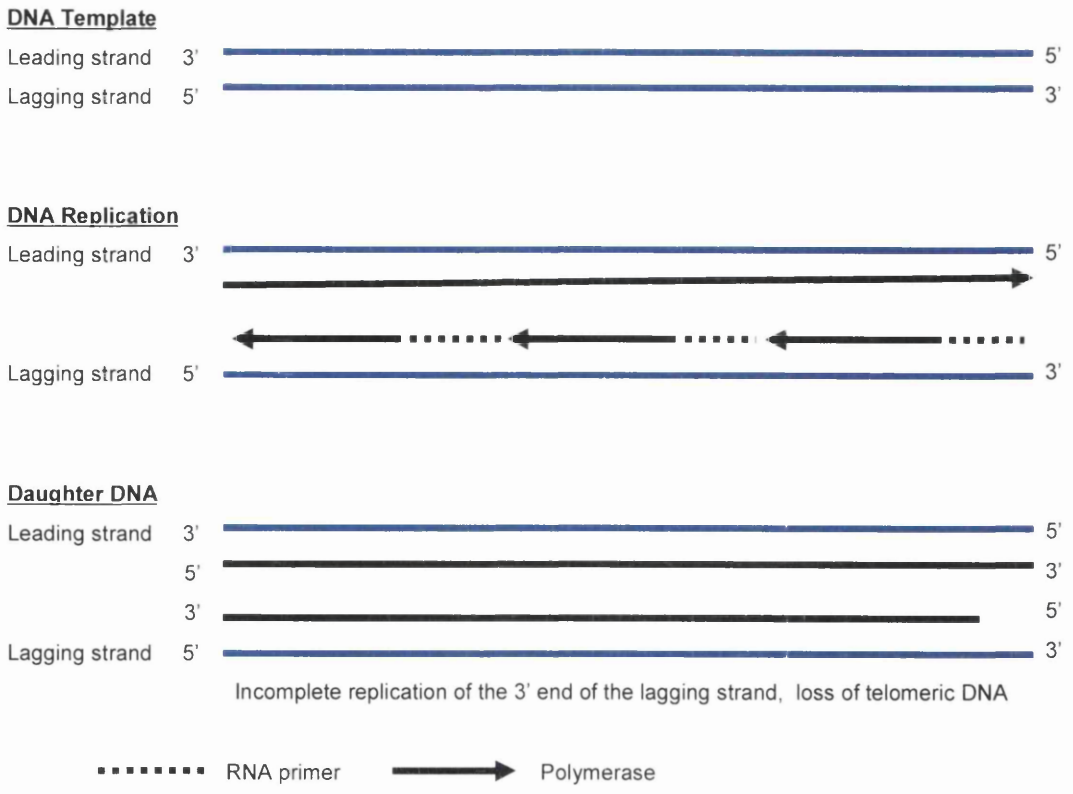



**Figure 1.3 The DNA end replication problem.**

Schematic illustration of the DNA end replication problem. The lagging strand is replicated discontinuously with RNA primers as initiation sites. These are continuously erased and the resulting gap is filled by the subsequent DNA polymerase. This leaves an unfilled gap at the 5' end of the daughter DNA of the lagging strand resulting in loss of telomeric DNA.

### 1.3.1.2 Telomerase components

The telomerase enzyme consists of two major components, a Telomerase reverse transcriptase (TERT) and a Telomerase RNA template (TR) (Blackburn 2000). These two components are adequate to execute telomerase activity in vitro although in vivo there are many other associated components that play an important role for function and regulation of telomerase activity (Blasco 2007). Of particular interest is the dyskerin protein (DKC1) which is mutated in the disease dyskeratosis congenita. Dyskerin stabilises the telomerase complex and mutations lead to short telomeres resulting in disease (Mitchell et al. 1999, Keith et al. 2002, Blasco 2007). Novel work has shown that the telomerase enzyme consists of two TERT molecules and two TR molecules together with one dyskerin molecule which acts as a stabiliser (Blasco 2007).

The TERT component of the telomerase enzyme functions as a reverse transcriptase to elongate the telomeres and uses the TR component as an RNA template for the telomeric repeats (Blackburn 1991, Blackburn 2000). The remaining components in the complex participate in stabilising and regulating the telomerase complex (Blasco 2007).

### 1.3.1.3 Telomerase's role in aging and disease

In humans telomerase is active during embryogenesis and in adult somatic cells the activity is low or absent resulting in the average telomere length being shorter in elderly compared to young people (Hornsby 2007). There are many factors that can influence the telomer length in cells such as obesity (Valdes et al. 2005), smoking (Valdes et al. 2005, Yim et al. 2007) and socio-economic status (Cherkas et al. 2006).

Congenital disorders in which the ability to maintain telomeres is dysfunctional result in degenerative diseases reflecting the inability of stem cells to renew tissues and also a premature death. The most commonly known human diseases associated with telomerase abnormalities are dyskeratosis congenita, aplastic anemia and idiopathic pulmonary fibrosis (Blasco 2007).

Some cancer cells have limitless replicative potential and as a consequence necessitate continuous telomerase activity to inhibit cells from entering cellular senescence. In this context telomerase activity has been detected in 90% of human cultured cancer cell lines (Kim et al. 1994, Kelland 2005). It is essential for a cell to

maintain telomere length for continuous cell cycling. In the absence of telomerase activity this is achieved by the alternative lengthening of telomeres (ALT) mechanism (Reddel 2001, Lundblad 2002, Reddel 2007, Nittis et al. 2008). This is a complex not well understood mechanism in which the telomeres are maintained by a form of homologous recombination. An interesting aspect of this is that this mechanism is more common in tumours of mesenchymal origin than epithelium derived tumours (Nittis et al. 2008).

It is possible for some tumours to develop in the absence of a telomere maintenance mechanism (Keith et al. 2002). These tumours are proposed to have telomere lengths that allow the cells to divide and the tumour to reach a certain size until the telomeres become critically short. These tumours will have a less aggressive phenotype than tumours with telomerase activity (Keith et al. 2002). It is also suggested that some tumour types could harbour both telomerase expressing and ALT cell types (Reddel et al. 2001, Yan et al. 2002, Reddel 2003). Even though telomerase expression leads to the ability of the cells to replicate continuously no deregulation in the cell cycle is observed and it is therefore not regarded as an oncogene (Harley 2002).

#### 1.3.1.4 Targeting telomerase expressing cells.

To utilise the increased telomerase activity for cancer cell gene therapy two promoters have been used; the TERT and the TR promoter mainly because of their central role in the function of the telomerase enzyme complex. TERT and TR promoters have been isolated and sequenced in humans but also in some mammalian species including dogs (Zhao et al. 1998, Cong et al. 1999, Takakura et al. 1999, Long et al. 2005).

#### 1.3.1.5 The human TERT promoter.

The human TERT (hTERT) promoter is GC-rich and lacks a TATA and a CAAT box. Several binding sites for transcription factors have been identified in the promoter (Cong et al. 1999, Takakura et al. 1999). The core promoter of hTERT is 181 base pairs upstream of the ATG for the hTERT transcription start site (Takakura et al. 1999). This promoter despite its shorter length than the full length promoter is still tightly regulated reflecting the telomerase activity in cells. An alternative recognition

sequence for the RNA polymerase to identify the transcription start site in absence of a TATA box in the hTERT promoter has been identified as the CCTCTCC sequence located 3 base pairs upstream of the ATG start site (Takakura et al. 1999). Expression of hTERT mRNA has been shown to reflect the telomerase activity in cells and therefore the TERT promoter activity must be tightly regulated in the cells to encompass this (Cong et al. 1999).

The mouse TERT promoter is different from the human promoter and telomerase is expressed in many tissues in this species throughout life making this an unreliable model for studying telomerase activity (Horikawa et al. 2005, Nasir 2008).

#### 1.3.1.6 The human TR promoter.

The human TR (hTR) promoter contains a CAAT site and a TATA box. The hTR promoter is a 867 base pair region upstream of the ATG start site (Zhao et al. 1998). A minimal promoter was isolated as being essential for promoter activity consisting of the first 272 base pairs upstream of the ATG start site. Upstream positive and negative elements regulate the activity of the hTR promoter. The mouse hTR promoter has a core promoter of 94 base pairs however there are no significant sequence homologies between the mouse and the human TR promoter, though some regulatory protein binding sites were present in both (Zhao et al. 1998)

The activity of the hTR promoter in relation to telomerase activity is debatable. Some show that the TR promoter is very tightly regulated by several mechanisms in accordance to the telomerase activity in the cell whilst others show that the promoter is more promiscuous being active despite no telomerase activity in the cells (Weinrich et al. 1997, Bodnar et al. 1998, Yin et al. 2004, Cairney & Keith 2008). The use of the TR promoter as a telomerase specific promoter to target gene expression to cancer cells is therefore questionable (Gu and Fang 2003).

It has been shown that transfection of cells with a TERT expressing plasmid allows for telomerase activity in cells and maintenance of telomeres and it is therefore suggested that the hTERT promoter activity is rate limiting for telomerase expression (Bodnar et al. 1998). This supports the use of this promoter for telomerase targeted gene therapy.

The TR and TERT promoters have been applied to gene therapy for cancer both as simple plasmid systems in which the promoters transcribe a suitable transgene and in

more complex systems including further regulatory mechanism and viral vectors (Irving et al. 2004, Bilsland et al. 2005, Wang et al. 2006, Bilsland et al. 2007).

#### 1.3.1.7 The canine TERT promoter.

The telomerase expression pattern in dog cells follow the pattern seen in humans as normal somatic cells do not show telomerase activity whilst stem cells and cancer cells do. Telomerase activity has been detected in up to 95 % of canine tumour samples (Yazawa et al. 1999). It has also been shown that there is a correlation between shortening of telomeres and age in dogs (McKevitt et al. 2002). The canine TERT gene (dogTERT) has been isolated and the gene shows many homologies with its human counterpart (Nasir et al. 2004). The dogTERT gene promoter has also been isolated and studied (Long et al. 2005). Approximately 5kb DNA was sequenced and analysed upstream of the dogTERT gene start site. This promoter region resembles the human region in that it is devoid of a CAAT and a TATA box sequence and that it is very GC-rich. Many of the transcriptional regulatory protein binding sites seen in the human promoter are present in the canine TERT promoter. The core dogTERT promoter region has been isolated as consisting of the proximal 314 base pairs upstream of the dogTERT gene. Inclusion of further base pairs upstream of the core promoter resulted in a decrease in promoter activity which could reflect the location of promoter repressor binding sites in this region (Long et al. 2005). The properties of the dogTERT promoter and the similarities in the telomerase regulation between the human and canine species suggests the dog as a good model for studying telomerase targeted gene therapy and also suggests that human TERT promoter driven therapies could be used cross species. It has also been shown that expression of the human hTERT gene in canine chondrocytes can increase the cellular lifespan without neoplastic transformation of the cells (Nicholson et al. 2007).

### 1.3.2 Improving efficacy of transgene expression.

The use of tissue or disease specific promoters in gene therapy allows the gene expression to be selectively targeted to cells in which the promoters are active. While these systems are promising with regard to selectivity, they are limited in their application due to the relative weak activity of the tissue and disease specific promoters. To overcome this obstacle a two step transcriptional amplification system has been

developed in which the weak signal from the tissue or disease specific promoter is amplified resulting in a high level of transgene expression. Other improvements which are applied to these systems are the incorporation of regulatory elements to be able to control transgene expression by induction.

### 1.3.2.1 Two step transcriptional amplification (TSTA) system.

The TSTA system is a two promoter system in which a relatively weak tissue or disease specific promoter transcribes a transcriptional activator protein which when expressed activates a second promoter which in turn transcribes a transgene as seen in figure 1.4. The second promoter is placed downstream of specific transcriptional activator binding sites. By binding of the transcriptional activator protein to the binding sites the second promoter becomes highly active resulting in a high level of transgene expression. In tissues where the first promoter is not active and hence the transcriptional activator protein is not expressed, the second promoter will be inactive and the transgene will not be transcribed.

Figure 1.4

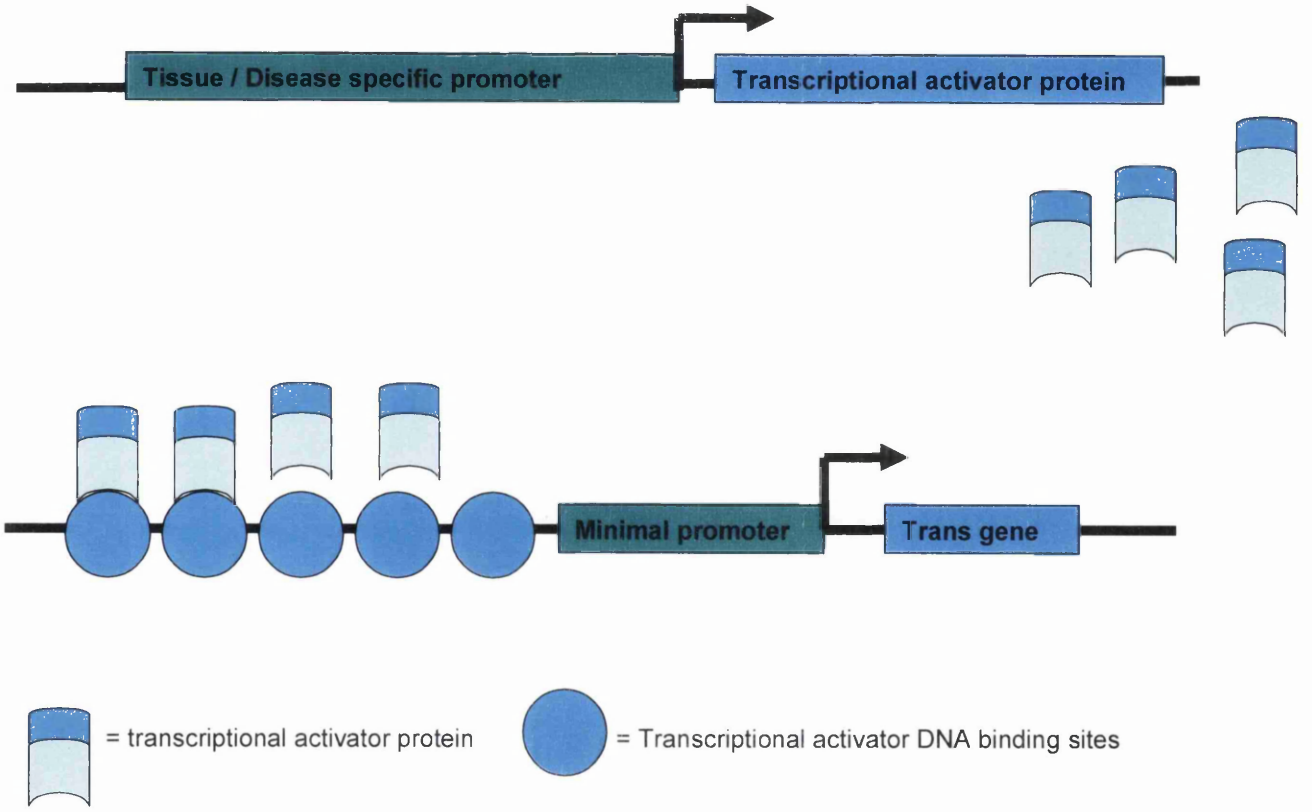



**Figure 1.4 Schematic illustration of the TSTA system**

The tissue / disease specific promoter drives the transcription of the transcriptional activator protein which when expressed in tissue where this promoter is active binds to binding sites upstream of a second promoter which is then strongly activated to transcribe a transgene.

### 1.3.2.2 The Gal4-VP16 transcriptional activator fusion protein

The Gal4VP16 transcriptional activator fusion protein has been used in TSTA systems. This is a fusion between the Gal4 DNA binding domain derived from the *Saccharomyces cerevisiea* yeast Gal4 protein and the acidic C-terminal domain of the VP16 transcriptional activator derived from human herpes virus type 1 (HSV-1). The Gal4 DNA binding domain is a protein consisting of 147 amino acids and it has been shown that this protein binds to a specific 17 base pair Gal4 binding sequence and in combination with VP16 activates transcription of downstream genes (Fang et al. 1998).

The VP16 is a regulatory protein responsible for stimulating transcription of immediate early genes in the HSV-1. The full length VP16 protein is 490 amino acids and can be divided into two domains; the N-terminal domain is responsible for targeting VP16 to specific promoter sequences while the C-terminal domain is an acidic domain that is responsible for stimulation of transcription (Flint & Shenk 1997). The C-terminal domain fused to a DNA binding domain can activate transcription at the binding site by promoting the assembly of RNA polymerase II at the site (Sadowski et al. 1988, Liu et al. 1999). The size of the VP16 C-terminal activation domain is 80 amino acids. This can be further divided into two sub domains of which the more C-terminal portion is important when used to stimulate promoters with only one DNA binding site, while the other part contributes to stimulation of promoters with more than one DNA binding site (Walker et al. 1993, Flint & Shenk 1997). The length of the VP16 fragment can vary in size but commonly the last 78 amino acids of the carboxyl-terminal of the VP16 are used (Sadowski et al. 1988).

Various promoters can be used to drive the transcription of the Gal4VP16 in the TSTA system. The second promoter used in the system which is responsible for transcribing the transgene is commonly a minimal Gal4 responsive promoter consisting of a TATA box derived from the adenovirus E1b region which is placed downstream of multiple Gal4 binding sites (Sadowski et al. 1992)

### 1.3.2.3 The use of the Gal4VP16 in the TSTA system

The Gal4VP16 fusion protein has been used in the TSTA system in conjunction with various disease and tissue specific promoters, transgenes and gene delivery vectors.

The hTERT promoter has been used in the TSTA system in conjunction with a Gal4VP16 fusion protein of which the VP16 consisted of the 78 amino acids of the carboxyl-terminal of the VP16 protein. The transcriptional activator in this system bound to a minimal Gal4 responsive promoter with 5 upstream Gal4 specific binding sites as seen in figure 1.5 a) (Fang et al. 1998). The system was delivered using an adenovirus vector system initially as a two vector system but later as a one vector system in which all the elements from the TSTA system were placed in the E1 region of one adenovirus vector (Gu & Fang 2003). As a transgene the system was tested first with a LacZ reporter gene and later with a sequence coding for a fusion protein between green fluorescent protein (GFP) and the full length TRAIL gene (Kagawa et al. 2001, Lin et al. 2002, Gu & Fang 2003) or a Bcl-2–associated X protein (BAX) gene (Gu et al. 2000, Kagawa et al. 2000). The system encoding the BAX gene was used as a two vector system since the toxic nature of the BAX gene made it difficult to produce viral vectors with the whole TSTA system due to the lethal effect on the cells with BAX expression (Kagawa et al. 2000). In summary from this work it was shown that the TSTA system was able to increase the transcriptional activity of a reporter gene 150 fold compared to using the hTERT to drive the transcription of the reporter gene on its own (Gu & Fang 2003). It was furthermore shown that using the TRAIL-GFP fusion protein cellular apoptosis was induced specifically in cancer cells both in vitro and in vivo in animal models with a good bystander effect and minimal toxicity to "off target" cells (Kagawa et al. 2001, Lin et al. 2002). The use of the system with the BAX gene did show significant apoptosis of normal hepatocytes suggesting that using the BAX gene in the system would cause pronounced side effects (Kagawa et al. 2000).

The prostate specific antigen (PSA) promoter and enhancer has been used with the TSTA system in order to target transgene expression to prostate cancer cells. This has been done using a Gal4VP16 fusion protein in which the VP16 consists of a tandem repeat of the VP16 protein base pairs 413-454 and in conjunction with a Gal4 responsive promoter with 5 upstream Gal4 binding sites as seen in figure 1.5 b). This was done in a single vector plasmid (Iyer et al. 2004, Hattori & Maitani 2006), adenovirus (Dzojic et al. 2007, Sato et al. 2008) and a lentivirus (Zhang et al. 2002) delivery system. Reporter genes were incorporated into these systems as transgenes used to monitor the tissue specific gene expression. From all these studies it was shown that using the PSA promoter and enhancer in conjunction with the Gal4VP16 and the TSTA system, cell specific gene expression was seen in prostate cells both in vitro and

in vivo in animal models (Zhang et al. 2002, Iyer et al. 2004, Dzojic et al. 2007, Sato et al. 2008). It was also shown that the TSTA system was stronger than a one step system with a 640 fold increase in transcriptional activity (Zhang 2002, Hattori & Maitani 2006). One study investigated the effect of incorporating the HSV-tk gene in the TSTA system with the PSA promoter and enhancer or a midkine promoter as tissue or disease specific promoters and showed selective inhibition of prostate tumour cell growth in vitro (Hattori & Maitani 2006). The midkine promoter used in this study is a promoter responsible for transcription of a growth factor and this promoter has been shown to be active in various cancer types (Hattori & Maitani 2006).

The TSTA system in conjunction with the Gal4VP16 fusion protein has also been used to transcribe two transgenes simultaneously (Ray et al. 2004). In this study 5 Gal4 binding sites were placed in between two Gal4 responsive promoters which each transcribed a reporter gene as seen in figure 1.5 c). A SV40 promoter was used to transcribe the Gal4VP16 protein in this study to prove the concept of the system without restricting transcription to a specific cell type. The study showed that both reporter genes were expressed in this TSTA system. This could be an advantage for monitoring gene expression in tissues in vivo (Ray et al. 2004).

**Figure 1.5**

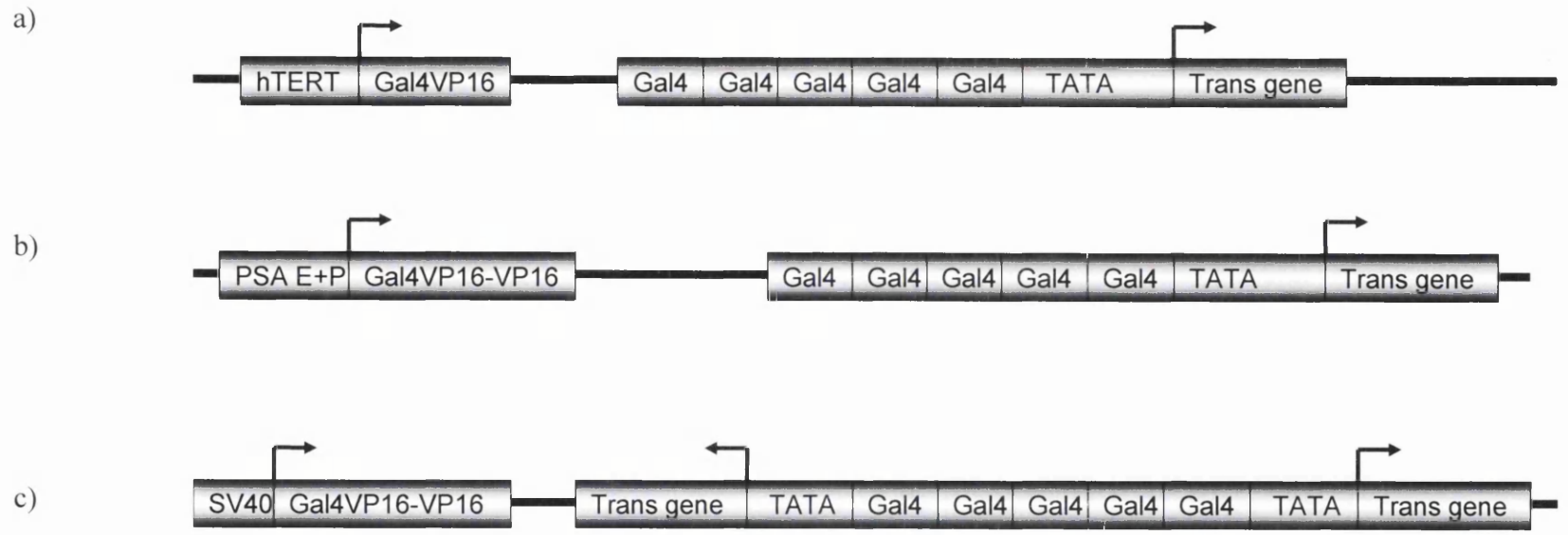



**Figure 1.5 Schematic models of different uses of the TSTA system with Gal4VP16.**

a) TSTA system with the human TERT promoter driving the transcription of the Gal4VP16 transcriptional activator fusion protein (Fang et al. 1998, Gu & Fang 2003)

b) TSTA system with the PSA promoter and enhancer driving the transcription of the Gal4VP16-VP16 transcriptional activator fusion protein (Zhang et al. 2002, Iyer et al. 2004, Hattori & Maitani 2006, Dzojic et al. 2007, Sato et al. 2008).

c) TSTA system with the SV40 promoter driving the transcription of the Gal4VP16-VP16 transcriptional activator fusion protein. Two Gal4 responsive promoters are present facilitating transcription of two transgenes (Ray et al. 2004).

### 1.3.2.4 Other transcriptional activators used with the TSTA system

Transcriptional activator fusion proteins other than the Gal4VP16 have been used in conjunction with the TSTA system. A Gal4p65 fusion protein consisting of the Gal4 DNA binding domain fused to the p65 transcription activation domain which is derived from mammalian cells has been studied (Liu et al. 2006, Liu et al. 2008).

A tetracycline responsive system in which the VP16 protein is fused to a tetracycline transactivator protein (tTA) has also been studied (Fitzsimons et al. 2001, Wack et al. 2008). This system is advantageous since it allows for regulation of transgene expression in vivo by tetracycline. Both the Gal4p65 and VP16tTA fusion proteins have shown promising efficiency in amplifying the transcriptional activity from a tissue or disease specific promoter (Fitzsimons et al. 2001, Wack et al. 2008, Liu et al. 2008).

In relation to the VP16tTA fusion protein it was shown that in order to save space the VP16 sequence could be replaced by a triple repeat of a 12 base pair minimal activation domain of the VP16 protein. This alternative shorter VP16 fragment should reduce some of the squelching effect induced by interactions with the transcriptional activator and the cellular transcription factors (Fitzimonz et al. 2001). No comparison has been made between the Gal4VP16, the VP16tTA and the Gal4p65 transcriptional activators and it is therefore not known if one is more potent than the other.

A TSTA system containing a cancer specific promoter, a tissue specific transcriptional activator and a tissue specific second promoter has been developed in order to target gene expression to lung cancer cells specifically (Fukazawa et al. 2004). This system illustrates the many possibilities to optimise the TSTA system for specific cancer types.

## 1.4 Cancer gene therapy

There are many types of gene therapy that have been applied to cancer treatment as noted in chapter 1.1. Two areas of cancer gene therapy are of particular interest for this thesis. These are effector gene therapy and oncolytic gene therapy. Oncolytic gene therapy which was not mentioned in chapter 1.1 is a gene therapy method which is only applied to cancer and is promising in the way that it circumvents two of the major

obstacles for gene therapy be namely transgene delivery and specificity of transgene expression.

### 1.4.1 Effector gene therapy for cancer

Effector gene therapy is quite a broad gene therapy approach. In effector gene therapy for cancer the goal is to make the target cells express a gene which will result in cancer regression either by a direct or indirect effect. The transgene applied can be over expression of a normal cellular gene or it can be expression of a gene that is unfamiliar to the cells.

Both endogenous and exogenous genes have been studied for this approach. A major focus has been on using genes coding for antiangiogenic peptides since sustained vascularity is essential for tumour growth and maintenance. Endostatin and angiostatin are two genes which have been used either alone or in combination to restrict tumour growth by gene therapy (Li et al. 2008).

A direct effect on cancer cells is often sought by expressing lethal genes directly in the cancer cells. Lethal genes can be targeted to cancer cells by cancer specific promoters as described in chapter 1.3. Several lethal genes have been studied for this use such as TRAIL, Caspase 8, FADD and BAX (Gu & Fang 2003). TRAIL is of particular interest for this thesis and a more detailed description of this protein's characteristics and use will follow.

#### 1.4.1.1 Tumour necrosis factor-related apoptosis inducing ligand (TRAIL)

Tumour necrosis factor-related apoptosis inducing ligand (TRAIL), also known as Apo2L, is an apoptosis-inducing protein which has received great attention for its ability to induce apoptosis in cancer cells while not harming normal cells (Wiley et al.1995, Kimberley & Screaton 2004, Koschny et al. 2007).

TRAIL is a member of the tumour necrosis factor (TNF) family. While other members of this family have been shown to induce severe toxicity by systemic administration, TRAIL appears to be less toxic (Duiker et al. 2006). The exact physiological role of TRAIL has not been determined but it is presumed that it plays a role in anti-tumour immunity. TRAIL knockout mice have no gross phenotypic characteristics; however upon inoculation with tumours they develop a heavier tumour

load than wild type mice (Sedger et al. 2002, Duiker et al. 2006). In longer term studies, aging TRAIL -/- mice develop more spontaneous lymphomas (Schaefer et al. 2007)

TRAIL is a type II transmembrane protein which has been shown to occur as a trimer (Kimberley & Screaton 2004). The protein is found to be expressed at variable levels in different normal human cells such as myocytes, hepatocytes and Leydig cells while being absent in other cells (Kagawa et al. 2001, Fulda & Debatin 2004, Spierings et al. 2004). The precise distribution of TRAIL expression in human tissues is not agreed and some studies show a much more restricted distribution than others (Daniels et al. 2005). The full length human TRAIL protein consists of 281 amino acids. In comparison the mouse TRAIL protein which has also been isolated concists of 291 amino acids. The mouse protein shares 65% amino acid homology with the human counterpart (Wiley et al. 1995).

#### 1.4.1.2 TRAIL receptors

In humans five TRAIL receptors (TRAIL-R) have been identified. TRAIL-R1 and TRAIL-R2 are death receptors which are located on the cell surface. They are type I trans-membrane proteins and consist of an extra cellular binding domain and a cytoplasmic death domain (Zhang et al. 2000, Spierings et al. 2004). TRAIL-R1 and TRAIL-R2 react upon binding of TRAIL by intracellular formation of the death-inducing signalling complex (DISC) (Fischer & Schulze-Osthoff 2005). This complex is formed by the cytoplasmic death domain of the receptors which interact with the death domain of the adaptor protein FAS-associated death domain (FADD) which in turn interacts with caspase 8 or 10 to complete the complex. The formation of DISC initiates the caspase cascade by cleavage of caspases 3, 6 or 7 which in turn leads to cell death by apoptosis (Fischer & Schulze-Osthoff 2005). In some cell types very low amounts of caspase 8 and 10 are activated. In these cells apoptosis occurs through a mitochondrial activation loop resulting in release of pro-apoptotic proteins such as cytochrome C from the mitochondria into the cytosol (Fulda & Debatin 2004). In mice only one death receptor has been identified (Cretney et al. 2007, Takeda et al. 2007).

The three other TRAIL receptors are decoy receptors which are incapable of inducing apoptosis.

TRAIL-R3 is an extra cellular linked protein and therefore does not possess an intracellular death domain (Zhang et al. 2000).

TRAIL-R4 is a type 1 trans-membrane protein but unlike TRAIL-R1 and TRAIL-R2 the intracellular death domain is truncated (Zhang et al. 2000).

The last receptor TRAIL-R is osteoprotegerin which is a soluble protein known to play a role in osteoclastogenesis (Speiring et al. 2004). The decoy receptors are mainly believed to inhibit apoptosis by competitive binding of the TRAIL ligand (MacFarlane 2003). This binding decreases TRAIL's availability to the death receptors and thereby causes TRAIL resistance as seen in normal cells. Studies have been done to localise the TRAIL receptors in different tissues and evaluate the effect of TRAIL when repressing individual receptors. It has been shown that while some cell types seem dependent upon the decoy receptors to protect them against TRAIL induced apoptosis, other cells use a different mechanism which seems to be effective downstream of caspase activation (Zhang et al. 2000, Kimberley & Screaton 2004).

#### 1.4.1.3 TRAIL receptor expression in vivo.

The distribution of the different TRAIL receptors varies between different cell types. In general the decoy receptor TRAIL-R3 has been shown to be expressed in a variety of human tissues whereas TRAIL-R4 has been shown to be expressed in all tissues tested. The death receptors TRAIL-R1 and TRAIL-R2 have been shown to be expressed in most tissues (Zhang & Fang 2005). A study comparing expression of TRAIL receptors in malignant and non-malignant tissue showed that TRAIL-R1 and TRAIL-R2 expression was increased in some malignant tissues while it was down regulated in others suggesting this to be the reason that some cancer cells are very responsive to TRAIL induced apoptosis while others seem more resistant (Daniels et al. 2005). Studies show great homology of TRAIL and TRAIL-receptor distribution between non-human primates and humans. Homology between the protein sequences of these constructs between the species has also been found and the human TRAIL protein binds and cross-reacts with the non-human primate receptors (Spierings et al. 2004). Even though the dog genome differs more from the human than the non-human primates it is reasonable to expect a similar cross-reactivity between the human TRAIL protein and the canine TRAIL receptors. A study used the combination of siRNA against the anti-apoptotic x-linked inhibitor of apoptosis (XIAP) gene and treatment with recombinant human TRAIL on multiple dog cell lines in vitro. This study also showed the ability of human TRAIL to induce apoptosis in dog cells, indicating that

there must be cross reactivity between these species and that TRAIL death receptors must exist in the dog (Spee et al. 2006).

#### 1.4.1.4 TRAIL for cancer therapy

The ability of TRAIL to induce apoptosis in transformed cells while not in normal cells has made this gene promising for cancer gene therapy (Wiley et al. 1995, Kimberley & Screaton 2004, Duiker et al. 2006, Koschny et al. 2007). This ability was initially believed to be due to the distribution of death receptors versus decoy receptors in cancer cells. A significant correlation between TRAIL susceptibility and expression of receptors on cancer cells has not been found (Zhang & Fang 2005).This also means that cancer cell resistance to TRAIL treatment is not correlated with the expression of decoy receptors. Other mechanisms of cancer cell resistance to TRAIL treatment have been proposed, such as dysfunctional death receptors due to mutagenesis, or dysfunction in any of the other important elements in the TRAIL-induced apoptosis pathway such as assembly of DISC and recruitment of FADD (Zhang & Fang 2005). It has also been shown that TRAIL can activate NFκB which in turn antagonises apoptosis by up-regulating the transcription of antiapoptotic genes (Shetty et al. 2002, MacFarlane 2003). An *in vitro* study also showed that expression of HPV16 E6 protein could protect HCT116 colon cancer cells against TRAIL-induced apoptosis. This effect was not seen in U2OS cells expressing the same protein, indicating that TRAIL might activate different pathways in different cell types (Garnett et al. 2007). Studies on hypoxia and cancer cell TRAIL resistance have also been performed. The results from these studies are conflicting whilst some suggest that hypoxia, which is commonly present in the tumour cell environment, inhibits TRAIL-induced apoptosis by blocking BAX translocation to the mitochondria (Kim et al. 2004a), others find a synergistic effect of hypoxia on TRAIL induced apoptosis in breast cancer cells (Fitzgerald et al. 2007). Some suggests that over expression of certain oncoproteins may play a role in sensitising tumour cells to TRAIL induced apoptosis, and in this aspect it was found that Myc sensitised cells to TRAIL induced apoptosis while hTERT and SV40 large T antigen did not (Huang & Sheikh 2007).

The data on normal cell resistance to TRAIL is diverse. Most studies indicate that while some normal human cells are refractory to TRAIL-induced apoptosis other cell types are categorised as partially resistant (Steele et al. 2006). Liver cells in

particular have been shown to be sensitive to TRAIL toxicity (Jo et al. 2000, Zheng et al. 2004).

Studies on the interaction between the TRAIL ligand and its receptors have shown that the trimeric TRAIL binds to 3 monomeric receptors leading to the suggestion of a ligand induced trimerisation model (Kimberley & Screaton 2004). It has also been suggested that the receptor trimerisation occurs under influence of a ligand independent extra cellular oligomerisation domain known as the pre-ligand assembly domain. These studies have led to multiple proposals to explain how some cell types and tumour types are resistant to TRAIL. A better understanding of this system is required before TRAIL can be seriously used for cancer cell killing (Kimberley & Screaton 2004).

A marked bystander effect has been shown with the transfection of the full length TRAIL gene into cells (Kagawa et al. 2001). This has been observed as the death of non-transfected cells surrounding transfected cells. This effect is not transferable with the media suggesting that it is not a secreted form of TRAIL that accounts for this effect (Kagawa et al. 2001).

To counteract the possible toxicity of the human wild type TRAIL in normal cells TRAIL has been used in gene therapy experimental trials under transcriptional control by tissue specific promoters (Lin et al. 2002).

The effects of different recombinant soluble forms of TRAIL have been studied in normal and cancerous cell lines. The soluble form of TRAIL consists of amino acids 114-281 of the TRAIL protein. Some studies however do use a larger fragment consisting of amino acids 95-281(Ma et al. 2005). Data indicate that the soluble form of TRAIL is more effective in killing cancer cells (Kim et al. 2004b, Kim et al. 2006). Further alterations have been done to make the soluble TRAIL protein even more efficient in cell killing. Adding a trimerisation signal in the form of an isoleucine zipper stabilises the trimerised form of soluble TRAIL which is needed for optimal death receptor binding (Kim et al. 2004b). Furthermore, the addition of a special cleavable secretion signal to avoid unwanted immune responses has shown great potential. This modified TRAIL construct has shown to be more efficient in tumour cell killing than the normal TRAIL protein and in addition is less toxic to normal cells (Kim et al. 2004b). Studies have shown that when transfecting this modified TRAIL gene into 293-T cells, the cell media can be transferred to a culture of TRAIL sensitive cells and

induce apoptosis. In the same study the TRAIL construct was transfected in to a range of normal cell types without showing signs of toxicity (Kim et al. 2004b). In practise this indicates that systemic administration of TRAIL could be performed with normal and cancer cells expressing and excreting TRAIL which in turn would only be able to induce apoptosis in cancer cells. The same TRAIL construct has been tested *in vivo* in mouse tumour models and it is shown that this construct is able to suppress tumour growth when injected intra-tumourally (Kim et al. 2006).

The major obstacle regarding TRAIL cancer gene therapy is its possible toxicity to normal cells and the possibility of cancer cells becoming resistant. Studies have compared the effectiveness of cancer cell killing by soluble TRAIL and adenoviral delivered full length TRAIL gene (Voekel-Johnson et al. 2002, Seol et al. 2003). These studies show that the full length TRAIL delivered by an adenoviral vector combination was able to induce apoptosis in cancer cell lines resistant to soluble TRAIL and showed minimal toxicity in normal cells (Voekel-Johnson et al. 2002, Seol et al. 2003). This indicates that controlled expression of the full length TRAIL gene might be a safe and effective treatment for cancer.

In summary the TRAIL gene has shown great potential for use in cancer gene therapy. TRAIL is competent when used on its own however to increase the cancer cell killing TRAIL can be used synergistically with other therapies such as chemotherapy and radiation therapy (Cretney et al. 2006).

### 1.4.2 Oncolytic gene therapy

Oncolytic viruses offer a great promise for cancer gene therapy. The reason is that this type of therapy utilises the properties of viruses to infect, replicate and induce cell death in cells while simultaneously releasing viral progeny which can then readily infect neighbouring cells. This strategy shows many promising advantages but also includes various risk factors such as immunogenic reactions and recombination of viruses (Ko et al. 2005, Liu & Kirn 2008).

Early in the 20$^{th}$ century it was observed that viral infections could alleviate or cure cancer, bringing forth the interest of using engineered viruses for cancer therapy (Liu et al. 2007). Two virus groups have been of particular interest for oncolytic cancer therapy due to their characteristics and pathogenesis. These are adenoviruses and herpes viruses, in particular herpes simplex virus type 1, which are double stranded

DNA viruses (Guo et al. 2008). Other virus types have also been used for oncolytic virus therapy and have been tested in late phase clinical trials such as vaccinia virus, Newcastle disease virus, and reovirus (Liu & Kirn 2008).

Some viruses need to be modified to be able to selectively infect and replicate in cancer cells while others have a natural phenotype that allows for replication in tumours only. The avian Newcastle disease virus depends on defective interferon signalling for successful replication and cell death and also the vaccinia virus is naturally oncolytic though by unknown mechanisms (Liu et al. 2007, Guo et al. 2008).

Other unmodified wild type viruses including adenovirus, reovirus, West Nile virus and mumps virus have been used for cancer therapy with variable response rates (Liu et al. 2007).

#### 1.4.2.1 Engineered viruses.

Adenoviruses and herpes simplex virus type 1 are typically modified for use as oncolytic viruses and to target their replication to cancer cells. This can be done either on a cell targeting level by modifying viral receptors to specific target cancer cells or at the transcriptional or translational level of viral gene expression by restricting this to cancer cells. As in other cancer gene therapy approaches it is important that the viral replication shows a high level of specificity for cancer cells but also has a high level of efficacy allowing for efficient killing of target cells. Early data from clinical trials showed that cancer therapy using oncolytic viruses is safe but did not show high treatment efficacy and there is an essential demand to improve current systems (Liu & Kirn 2008). The development of oncolytic viruses for cancer therapy has gone from unmodified wild type viruses, to deletion mutants in which replication depends on phenotypic alterations in the cancer cells, to transcriptionally regulated viruses in which transcription of essential viral replication elements is under transcriptional control of a cancer or tissue specific promoter to the latest armed oncolytic viruses which as well as inducing cell lysis also encode therapeutic or suicide genes (Liu & Kirn 2008). The oncolytic viruses offer a great plasticity and an opportunity to engineer viruses which are optimised to target certain cancer types. Some engineered viruses have several cancer and tissue specific promoters incorporated to increase the specificity for certain cancer types (Wang et al. 2008), and some are armed with several therapeutic genes such as the Ad5-CD/TKrep virus which on top of having a E1B deletion making it

dependant on p53 defects for replication, also encodes the two suicide genes thymidine kinase and cytosine deaminase making it able to convert two pro-drugs into active cytotoxic metabolites to kill the cancer cells (Barton et al. 2006). Many clinical trials have been performed using viruses as single agents or in combination with chemotherapy, radiotherapy or surgery and it has been shown that virus therapy has a synergistic or additive effect when used in combination with other therapies. Several different routes of administration have been used and it is now known that circulating host antibodies do not have an effect on the viral treatment efficacy when virus is administered locally and also that the immune system can sometimes improve the efficacy of the virus treatment (Liu & Kirn 2007). The first genetically engineered cancer selective virus to be used in human cancer treatment was the ONYX-015. This human adenovirus with a E1B and E3 deletion showed some antitumoural effect in clinical trials however the efficacy was too low. In 2005 the first oncolytic virus was approved for marketing for use in cancer therapy in China. This H101 oncolytic adenovirus which has obtained cancer selectivity by deletion in the E1B region resembles the earlier ONYX-015 virus but has retained the viral E3 region and thereby has improved cytolytic effect. Results from phase III clinical trials with this virus in combination with chemotherapy showed increased tumour response rate leading to the approval of the virus for clinical use as a combination therapy for cancer (Liu et al. 2007, Guo et al. 2008).

Rodent models are generally used as preclinical models for studying human oncolytic virus therapy, however, these models are limited by the size of the animals, the difference in immune response and the species specificity of the viruses making better models necessary for future progress (Hemminki et al. 2003, Zhu et al. 2006).

#### 1.4.2.2 Conditionally replicative adenoviruses (CRAds)

CRAd is a term used specifically about adenoviruses and often refers to adenoviruses in which the transcription of genes essential for viral replication have been put under the control of a disease or tissue specific promoter allowing the virus to replicate and induce lysis in target cells only. This if often accomplished by replacing the E1 or E4 promoters with exogenous promoters.

The OBP-301 virus and the CRAd-CXCR4.RGD virus are good examples of CRAds used to target cancer cells. The OBP-301 virus also known as telomelysin is a CRAd which has been made selective for replication in cancer cells by replacing the promoter responsible for transcription of the E1 adenoviral gene region with the human telomerase promoter hTERT (Huang et al. 2008). This virus has shown anticancer activity against different human cancer cells in vitro. It was also shown in a nude mouse tumour model that the virus when applied intratumourally could eradicate tumour cells and also showed an oncolytic effect on distant untreated tumour sites (Huang et al. 2008).

The CRAd-CXCR4.RGD is a virus in which the promoter responsible for the transcription of the adenoviral E1 region has been replaced with a CXCR4 promoter (Zhu et al. 2007b). This promoter has been shown to be active in multiple cancer cell types while remaining silent in normal cells. This CRAd has further been modified to increase its infectivity by a capsid modification. This virus was shown to be able to replicate and induce cytotoxicity in multiple cancer cell lines in vitro and also to be able to replicate in excised human lung tumour slices accessed in vitro (Zhu et al. 2007b).

The viruses described above are both examples of CRAds which could potentially be used for cancer therapy in humans. The extrapolation of data from preclinical trials to clinical trials in humans is difficult due to the lack of good animal model systems for testing species specific CRAds.

#### 1.4.2.3 CRAds and immunity.

The pre-existing host immune response against adenoviruses is a major concern in adenovirus based gene therapy. The mode of application could have a great impact on the efficacy of the treatment. Local oncolytic therapy such as intra tumoural treatment is not initially affected by circulating neutralizing antibodies however the innate immune response is still a concern (Hay 2003). In studies using the herpes simplex virus type 1 as an oncolytic virus to target sarcoma cells in an immune competent mouse model, it was shown that seropositive mice had a better antitumour response than serom negative mice (Zhu et al. 2007a). In this experiment the mice were vaccinated against herpes simplex virus and subsequently inoculated with sarcoma cells which were treated by intra tumoural injection with an oncolytic herpes simplex virus (Zhu et al. 2007a). These results indicate that for gene therapy in cancer the immune response might

participate in eliminating tumour cells and the pre-existing host immune response against viruses can have a complimentary effect on cancer therapy.

### 1.4.2.4 Studying CRAds in animal models.

CRAds for targeting cancer cells have been developed using the CAV-2 virus. This has not been done as an attempt to treat human patients but in order to investigate in vivo effects of a CRAd in a comparative naturally occurring immune competent animal model system (Hay 2003, Hemminki et al. 2003). The many similarities between human and canine cancer pathology and epidemiology have made the canine pet population a valuable model for studying cancer and cancer therapy (Ostrander et al. 2000, Bergman et al. 2003, Hemminki et al. 2003, Hansen & Khanna 2004, Bergman et al. 2006, Palonni & Khanna 2007).

Viral replication of human adenovirus in canine cells has been reported but no cell killing effect was observed, indicating that the cytopathic effect exerted by the virus is species specific (Ternovoi et al. 2005). This is a particular problem with regard to the use of preclinical rodent animal models for testing human CRAd's. The rodents will often be immunocompromised animals with human tumour xenografts which grow *in situ* and form tumours. When testing a human CRAd in these animal models, the CRAd can replicate in the human derived tumour cells but will not be able to replicate in the normal rodent cells due to the species difference. Therefore the model will not give valuable information about the CRAds safety or the risk of killing normal cells in the host. The host immune response to adenovirus vectors is an important factor when testing both adenovirus vectors but also CRAds in vivo. The rodent preclinical models often have an immunosupressed phenotype such as SCID and therefore no information regarding the host immune response is achieved by studying these models. Another aspect is the xenograft derived tumours which might not be representative with regard to architecture and tumour environment (Hay 2003, Hemminki et al. 2003, Le et al. 2006). Using an animal model such as the dog with spontaneously occurring cancers for studying the effect of a CRAd *in vivo* is more informative however in order for this to succeed the CRAd must be derived from a canine adenovirus (Hemminki et al. 2003).

### 1.4.2.5 CAV-2 based CRAd

A CRAd based on CAV-2 in which the E1a gene of the virus is under transcriptional control of the osteocalcin promoter has been created (Hemminki et al. 2003, Le et al. 2006). This CRAd was developed to specifically replicate in osteosarcoma cells in which the osteocalcin promoter has been shown to be active. The choice to use the CAV-2 virus was based on this virus' pathophysiological similarities with the human adenovirus serotypes 2 and 5 which are commonly used for gene therapy in humans (Hemminki et al. 2003). As in humans where 90 % of the population are seropositive with antibodies against human adenoviruses (Russell 2000, Muruve 2004) then dogs, as mentioned previously, are commonly vaccinated against CAV-1 using attenuated CAV-2 and therefore the immunological status in this model system resembles the human condition (Hemminki et al. 2003). The CAV-2 based CRAd has been developed and tested in various normal and osteosarcoma cell lines and in mouse models in vivo (Hemminki et al. 2003, Le et al. 2006). Further improvements have been made to increase the infectivity of the virus in osteosarcoma cells, by incorporating a polylysine polypeptide to the viral fiber knob which has been shown to increase virus transduction (Le et al. 2006). The canine CRAd system has been taken forward and has been tested in healthy dogs to determine if any virus associated cytotoxicity could be detected. No adverse effects were observed in any of the dogs after intravenous systemic administration of the virus. Furthermore, no replication of the virus was observed, indicating that the virus was not able to replicate in normal healthy canine cells (Smith et al. 2006). The system was therefore taken further forward to be tested in clinical canine osteosarcoma cases. Canine patients received a systemic injection of the CAV-2 based CRAd 48 hours after limb amputation due to osteosarcoma. The viral load was monitored for 5 days and viral particles could be detected in the blood stream for at least 48 hours after administration. The patients received chemotherapy and were regularly monitored. At the time of publication the 4 patients were still alive indicating a possible beneficial effect with the CAV-2 CRAd (Smith et al. 2007).

## 1.5 Cancer and comparative oncology

Cancer is a disease of age and therefore it is predicted that as the lifespan of the population is extended due to better medical care and decrease in other particularly infectious diseases that the cancer incidence will increase. (Parkin 2001, Ma & Yu 2006, Higginson & Costantini 2008). In humans, in certain age groups in the developed

world, cancer is the leading cause of death (Jemal et al. 2007). The lack of gold standard curative treatments for cancer and the morbidity and mortality of the disease has made cancer a candidate disease for developing new drugs and treatment protocols.

It is not only in the human species that cancer is a common cause of death. The canine population has during the last years gained a more central position in the household in the developed world. Vaccination and regular veterinary check ups have resulted in an increase in the average life expectancy of the dog with accompanying increase in age related diseases such as arthritis and cancer.

Studies indicate that the same cancer forms are of relevance in the canine population as in the human population and also that it is the most common disease to cause death in the canine pet population in the developed world (Bonnett et al. 1997, Mitchell 1999, Dobson et al. 2002, Proschowsky et al. 2003, Hansen & Khanna 2004, Merlo et al. 2008).

As in humans there are many factors that lead to the development of cancer in dogs. Several breeds are genetically predisposed to certain forms of cancer because of the intensive inbreeding that has occurred in the canine population. Mating between close relatives is not uncommon which increases the risk of certain recessive disease genes being expressed (Ostrander et al. 2000).

As in humans, environmental exposure to carcinogens, lifestyle, diet and activity level also play major roles in the development of cancer in the dog. For instance it has been shown that there is increased risk of lung cancer and cancer in the nasal cavity in dogs that are exposed to passive smoking in their home environment (Reif et al. 1998).

Epidemiological studies in human cancer and the corresponding canine cancer form showed that there was a connection between cancer cases in a spatial and temporal manner between humans and canines indicating that factors causing cancer in humans affect the canine population as well (O'Brian et al. 2000). Due to the shorter lifespan of the dog and hence the earlier cancer development, one could regard at the canine population as a "canary in a mine" reflecting environmental carcinogens.

The conventional cancer treatment methods used for cancer in canines are similar to those employed in human cancer treatment. One aspect in which canine cancer treatment differs from human is the aggressiveness of the treatment. In humans the goal of cancer treatment is to cure the disease and prolong the lifespan of the patient. In the canine patient cancer treatment is less aggressive and rather palliative than

curative. The perception of life quality in animals is different from humans and therefore severe side effects from medication are less tolerable in animals compared to humans. The general life span of a dog is also shorter than the human life span and therefore a treatment that increases the lifespan a couple of months or even years is a long prolongation relative to the dog's natural lifespan.

The high prevalence of cancer in the canine population and the many similarities in lifestyle between the human and canine pet population has made the dog an ideal comparative model system for human cancer. The relatively shorter life span of the dog and the shorter generation time has made the dog a suitable model for studying genetic relations in cancer development since many generations can be alive at the same time point. The full dog genome was amongst the first to be fully sequenced allowing for comparison with the human genome and related disease genes (Ostrander & Wayne 2005). Many dog breeds are inbred and genome analysis for cancer related gene mutations therefore need far fewer probes than for a similar approach in humans. It has now been shown that many human and canine cancer types share the same phenotypic and histopathological characteristics. Similarities between the histopathology of canine and human mammary cancers have been shown. From this it was concluded that these lesions which are highly prevalent in females of both human and canine origin share many similarities and that canine breast cancer is a good model for studying breast cancer progression, treatment and prevention in general (Antuofermo et al. 2007).

Rodent models are often used as preclinical models in human cancer research. However rodent physiology, lifespan and the environment in which they are kept under laboratory settings differ significantly from humans. Dogs being physically both in size, but also in regard to metabolism, more similar to humans are sometimes used in experimental settings to study cancer development. Working with dogs in a laboratory setting is both very expensive and removes the advantage of the animals sharing an environment with humans and thereby adds distance to the translation and comparison with humans. Working with experimental animals in a laboratory, regardless of species, also gives rise to ethical implications (Bukowski & Wartenberg 1997). It is difficult to maintain laboratory animals in a stress free environment and therefore these animals could release stress factors or other substances that could mislead the interpretation of experimental results (Eriksson et al. 2004, Everitt & Schapiro 2006).

The canine population in the UK is estimated to be 6 million and in the USA about 55 million. This gives rise to a large naturally occurring population at risk for spontaneously developing cancer (Hansen & Khanna 2004, Starkey et al. 2005). This opens the possibility for epidemiological studies and clinical trials to be performed using domestic pet dogs, and removes the ethical dilemma encountered with experimental animals. Clinical trials for a new therapeutic agent in a dog study require about 1-3 years while in comparison a human trial takes 5-15 years (Hansen & Khanna 2004). Several therapeutic protocols have already been developed in dogs and then later transferred to humans. A good example is the limb spare therapy for osteosarcoma which was originally developed for use in dogs however is now routinely used in both humans and dogs (Withrow et al. 1993, Hansen & Khanna 2004).

A DNA vaccine containing the human tyrosinase antigen has been developed and clinically tried for treatment of canine malignant melanoma with a high success rate. In this case the human xenogeneic antigen was used to stimulate a better immune response in the dog (Bergman et al. 2003, Bergman et al. 2006, Bergman 2007). After the success in this canine trial a similar trial was initiated in humans. This trial showed good effect of the vaccine with only minor side effects. Furthermore the human study augmented the use of xenogeneic DNA since the patients in the human trial received boosters with mouse tyrosinase and human tyrosinase DNA (Wolchok et al. 2007).

Since the size and physiology between humans and canines is similar it is easy to transfer treatment protocols between these species (Hansen & Khanna 2004). Methods developed in experimental animals are also more easily applied to pet animals which function as a transition step before applying the method to humans (Cook 2007).

The use of pet animals however does not eliminate the use of simple *in vivo* rodent models to check for initial carcinogenicity or toxicity (Mack 2006).

### 1.5.1 Comparative medicine and gene therapy models.

The canine population is a valuable model system for studying cancer and is a potential population in which to test upcoming gene therapy drugs prior to human trials. There is a whole range of other non cancerous genetic and acquired disorders for which gene therapy is being developed and for which the naturally occurring diseases in the dog will likewise be a good model systems. Gene therapy for skin disorders as an example has received a lot of attention due to the easy accessibility of this organ making

topical gene therapy treatment an option. Many skin diseases are of genetic origin, either caused by mutations or inheritance and their effects can have detrimental and fatal outcomes. Ichthyosis and epidermolysis bullosa are two cutaneous disorders which occur in both humans and canines and for which gene therapy is being considered as a treatment option (Hargis & Ginn 2001, Hengge 2005).

Degenerative joint disease is another disease which is currently being exploited for treatment with gene therapy (Evans et al. 2004). The geriatric canine population has a high prevalence of osteoarthritic disease and is therefore a good candidate for studying gene therapy in a spontaneous *in vivo* model. Other musculo-skeletal disorders of non genetic origen such as fractures, ligament and cartilage damage often traumatically induced, are also being exploited for gene therapy purposes. In this manner canines which frequently suffer from traumatic injuries from road traffic accidents are likewise a good model for studying the effect of gene therapy *in vivo* (Evans et al. 2004, Carofino & Lieberman 2008). Duchenne's muscular dystrophy which is a genetically X-linked disorder causing premature death in humans, has a counterpart in the canine population and gene therapy trials have already been carried out in dogs in which a functional dystrophin gene was restored and trials are now ongoing in humans (Tsai et al. 2007). Ophthalmic disorders are being exploited for gene therapy. The eye is a good organ for gene therapy due to the blood retina barrier allowing the eye to avoid the immune system, the easy accessibility of the organ, the small volume of the organ and the ease with which it can be monitored by visual inspection. Dogs suffer from retinal degenerative disease similar to humans and gene therapy trials in dogs were able to restore eyesight which has led to consideration of similar trials in humans (Rex 2007, Tsai et al. 2007). Another genetic disorder shared between humans and canines is haemophilia B. This disease has been exploited for gene therapy treatment and in dogs expression of a therapeutic gene by gene therapy has already been tested providing evidence proof that the method can potentially be used in humans (Tsai et al. 2007).

The large canine pet population in the developed world, the motivation of compliant owners to treat their animals and the presence of high standard veterinary hospitals with state of the art equipment provides a good base for treating dogs with gene therapy for various disorders in order to cure the disease in this species and to function as a preclinical model for human trials.

## 1.6 Objectives of this project.

The efficacy of gene therapy is restricted *in vivo* due to challenges in restricting transgene expression to target cells, assuring adequate therapeutic levels of transgene expression and efficacy of *in vivo* delivery systems. The aim of this thesis is to target each of these problem areas in order to create better gene therapy systems. This work also focuses on using the canine population as a representative naturally occurring spontaneous animal model for studying cancer gene therapy.

The major aims of this thesis are:

I. To develop a TSTA system which is more potent than the commonly used systems.

II. To target transgene expression to cancer cells and in conjunction with the TSTA system induce cell death in these.

III. To develop a CRAd based on the CAV-1 virus to be used for treating canine cancer and as a model system for human cancer gene therapy including the developed TSTA system.

# Chapter 2

## Material and Methods.

### 2.1 Materials

#### 2.1.1 Antibodies

**Abcam Plc (Cambridge, United Kingdom)**

Rabbit polyclonal IgG anti human adenovirus type 5.

Rabbit polyclonal IgG anti TRAIL

Rabbit polyclonal IgG anti VP16 tag

**Acris Antibodies GmbH (Hiddenhausen, Germany)**

Sheep polyclonal IgG anti purified canine IgG, Horseradish peroxidise conjugated.

**New England Biolabs Ltd (Hertfordshire, United Kingdom)**

Rabbit anti cleaved caspase 3 (Asp 175)

Rabbit anti caspase 3

Rabbit anti cleaved PARP (human)

**Sigma Aldrich Company Ltd. (Dorset , United Kingdom)**

Goat anti Rabbit IgG, peroxidise conjugated.

Mouse anti gammatubulin clone GTU-88

Rabbit anti mouse IgG (whole molecule), peroxidise conjugated.

### 2.1.2 Bacteriology

#### 2.1.2.1 Antibiotics and selection indicators

**Sigma Chemical Co Ltd (Dorset United Kingdom)**

Ampicillin

Streptomycin

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL)

#### 2.1.2.2 Competent cells

BJ5183s cells were a kind gift from Dorothy Montgomery, University of Glasgow.

JM-109 cells were a kind gift from Dr Philippe Gobeil, University of Glasgow.

**Invitrogen Ltd (Paisley, United Kingdom)**

One Shot® TOP10 Electrocompetent™ *E. coli*

MAX Efficiency® Stbl2™ Competent Cells

Subcloning Efficiency™ DH5α™ Competent Cells

#### 2.1.2.3 Growth medium

**Institute of Comparative Medicine Central services (University of Glasgow, United Kingdom)**

Luria Broth (L-Broth), 1 litre: 10 g Tryptone, 10g NaCl, 5g yeast extract, 1000 ml $ddH_2O$.

Luria Bertai agar, 1 litre: 10g tryptone, 10g NaCL, 5g yeast extract, 15g bacteriological agar, 1000 ml $ddH_2O$.

Super Optimal Catabolite repression broth (SOC) 1 litre: 20 g Tryptone, 5 g yeast extract, 0.5 g NaCl, 2.5 ml 1M KCl, 1000 ml $ddH_2O$.

### 2.1.3 Cell Culture

#### 2.1.3.1 Cell lines

**293-T:** Human Embryonic Kidney 293T cells are derived from the 293 cell line in which the SV40 T-antigen has been inserted. 293-T cells exert a high level of telomerase activity (Nasir et al. 2001). This cell line was a kind gift from Dr. Brian Willett, University of Glasgow.

**C33a:** C33a cells are derived from a human papilloma virus negative cervical carcinoma. C33a cells are telomerase positive (Kyo et al. 1998). This cell line was obtained from Cancer Research UK (Cancer Research United Kingdom (CRUK), London).

**GM-847:** GM-847 cells are SV40-immortalized human fibroblasts. The cell line maintains its telomerase by an alternative lengthening of telomeres (ALT) mechanism (Perrem et al. 2001). GM-847 cells were a kind gift from Professor Nicol Keith, Beatson Institute, Glasgow.

**MCF-7:** MCF-7 cells derived from a Human Caucasian breast adenocarcinoma. This is a telomerase positive cell line (Raymond et al. 1999). MCF7 cells were purchased from Cancer Research UK (CRUK, London United Kingdom).

**MDCK:** Madin-Darby Canine Kidney (MDCK) is a canine epithelial cell line derived from kidney tissue. This is a telomerase positive cell line (Nasir et al. 2001). MDCK cells were originally bought from ECACC (ECACC 85011435)

**MDCK P 3.1:** MDCK P 3.1 cells are derived from the MDCK cell line and are stably expressing the canine adenovirus E1 gene under Geneticin (Invitrogen) selection. These cells were a kind gift from Dorothy Montgomery, University of Glasgow.

**MDCK Rvd 1.9:** MDCK Rvd 1.9cells are derived from the MDCK cell line and are stably expressing the canine adenovirus E1 gene under Geneticin (Invitrogen) selection. These cells were a kind gift from Dorothy Montgomery, University of Glasgow.

**MRC5:** MRC5 is a primary foetal fibroblast cell line derived from humans. MRC5 cells are telomerase negative cell and it is not an immortal cell line (Keys et al. 2004). The cell line was purchased from CRUK (CRUK, London United Kingdom).

**Primary canine chondrocytes:** These cells were primary cells derived from the femur of a white Alsatian dog. Cells were a kind gift from Dr. Iain Nicholson, University of Glasgow.

**Primary canine fibroblasts:** These cells were primary cells which were cultured from an abdominal skin sample from an euthanized dog. Cells were dissociated from a canine abdominal skin sample and cultured as described in methods.

**Primary canine fibrosarcoma** These cells were primary cells which were cultured from an excised fibrosarcoma from the Small Animal Hospital, University of Glasgow. Cells were dissociated from the tumour mass and cultured as described in methods.

**Primary canine sertoli cell tumour** These cells were primary cells which were cultured from an excised sertoli cell tumour from the Small animal hospital, University of Glasgow. Cells were dissociated from the tumour mass and cultured as described in methods.

**U2OS:** U2OS cells are oesteosarcoma cells, expressing wild type p53. This is a telomerase negative cell line which uses the ALT mechanism to maintain its telomeres (Savage et al. 2005). U2OS cells were purchased from CRUK (CRUK, London United Kingdom).

### 2.1.3.2 Media and additives

**Invitrogen Ltd (Paisley, United Kingdom)**

DMEM 1 x w/GlutaMAX™/Glucose Na Pyr

Distilled water

Foetal bovine serum

Fungizone® antimycotic liquid

Geneticin® selective antibiotic

GIBCO™ Hanks' Balanced Salt Solution (HBSS) (1X) liquid, no calcium chloride, magnesium chloride, or magnesium sulphate

HEPES buffer solution

L-Glutamine 200 mM (100x), liquid

Non-essential amino acids

Opti-MEM® I Reduced Serum Media

Penicillin-Streptomycin Solution

RPMI Medium 1640 (1X), liquid

Trypan blue stain

Trypsin-EDTA (1x) 0.05% trypsin 0.53mM EDTA·Na

### 2.1.3.3 Transfection reagents

**Amaxa Biosystems (Cologne, Germany)**

Nucleofector™ II device

Cell line Nucleofector Kit L

**Cambridge BioScience Ltd (Cambidge, United Kingdom)**

TransIT LT1 Transfection Reagent

### 2.1.4 Chemicals and Reagents

**Sigma Chemicals Co. Ltd (Dorset, United Kingdom)**

3′,5′,5″-Tetrabromophenolsulfophethalein (Bromphenol Blue)

4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)

3-Hydroxy-4-(2-sulfo-4-[4-sulfophenylazo] phenylazo)-2, 7-naphthalenedisulfonic acid sodium salt (Ponceau S)

Acetic acid sodium salt (Sodium acetate)

Bicinchonoinic Acid (BCA) solution

Boric Acid ~99%

Calcium Chloride

Copper (II) sulphate (pentahydrate 4% (w/v) solution)

Ethidium bromide

Nonidet P-40 (NP40)

Paraformaldehyde

Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

Poly-L-lysine

Sodium Acetate

Sodium Chloride

Tween 20 (Polyoxethylene sorbitan nonolaurate)

Tris Base (2-Amino-2-(hydroxymethyl)-1, 3-propanediol)

**University of Glasgow Stores (Glasgow, United Kingdom)**

Crude ethanol

**VWR International (Leicestershire, United Kingdom)**

Absolute 99.7-100% AnalaR® ethanol

AnalaR® Sodium Hydroxide pellets

AnalaR® Glycine molecular biology grade

AnalaR® D-(+)-Glucose

AnalaR® Glycerol

Crystal Violet

Dimethyl sulfoxide (DMSO)

Ethylene diamine tetra acetate (EDTA) disodium salt

Hydrochloric acid

Methanol AnalaR®

Propan-2-ol (Isopropanol) Molecular Biology Grade

Sodium dodecyl sulphate (SDS) AnalaR®

### 2.1.5 Enzymes

All enzymes were supplied with optimal reaction buffers and supplements if required.

#### 2.1.5.1 Ligase

**Cambrex Bioscience (Wokingham, United Kingdom)**

Takara DNA ligation Kit (version 2.1)

#### 2.1.5.2 Phosphatase

**New England Biolabs (Hitchin, United Kingdom)**

Antarctic Phosphatase

**Promega Ltd (Southampton, United Kingdom)**

TSAP Thermosensitive Alkaline Phosphatase

#### 2.1.5.3 Polymerase

**Invitrogen Ltd (Paisley, United Kingdom)**

Platinum ® Taq DNA polymerase

T4 DNA Polymerase

Taq DNA Polymerase, Native + W1

**Merck Chemicals Ltd (Nottingham, United Kingdom)**

KOD Hot Start DNA Polymerase

**Promega Ltd (Southampton, United Kingdom)**

*Pfu* DNA Polymerase

### 2.1.5.4 Restriction enzymes

**Invitrogen Ltd (Paisley, United Kingdom)**

BamH I

Bgl II

Dpn I

EcoR I

EcoR V

Hind III

Kpn I

Nhe I

Sma I

Xba I

Xho I

**New England Biolabs (Hitchin, United Kingdom)**

Fse I

Fsp I

I-Sce I

Pme I

**Promega Ltd (Southampton, United Kingdom)**

I-Ppo I

### 2.1.6 Kits

**Applied Biosystems (Warrington, United Kingdom)**

BigDye® Terminator v3.1 Cycle Sequencing Kit

RNAqueous® Kit

**Invitrogen Ltd (Paisley, United Kingdom)**

Charge Switch ® Plasmid ER mini prep Kit

Charge Switch ® PCR Clean-Up Kit

PureLink™ Gel Extraction Kit

Purelink™ Plasmid maxi prep kit

SuperScript™ III Reverse Transcriptase kit

TOPO TA Cloning® Kit (with pCR®2.1-TOPO® and TOP10 cells Electrocomp™)

**Millipore (Herts, United Kingdom)**

TRAPeze® ELISA Telomerase Detction Kit

**Promega Ltd (Southampton, United Kingdom)**

DeadEnd™ Fluorometric TUNEL System

Luciferase Assay System

Reporter Lysis Buffer (5x solution)

**Qiagen Ltd (Crawley, United Kingdom)**

QIAprep® Spin Miniprep Kit

Quiagen Large Construct Kit

**Fisher Scientific UK Limited (Leicestershire, United Kingdom)**

NucleoBond® Xtra Maxi Plus Kit

NucleoBond® Xtra Midi Kit

### 2.1.7 Miscellaneous

**Applied Biosystems (Warrington, United Kingdom)**

Hi-Di™ Formamide

**Bio-Rad Laboratories Inc. (Hertfordshire, United Kingdom)**

Certified Molecular Biology Agarose

**Promega Ltd (Southampton, United Kingdom)**

dATP 100mM (40μmol)

dCTP100mM (40μmol)

dGTP100mM (40μmol)

dTTP 100mM (40μmol)

**Invitrogen Ltd (Paisley, U.K.)**

100 bp DNA Ladder

1Kb DNA Ladder

λ DNA/Hind III Fragments

Agarose (ultrapure electrophoresis grade)

See Blue® Pre-stained protein standard

**Morrisons Supermarket (Anniesland, United Kingdom)**

Marvel dried skimmed milk

**Roche Biosystems (Basel, Switzerland)**

Complete Protease Inhibitor Cocktail Tablets

**Thermo Labsytems Corporation (Warwickshire, United Kingdom)**

Luminoskan Acsent Luminometer and Ascent software (version 2.4.2)

**Ultra Violet Productions (UVP) (Cambridge, United Kingdom)**

UVP Multi-doc gel documentation system

High performance ultra-violet transilluminator (UVP VB-26)

**Vector Laboratories, Ltd., (Peterborough, United Kingdom)**

VECTASHIELD® Mounting Medium with DAPI

## 2.1.8 Oligonucleotides 5'-3'

### 2.1.8.1 Oligonucleotides for PCR (5'- 3')

| Primer | Sequence | Purpose |
|---|---|---|
| HINDIII-GAL4 | CCGAAGCTTACGACTCACTATAGGCTAGC | Cloning of Gal4VP16 down stream of telomerase promoters. |
| 3'VP16(XBAI) | CGGTCTAGATCACTCGTCAATTCCAAG | Cloning of Gal4VP16 down stream of the telomerase promoters. |
| 5'HINDIIImay2006 | CGGAAGCTTATGTCGACGGCCCCCCCGACC | Cloning of VP16E2 down stream of the hTR promoter. |
| 3'E2JULY2006 | CGCGTCTAGATCAGAAGTCCAAGCTGGC | Cloning of VP16E2 down stream of the hTERT promoter. |
| 5'VP16JULY2006 | CCGGAAGCTTATGTCGACGGCCCCCCCGACC | Cloning of VP16E2 down stream of the hTERT promoter. |
| 3'E2XBAI | CGCTCTAGATCAGAAGTCCAAGCTGGC | Cloning of VP16E2 down stream of hTR promoter. |
| 5'HINDIII-TRAIL | CGCAAGCTTGCCACCATGGCTATGATGGAGGTC | Cloning of TRAIL downstream of 3'bpTATA or CMV promoter. |
| 3'XBAI-TRAIL | CGCTCTAGATTAGCCAACTAAAAAGGCCCC | Cloning of TRAIL downstream of 3'bpTATA or CMV promoter. |
| 3'BGLII E2 | CCGAGATCTTCAGAAGTCCAAGC | Cloning of hTERT-VP16E2 into one vector system. |
| 5'BGLII HTERT | CCGAGATCTGTCCGGCATTCGTGG | Cloning of hTERT-VP16E2 into one vector system |
| 5'BGLII HTR | CCGAGATCTAGCTACTCAGGAGGC | Cloning of hTR-VP16E2 into one vector system |
| Leftend 5'EcoRV | CCGCGATATCGTTTAAACCGATCAAGGCGAGTTAC | Cloning of leftend CAV-1 |
| 3'bpwith-r-sites | CCGCGTCGACGTTTAAACCCCGGGCACCAAATCCGCACTGCT | Cloning of 3'bpTATA into virus |
| 5'bpxhol | CCGCCTCGAGATCCACCGAAAACG | Cloning of 3'bpTATA into virus |
| Leftend3'XHOI | CCGCCTCGAGGCCGGGTGTGGAAAATTGG | Coning of leftend CAV-1 |
| Pcg-pmeI-2 | CCGCGTTTAAACTAGTTATTAATAGTAATC | Cloning of CMV-VP16 into E3 region |
| 3'PMEIE2 | CCGGTTTAAACTCAGAAGTCCAAGC | Cloning of CMV-VP16 into E3 region |
| polyAAA 5'April | CCGGGTCGACCGGCCGCAATAAAATATC | Cloning of PolyAAA |
| polyAAA 3'April | GGCCCTCGAGGGTACCTATCGATAGAG | Cloning of PolyAAA |
| Leftend 3 | GAAAAGTGCCACCTGACGTC | Pcr of mutant and wild type virus |
| Leftend3'XHOI | CCGCCTCGAGTTCTCGCGGGTGCGGTCCTG | PCR of mutant and wild type virus |

### 2.1.8.2 Oligonucletides for Sequencing (5'to 3')

| Primer | Sequence | Purpose |
|---|---|---|
| Vp16e2seq1 | GGATCCGGACTCGTCAATTCC | For sequencing of VP16 |
| Vp16e2seq2 | GAAGTCGGCCATATCCAGAGC | For sequencing of VP16 |
| Vp16e2seq3 | GCTAACCAGGTAAAGTGCTATCG | For sequencing of E2 |
| T-7 | TAATACGACTCACTATAGGG | Sequencing of T-7sites |
| GLPRIMER2 | CTTTATGTTTTTGGCGTCTTCC | Sequencing of GL2 sites |
| RVPRIMER3 | CTAGCAAAATAGGCTGTCCC | Sequencing of RV3 sites |
| 5'virus det | GACGATCTGCAGCTTCCAG | Sequencing of CAV-1 E1 region |
| 3'virus det | GACTATGTGCGATAGTGC | Sequencing of CAV-1 E1 region |
| SEQHTERTREV | CGGGCTCCGGGCACCACG | Sequencing of hTERT |
| SEQHTRREV | GATTCTCGTGTCTCAGCC | Sequencing of hTR |
| SEQE2REV | GGACCGTCCCGTACCCAAC | Sequencing of VP16E2 with double VP16 |

### 2.1.8.3 Oligonucleotides for sequencing, isolation and cloning of dog TRAIL (5'to 3')

| Primer | Sequence | Target |
|---|---|---|
| TRAIL3'upstr | GAATAGAGTCCATATTC | Upstream of dog TRAIL TAA |
| TRAIL5'ATG | GGCAGCGAGATGCAGGCC | dogTRAIL start site ATG |
| TRAIL5'DS | CAGGACAAGTACTCC | Downstream of dogTRAIL ATG |
| TRAIL3'TAA | TTAGCCGATTAAAAAGGC | dogTRAIL end site TAA |

### 2.1.8.4 Oligonucleotides for site directed mutagenesis

| Primer | Sequence | Target |
|---|---|---|
| 3'MUT E2 | CTTCCTCTGTGGCGTCGGGCGAC | E2 serine 301 mutation |
| 5'MUT E2 | GTCGCCCGACGCCACAGAGGAAG | E2 serine 301 mutation |

### 2.1.9 Plasmids and cosmids

**3'Bp-TATA:** This is a PGL3-Basic vector into which six E2 binding sites have been cloned in using the Bgl II restriction site. Downstream from this a minimal BPV-4 promoter has been cloned with a 5' deletion leaving only 3 base pairs upstream from the TATA box. This promoter was cloned in to the vector using Bgl II and Hind III restriction sites. This vector was constructed by Dr. Keith Vance (Vance et al. 2001).

**dogTERT-hTRAIL:** This vector is based on the pCR3.1-TRAIL vector (Invitrogen). In this construct the Cytomegalovirus promoter has been deleted and instead the canine telomerase promoter has been cloned in together with the full length 843 bp human TRAIL gene which was pcr amplified from the pORF-hTRAIL vector (Invivogen). This vector was constructed by Dr. Sam Long, Glasgow University.

**fΔE1ΔE3:** This is a cosmid containing the canine adenovirus type 1 (CAV-1) genome with the virus E1 and E3 coding regions removed. The cosmid is based on the Supercos cosmid (Stratagene) This cosmid was provided by Dorothy Montgomery, Glasgow University.

**LASC:** This is a cosmid containing the complete canine adenovirus type 1 (CAV-1) genome. The E3 region has been removed from this cosmid. This cosmid was provided by Dorothy Montgomery, Glasgow University.

**pBind-Gal4VP16:** This vector is based on the P-Bind vector (Promega). This vector contains the 147 amino acid yeast Gal4 DNA binding domain under transcriptional control of a CMV promoter. It has a VP16 fragment consisting of amino acids 410-487 of the full length VP16 protein. This has been cloned into the pBind vector downstream from the Gal4 site using the restriction sites BamH I and Kpn I.

**pCG-VP16E2:** This vector contains the Cytomegalovirus promoter which transcribes a fusion protein made up by amino acids 410-487 of the full length VP16 protein and 161 amino acids of the BPV-1 E2 gene including the full length DNA binding domain. This plasmid was a gift form Dr. Mart Ustav (Estonian Biocenter).

**pCR 2.1-TOPO:** This is a commercial plasmid that comes with the TOPO TA Cloning kit (Invitrogen). The plasmid is 3.9kb and contains an ampicillin resistance gene as well as a kanamycin resistance gene. A multiple cloning site is present in this plasmid and binding sites are present for use of the T-7 and M13 sequencing primers.

**pDNA:** This vector contains the cytolomegalus promoter upstream from a multiple cloning site. This vector was provided by Ed Dornan (Glasgow University).

**pDNAVP16E2:** This vector is based on the pDNA vector in which the VP16E2 sequence has been inserted downstream of the CMV promoter using the EcoR I and Xho I restriction sites.

**pGL3-Basic:** This vector contains the firefly luciferase gene and a multiple cloning region but lacks promoter and enhancer activity (Promega Ltd. Southampton, United Kingdom).

**pGL3 control:** This vector contains SV40 promoter and enhancer sequences driving the expression of firefly luciferase gene (Promega Ltd. Southampton, United Kingdom).

**pGL3hTP19:** This vector is based on the pGL3-Basic vector (Promega). It contains the 536 bp human telomerase reverse transcriptase (hTERT) minimal promoter fragment cloned in using the Xho I and Hind III restriction sites. This promoter controls the transcription of the downstream firefly luciferase gene. This vector was a gift from Professor Nicol Keith, Beatson Institute, Glasgow.

**pLh2023:** This vector is based on the pGL3-Basic vector (Promega). It contains the 867 bp human telomerase RNA template (hTR) minimal promoter fragment cloned in using the Xho I and Hind III restriction sites. This promoter drives the transcription of the downstream firefly luciferase gene. This vector was a gift from Professor Nicol Keith, Beatson Institute, Glasgow.

**pTK-Gal4-6xE2:** This vector contains 5 Gal4 binding sites and 6 E2 binding sites upstream of a TK promoter transcribing a luciferase gene. This was constructed by Dr Roni Wright (Glasgow University) by cloning 5 Gal4 binding sites which were amplified by PCR from the pG5Luc vector, into the tk 6E2 vector (Vance et al.1999) using the KpnI and NheI restriction sites.

**pSwaPme:** this vector is a modified version of the pBluescript vector (Stratagene, Cheshire UK). This vector has been modified to contain a multiple cloning site with two Swa I sites and two Pme I sites. This vector was provided by Dorothy Montgomery, Glasgow University.

**PV2 x4E2:** The PV2 x4E2 plasmid was made previously (Vance et al. 1999). This plasmid contains a mutated minimal promoter derived from the BPV-4 promoter responsible for transcription of a firefly luciferease reporter gene and has 4 upstream E2 binding sites

**PV2 x8E2:** As PV2 x4E2 but with 8 upstream E2 binding sites.

**PV2 x12E2:** As PV2 x4E2 but with 12 upstream E2 binding sites.

### 2.1.10 SDS-Polyacrylamide Gel Electrophoresis and western blotting

#### 2.1.10.1 Buffers and solutions

**Invitrogen Ltd (Paisley, United Kingdom)**

NuPAGE® Antioxidant

NuPAGE® MES SDS Running Buffer (20X)

NuPAGE® Sample Reducing Agent (10X)

NuPAGE® Transfer Buffer (20X)

#### 2.1.10.2 Chemiluminescence film

**Perbio Science UK Ltd (Northumberland, United Kingdom)**

CL-XPosure™ Film

#### 2.1.10.3 Developing solution

**GE Healthcare UK Ltd (Buckinghamshire, United Kingdom)**

ECL Plus™ Western Blotting Detection Reagents

#### 2.1.10.4 Gels and membranes

**Invitrogen Ltd (Paisley, United Kingdom)**

NuPAGE® 4-12% Bis-Tris 12 well 1.0 mm gels

NuPAGE® 4-12% Bis-Tris 10 well 1.0 mm gels

#### 2.1.10.5 Protein transfer systems

**Bio-Rad Laboratories Inc. (Hertfordshire, United Kingdom)**

Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell

**Invitrogen Ltd (Paisley, United Kingdom)**

iBlot ™Transfer Stack, Mini (Nitrocellulose)

iBlot™ Transfer Stack, Regular (Nitrocellulose)

iBlot™ Gel Transfer Device (UK)

Nitrocellulose membrane, 0.2 μm pore size

Nitrocellulose membrane, 0.4 μm pore size

## 2.2 Methods

### 2.2.1 Molecular biology protocols

#### 2.2.1.1 Chemical transformation of competent bacteria.

For chemical transformation of E.coli (Subcloning Efficiency™ DH5α™ Competent Cells, Invitrogen) the following protocol was used. Cells stored at -80°C were thawed on ice. 5 µl ligation or 1 µl (1ng/µl) plasmid DNA was added to a 30 µl aliquot of competent E.coli cells in a sterile microcentrifuge tube. The tubes were then left on ice for 30 minutes. Cells were heat shocked at 42°C for 1 min. After this 100 µl SOC medium was added to each tube and the tubes were incubated at 37°C for 1 hour with rotation at 225 rpm. The contents of each tube were then spread on L-agar plates with the appropriate selective antibiotic, allowed to dry then inverted and incubated over night at 37°C.

For chemical transformation of JM-109 cells the protocol was as follows. Cells stored at -80°C were thawed on ice. 100 µl cells were mixed with 7 µl ligation or 1ng plasmid DNA in a sterile microcentrifuge tube and left on ice 1 hour. Cells were heat shocked at 46°C for 50 seconds. After this they were put back on ice for two minutes and following 700µl SOC medium was added to each tube and the tubes were incubated at 37°C for 1 hour with rotation at 225 rpm. 300µl of each cell suspension was then spread on L-agar plates with the appropriate selective antibiotic, allowed to dry then inverted and incubated over night at 37°C.

Stbl 2 cells where transformed according to manufacturers manual (MAX Efficiency® Stbl2™ Competent Cells, Invitrogen).

#### 2.2.1.2 Small scale preparation of plasmid DNA (Miniprep).

Transformed bacteria were grown on selective antibiotic plates. A single colony was used to inoculate 2ml L-Broth with antibiotic to select growth of plasmid carrying cells. The culture was allowed to grow for 10-12 hours at 37°C with rotation at 225 rpm. 1.5 ml culture was then pelleted by centrifugation in a sterile microcentrifuge tube at 20800 x g for 10 minutes at room temperature. The plasmid DNA was prepared using

the Invitrogen Charge Switch ER mini kit according to manufacture's manual. Purified DNA was resuspended in 50 µl elution buffer.

### 2.2.1.3 Large scale preparation of plasmid DNA (Maxiprep).

Colonies were selected from agar plates and grown up in 2ml L-Broth with 100 µg/ml ampicillin over night 37C°with rotation at 225rpm. This culture was then either prepared as a small scale DNA preparation as described (2.2.1.2) or was amplified without this step. 100 µl of the 2 ml bacterial culture was inoculated into 100ml L-Broth with antibiotic. The culture was allowed to grow over night at 37°C with rotation at 225 rpm. The bacteria culture was transferred into two 50 ml tubes and the bacteria cells where pelleted by centrifugation at 2000 x g for 15 minutes. The plasmid DNA was then prepared using the Purelink Highpure plasmid maxiprep kit (Invitrogen) according to manufacturer's manual.

### 2.2.1.4 DNA clean-up with phenol chloroform isoamyl alcohol followed by ethanol precipitation.

Restriction digest and blunt ending reactions were cleaned with phenol chloroform isoamyl alcohol and then ethanol precipitated. An equal volume of phenol: chloroform: isoamyl alcohol (24:25:1 v/v/v) was added to a microcentrifuge tube containing the DNA and the tube was vortexed. The sample was then centrifuged for 15 minutes at room temperature at 20800 x g. This procedure resulted in separation of the aqueous and organic phases. The top aqueous phase containing the DNA was transferred to a new microcentrifuge tube and 1/10th the volume of 3M sodium acetate, pH 5.2 was added together with 2x volume 100% ethanol. The DNA was then allowed to precipitate at –20°C for 30-60 minutes. Following this the samples were centrifuged for 30 min, 20800 x g at 4°C. The ethanol was decanted and discarded and the pellet was washed by adding 100 µl 70% ethanol. An additional centrifugation was performed for 20 min under same condition as the previous one. The ethanol was removed and the remaining pellet was allowed to air dry and then resuspended in dd$H_2O$ or buffer (TE buffer, Invitrogen).

### 2.2.1.5 DNA gel electrophoresis on agarose gels.

DNA gel electrophoresis was performed on 1% agarose gels. These gels were made by dissolving powdered agarose in TBE Buffer (10 x TBE: 900mM Tris base, 900mM boric acid, 25mM EDTA, pH8.0) by heating. Ethidium bromide was added to visualise the DNA at a concentration of 0.25μg/ml. DNA was mixed with $1/10^{th}$ volume loading buffer (10 x loading buffer: 65% (w/v) sucrose, 10mM Tris-HCl pH 7.5, 10mM EDTA, 0.3%(w/v) bromophenol blue). DNA samples were loaded onto the gel and as a comparison for size a 100 bp ladder (Invitrogen) and a 1kb ladder (Invitrogen) was run in parallel. The gel was run in an electric field at approximately 80 Volts for 1 hour. DNA bands were visualised under a UV light and photographed using a UVP Gel Documentation System (UV Productions). For large cosmids a phage Hind III ladder was used to allow for determination of large size DNA fragments.

### 2.2.1.6 Restriction endonuclease digest of DNA.

The required amount of DNA was added to a sterile microcentrifuge tube. Restriction enzyme buffer was added at 1/10th of the final reaction volume. 1μl of each restriction enzyme was added and the reaction was completed with $ddH_2O$ to the desired volume. The digest was incubated at the optimal temperature for the chosen restriction enzymes for 1-2 hours for diagnostic purposes, 2-3 hours for plasmid DNA for cloning and 3-18 hours for PCR products.
Restriction endonuclease activity was ended by cleaning the DNA with phenol chloroform isoamyl alcohol and following ethanol precipitation or by heat inactivation at 65˚C for 20 minutes.

### 2.2.1.7 Phosphatase Treatment of restriction digested vectors for cloning.

Plasmids cut with restriction endonucleases were phosphatase treated prior to gel or phenol chloroform extraction, to prevent vector religation. This was performed by using either Antartic Phosphatase (New England Biolabs) or Thermosensitive Alkaline Phosphatase (Promega). Antartic phosphatase is derived from recombinant E.coli carrying the gene encoding alkaline phosphatase (AP) isolated from the psychrophilic strain TAB5. This enzyme shows high activity at low temperatures and is heat sensitive

(Rina et al. 2000). Phosphatase reaction buffer was added to the reaction in a volume of $1/10^{th}$ of the total. 1-2µl phosphatase was added and the reaction was incubated at 37°C for 30-45 min. Following this the reaction was cleaned with phenol chloroform isoamyl alcohol and following ethanol precipitation as described (2.2.1.4).

### 2.2.1.8 Blunt ending of restriction enzyme digested DNA

DNA was digested with the appropriate restriction enzyme as described (2.2.1.6). Following this the reaction was cleaned by phenol chloroform extraction and ethanol precipitation. The DNA was resuspended in 50µl $ddH_2O$, 2µl T4 DNA polymerase, 20 µl T4 DNA polymerase 5x buffer, 5 µl 2mM dNTP mixture and 23 µl $ddH_2O$ was added to the DNA and the reaction was incubated at 11°C for 15 minutes. The reaction was cleaned up with phenol chloroform isoamyl alcohol followed by ethanol precipitation as described (2.2.1.4) and could then be used in normal ligation reactions. If it was a plasmid that was blunt ended then this was phosphatase treated (2.2.1.7) prior to ligation.

### 2.2.1.9 Gel purification of DNA

For DNA gel purification DNA was loaded and run on a 1% agarose gel as described (2.2.1.5). The DNA was visualised under low energy UV exposure to avoid DNA damage. The DNA band of interest was excised from the agarose gel using a sterile scalpel and transferred to a microcentrifuge tube. The DNA was then purified using the PureLink™ Gel Extraction Kit (Invitrogen) according to the manufacturer's instructions. For cloning where a higher purity DNA was required, certified molecular biology agarose (BioRad Laboratories Inc.) was used.

### 2.2.1.10 DNA Ligation

DNA ligations were carried out by mixing 0.05-0.1µg linearised plasmid DNA with cut insert DNA with compatible ends. This was done in a plasmid versus insert ratio of 1:4 to a total volume of 5µl. To this mixture 5 µl ligase was added (Takara solution I DNA Ligation Kit ver.2.1, Amersham). Ligations were incubated at 16°C for

40-60 min according to the manufacturer's instructions. For complicated cloning or for blunt end cloning ligations were incubated overnight at 16°C.

### 2.2.1.11 TOPO TA Cloning.

TOPO TA Cloning was performed using TOPO TA Cloning system with pCR 2.1-TOPO vector and TOP10 electrocompetent cells according to the manufacturer's manual (Invitrogen).

### 2.2.1.12 Polymerase Chain Reaction (PCR)

PCR reactions were performed in a total volume of 50µl in a 0.5 ml flat cap tube. PCR products to be used for cloning were generated using the KOD Hot Start polymerase (Merck Chemicals Ltd.).

1ng template DNA was mixed with 10pmol of 3' and 5' primer, 5µl 2 mM dNTP mixture, 1.5µl 25 mM $MgSO_4$, 5µl KOD hot start 10x buffer, 1µl KOD hot start polymerase and the reaction was completed with distilled water until 50µl. Amplification was carried out using a MJ Research PTC200 gradient cycler. Cycle conditions were adjusted according to size of PCR products, primer length and melting temperature.

For cloning using the TOPO TA cloning kit (Invitrogen) PCR reactions were set up using Platinum Tag DNA polymerase with supplied buffers and supplements according to manufacturer's protocol (Invitrogen).

Diagnostic PCR reactions were set up similarly using Taq DNA polymerase (Invitrogen) with supplied buffers and additives according to manufacturer's protocol.

### 2.2.1.13 PCR clean up.

PCR products were cleaned by using ChargeSwitch® PCR Clean-Up Kit (Invitrogen) according to manufacturer's protocol.

### 2.2.1.14 Site directed mutagenesis

5' and 3' primers for site directed mutagenesis were designed as 23 base pairs long primers containing the desired point mutation and annealing to the same DNA sequence on opposite strands of the plasmid. Primer sequence is shown in section 2.1.8.4.

Site directed mutagenesis was performed as a normal PCR reaction using the *pfu* polymerase and buffer (Promega) however extension times were increased to 10 minutes and the PCR cycle was only repeated 18 times. PCR products were digested 1 hour with 1µl Dpn I in 37°C waterbath to remove the parental methylated template DNA. Following this the DNA was cleaned by phenol chloroform isoamyl alcohol and ethanol precipitation and resuspended in 10–20 µl distilled water. 5-10 µl DNA was transformed into JM-109 cells. Colonies were grown up and amplified and DNA prepared using Charge Switch® Plasmid ER mini prep Kit (Invitrogen). Clones containing the correct mutation were identified by DNA sequencing.

### 2.2.1.15 DNA sequencing.

The reactions were set up in 0.5 ml flat cap tubes in a total volume of 20 µl. Approximately 500 ng plasmid DNA was mixed with 3.6pmol primer, 2µl Big Dye Terminator Reaction mix and 4µl 5 x Big Dye Buffer (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) and distilled water was added to a total volume of 20 µl.

Sequencing reactions were performed on a MJ Research PTC200 gradient cycler. Samples were heated to 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. This cycle was repeated 25 times. The reactions were then purified by ethanol precipitation. The DNA volume was made up to 100µl with distilled water in a sterile microcentrifuge tube. 200µl 100% ethanol and 1 µl 3M Sodium Acetate, pH 5.2 was added to the tube and it was incubated on dry ice for 30 minutes. Then this the tube was centrifuged for 30 minutes, 20800 x g at 4°C. The ethanol was decanted and the pellet was washed in 150 µl 70% ethanol. An additional centrifugation step was performed for 20 minutes under the same conditions as the previous one. The purified extract was

dried under a vacuum and then resuspended in 20µl Hi-Di formamide (Applied Biosystems). The samples were then transferred to a 96-well plate and analysed using an ABI 300 automatic sequencer.

### 2.2.1.16 RNA isolation from dog blood.

1.5 ml canine blood samples in EDTA sampling tubes were spun at 2000 x g for 15 minutes at room temperature. The plasma layer was removed with a Pasteur pipette. The buffy coat layer was removed and transferred to a sterile micro centrifuge tube. RNA was isolated using the RNAqueous kit (Applied Biosciences) according to the manufacturer's protocol. The RNA concentration was measured by spectrophotometer and RNA was frozen at -20°C until further use.

### 2.2.1.17 Reverse transcriptase PCR

SuperScript™ III Reverse Transcriptase kit (Invitrogen) was used to perform this reaction.

16 µl RNA isolated from canine blood was mixed with 2 µl 10 mM dNTP mixture and 2 µl 3' primer 2mM in a sterile 0.5ml micro centrifuge tube. The mixture was then incubated at 65°C for 5 minutes and then incubated on ice for 1 minute. 4 µl RT 10x buffer, 8 µl $MgCl_2$ 25mM, 4 µl DTT 0.1 M , 2 µl RNase OUT and 2 µl SuperScript™ III Reverse Transcriptase made up as a mix was added to each sample as a master mix. Controls were set up without template or without reverse transcriptase.

The mixture was incubated at 50°C for 50 minutes and then at 85°C for 5 minutes. Next 2 µl RNAse H was added to each sample to remove the RNA and the sample was incubated at 37°C for 20 minutes and subsequently the reactions were ready to be used as template for PCR reactions.

### 2.2.1.18 PCR reaction using template from reverse transcriptase PCR products

PCR reactions were set up as described (2.2.1.12) using 2-4 µl cDNA from reverse transcriptase reactions as template for the reaction. Taq polymerase was used for the reactions so PCR products could be cloned directly into the pCR 2.1-TOPO vector using the TOPO TA Cloning system (Invitrogen).

### 2.2.2 Cell culture protocols

#### 2.2.2.1 General growth of cells

All cell culture experiments were carried out in a room specifically for the purpose and under strict sterile conditions. Cell culture work was performed inside a flow hood (Class II Microbiology Safety Cabinets, Gelaire BSB4). 293T, C33a, GM847, MCF7, MDCK, MRC5 and U2OS cell lines were grown in DMEM supplemented with 10% FCS, 1% penicillin and streptomycin solution in an atmosphere at 37°C containing 5% $CO_2$ (v/v) (Napco model 5410, Napco Scientific). Cells were trypsined and split 1/10 when they reached 90% confluence.

#### 2.2.2.2 Subculturing cells

When cells were reaching confluence in their flask they were washed twice with PBS and then trypsinised by adding sufficient trypsin EDTA solution to cover the cells. The cells where then incubated at 37°C for approximately 5 minutes until they had detached. The cells where then washed off the flask with warm growth medium and transferred to a sterile universal tube and pelleted by centrifugation for 5 minutes at 170 x g at room temperature. The pellet was washed with PBS and the centrifugation was repeated. The cells were then resuspended in growth medium, counted using a haemocytometer and then seeded into a flask at the required concentration or seeded into cell plates for assays. For TUNEL staining, cells were seeded on sterilised glass cover slips in a 6 well plate.

#### 2.2.2.3 Dissociation of primary cells from tissue.

Tumour tissue was obtained directly after resection from canine patients at the Small Animal Hospital, University of Glasgow. Normal skin samples were obtained from euthanized dogs from the Veterinary Anatomy department, University of Glasgow with ethical approval. To isolate cells of interest, a representative piece of tissue

approximately 0.5-1cm$^3$ was placed in a Petri dish and dissected into 2-3mm pieces with a scalpel. The tissue was washed twice in a balanced salt solution without calcium and magnesium (Invitrogen). A 0.25% trypsin solution made up in the same balanced salt solution was added to the tissue (approximately 1ml solution for every 100 mg tissue). The tissue was incubated for 14-18 hours at 4°C in trypsin solution, and for a further 30 minutes at 37°C. Warm DMEM or RMPI medium was added and the tissue was mechanically manipulated by pipetting. Tissue and media were then filtered through a 100μm cell strainer (BD Biosciences) to remove unwanted tissue pieces. The cell suspension was centrifuged for 5 minutes at 170 x g and resuspended in 10ml medium. A viable cell count was performed by making a 1:1 solution of cell suspension and Trypan blue stain (Invitrogen). Cells were then seeded in T-25 flasks at approximately 5x10$^5$ cells per flask. Primary cells were fed every 3-5 days and were maintained in either RMPI or DMEM with added Glutamax 1%, Fungizone 1%, penicillin and streptomycin solution 1% and ciprofloxacin solution 0.1%.

**2.2.2.4 Telomerase repeat amplification PCR (TRAP) assays and freezing of cell pellets prior to assay.**

Cells growing in tissue culture flasks were washed twice in PBS and enough trypsin to cover the cells was added to the cells. The trypsin treated cells were incubated at 37°C for 5 minutes which allowed the cells to lose their adherence to the culture flask. The cells were washed off the flask using normal cell medium and pelleted by centrifugation at 170 x g for 5 minutes at room temperature. The supernatant was removed and the cells were resuspended in PBS and counted using a haemocytometer. 1x10$^6$ cells in PBS were transferred to an Ependorf tube and the cells were pelleted by centrifugation at 110 x g for 5 min. Following this the supernatant was removed and the cell pellets were frozen immediately at -80°C.

TRAP assays were performed on cell pellets, using the TRAPeze ELISA telomerase detection kit (Millipore) according to manufacturer's instructions including strict separation of working areas to avoid contamination of samples. Results were detected by gel electrophoresis. Gel electrophoresis was carried out by running 10 μl of PCR product mixed with 2 μl Bromophenol blue 10x dye on a polyacrylamide gel for 1 hour at 150 volts. The gel was then stained with Ethidium bromide 0.25μg/ml for 10-20

minutes. TRAP ladders bands were visualised under a UV light and photographed using a UVP Gel Documentation System (UV Productions).

### 2.2.2.5 Transfection of cells using calcium phosphate

Cells were plated at a concentration of $2x10^5$ cells ($3x10^5$ for MRC-5 and C33a cells due to their smaller size) in a 60mm tissue culture dish and incubated for 24 hours to allow cells to adhere and grow. A mixture was made up by adding a solution of 500 µl plasmid DNA in 250 mM $CaCl_2$ dropwise, into 500 µl 2 x HEPES buffered saline (pH 7.05 ) under rotation. The mixture was left for 15 minutes to allow for formation of a precipitate. 500 µl of the mixture was then added dropwise into the media of each of the tissue culture cell plates. If tranfections were performed in duplicate, the remaining 500µl was added onto a second plate. For single tranfections the remainder was discarded. The cells were incubated with the precipitate for 14-18 hours after which time they were washed twice with PBS and re-incubated in fresh growth media. Approximately 24 hours later (unless otherwise stated) the cells were harvested. As a positive control in luciferase assays two plates were always transfected with 1 µg pGL3-Control vector. This vector contains the firefly luciferase gene under transcriptional control by the SV40 promoter and therefore exhibits a high level of transcriptional activity in many cell lines.

### 2.2.2.6 Transfection of cells using Trans-IT

Cells were plated at a concentration of $2x10^5$ cells for each 60 mm tissue culture plate. They where incubated for 24 hours to allow for adherence and growth. A transfection mixture was made by adding Trans-IT transfection reagent to Optimem medium (Invitrogen) in a microcentrifuge tube. The amount of Trans-IT lipid transfection and Optimem medium depended on the amount of DNA being transfected. In general 3µl lipid transfection reagent was added into 97µl Optimem medium for each 1µg DNA being transfected. The lipid transfection reagent-Optimem mixture was flicked and incubated for 5 min at room temperature. DNA was then added and the tube was flicked and further incubated for 25-35 minutes at room temperature. The transfection mixture was then added directly into the media in the tissue culture plate

and incubated for a further 36 hours (unless otherwise stated) before the cells were harvested with no change of media in between.

### 2.2.2.7 Luciferase assay

Approximately 40 hours after transfection, transfected cells were washed twice with PBS. PBS was carefully removed using a Pasteur pipette and 300 µl Reporter lysis buffer (Promega) was added to each 60 mm cell plate and the cells were incubated for 10 minutes at room temperature before the cell lysate was then scraped from the plate using a cell scraper and transferred to a sterile 1.5 ml microcentrifuge tube. The lysate was then centrifuged for 10 min at 20800 x g, at 4°C to pellet the debris, and the supernatant was transferred to a fresh tube and stored on ice.
80 µl of each harvested cell supernatant was transferred into an opaque 96 well plate and assayed for luciferase activity using a Luminoscan Ascent luminometer and Ascent software with the Luciferase Assay System substrate (Promega). 80 µl of Luciferase assay system substrate diluted 1:3 was used for each sample. The luciferase expression readings were adjusted by accounting for protein content in each sample by performing a BCA-$CuSO_4$ protein assay on the cell lysate as described (2.2.3.2).

### 2.2.2.8 TUNEL staining.

$2x10^5$ U2OS and 293-T cells were plated out in 6 well plates in which a glass coverslip was placed in each well. For the 293-T cells the coverslips were treated with poly-L-Lysine prior to plating of cells to allow for better attachment. 24 hours after plating U2OS and 293-T cells were transfected using Trans-IT and calcium phosphate respectively. 24 hours later cells on cover slips were fixed in 4% formaldehyde in PBS. At this stage the cells could be stored for up to 2 weeks. TUNEL staining of the cells on coverslips was performed using the DeadEnd™ Fluorometric TUNEL System (Promega) according to manufacturer's protocol for adherent cell lines. This kit stains apoptotic cells in which the DNA is fragmented by catalytically incorporating fluorescein-12-dUTP at 3′-OH DNA ends using the terminal deoxynucleotidyl transferase recombinant enzyme (rTdT).

After staining, coverslips were mounted and counterstained using Vectashield mounting medium with DAPI stain (Vector Laboratories). Stained coverslips were visualised using a fluorescent microscope and 5 random pictures were taken of cells using a filter with an excitation spectrum of 594 nm to excite and visualise the flourescein stain and a filter with an excitation spectrum of 358nm to excitate and visualise the DAPI stain. The number of DAPI stained and flourescein stained cells for each exposure was quantified and the percentage of DAPI stained cells which were also fluorescein stained was determined. The difference in flourescein staining between groups was determined by statistical analysis using Yates corrected chi square analysis.

### 2.2.3 Protein preparation and analysis

#### 2.2.3.1 Preparation of protein extracts for western blots

Cells were transfected using either calcium phosphate or Trans-IT as described in 2.2.2.5 and 2.2.2.6. Cells were harvested 40 hours after transfection or at the times stated. At harvest, cells were washed twice with PBS and then trypsinised by adding 1 ml trypsin EDTA to each 60mm tissue culture plate. The cells were incubated with the trypsin for approximately 3-5 minutes until they detached. The cells were then washed off the plate using normal cell growth medium and transferred to a universal tube. The cells were then pelleted by centrifugation at 170 x g for 5 minutes at room temperature. The supernatant was then removed and the pellet resuspended in 1 ml ice-cold PBS and transferred to a sterile microcentrifuge tube. The cells were pelleted by centrifrugation at 420 x g for 5 minutes at room temperature. The supernatant was discarded and the pellet was then either frozen at -80°C until further use or resuspended in 100 µl lysis buffer (0.5% NP40, 150mM NaCl, 50mM Tris pH 8.0 containing 1/25 volume protease inhibitor cocktail tablet) and put on ice to incubate for 30 minutes. After incubation the lysed cells were centrifuged for 30 minutes at 20800 x g at 4°C and the supernatants were transferred to a new sterile tube. The protein content of the protein lysates were measured by a BCA-$CuSO_4$ protein assay and were then ready to be used for polyacrylamide gel electrophoresis and Western blot analysis.

### 2.2.3.2 BCA-$CuSO_4$ Protein assay

To account for diversity in cell numbers, protein concentration was determined to reflect cell quantity. This was done using bichinchoninic acid ((BCA), Sigma)) and copper II sulphate pentahydrate 4% w/v solution (Sigma).

A volume of 10 µl harvested cell lysate was added to wells of a transparent 96 well plate. Bovine serum alkaline protein solutions at known concentrations were added to 6 wells at different concentration to be used as a protein standard for determining the final concentration in the samples. 200µl developing solution were then added (5ml BCA mixed with 100µl copper II Sulphate pentahydrate. The plate was incubated at 37°C for 30-60 minutes. The protein in the lysates reduces Cu(II) to Cu(I) which then reacts with BCA to form a purple complex, in a concentration specific manner such that the colour intensity reflects the amount of protein in the sample. The light absorbance of each sample was measured at 562nm using a Dynatech MR7000 automated plate reader. Based on the known protein content in the bovine serum alkaline protein samples a standard curve could be conducted and the protein concentrations extrapolated.

### 2.2.3.3 Polyacrylamide gel electrophoresis (PAGE).

Protein concentrations were determined using a BCA/$CuSO_4$ assay as described (2.2.3.2). Equal amounts of protein in a volume of 13µl in $ddH_2O$ were mixed with 2µl sample reducing agent (NuPAGE sample reducing agent (10x), Invitrogen) and 5 µl sample buffer (NuPAGE LDS sample buffer (4x), Invitrogen) in a sterile microcentrifuge tube and then incubated on a heat plate at 70°C for 10 min.

A precast gel (NuPAGE® Novex Bis-Tris Gels, Invitrogen) was transferred to a tank and mounted in 1x buffer (NuPAGE MES SDS Running Buffer 20x, Invitrogen, (50ml in 1 litre)). 400 µl antioxidant was added to the tank. Each sample was then loaded into the wells of the gel. In parallel with the samples a standard protein ladder (SeeBlue Plus 2 Prestained Standard, Invitrogen) was loaded to determine the sizes of the proteins in the samples. The tank in which the gel and buffer was sited was then connected to a power source and run at 200V until the mobile front reached the bottom of the gel. The gel was then ready to be used for Western blotting.

### 2.2.3.4 Western blot.

Proteins in polyacrylamide gel were transferred to a nitrocellulose membrane using either a semidry blotter (Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad) or the iBlot protein transfer system (iBlot™ Gel Transfer Device, Invitrogen). The protocol for using the semidry blotter for protein transfer was as follows. The gel was soaked in 1x transfer buffer with 10% methanol (NuPAGE Transfer Buffer 20x, Invitrogen) together with a nitrocellulose membrane and 6 pieces of filter paper. These where all transferred to the semidry blotter as a sandwich consisting of 3 pieces of filter paper, then the nitrocellulose membrane, then the polyacrylamide gel and then 3 pieces of filter paper again. It was ensured that no air bubbles were sited between the layers. The semidry blotter was then assembled and an electric supply was connected. The proteins were left to transfer to the membrane for one hour at 10V.

The method for protein transfer using the iBlot protein transfer system was carried out according to themanufacturer's instructions but the polyacrylamide gel was soaked for 10 min in 2x transfer buffer with 10% methanol (NuPAGE Transfer Buffer 20x, Invitrogen) prior to transfer.

After protein transfer using either system the membrane was stained with Ponceu S (Sigma) to confirm complete protein transfer. The membrane was then transferred to a container and blocked in 5% Marvel in PBS-T (1 x PBS 0.1% tween) for an hour at room temperature or overnight at 4°C. The membrane was then gently washed with PBS-T and then incubated for one hour with a primary antibody diluted in 5% Marvel PBS-T under gentle rotation. Next the membrane was washed 4 times in PBS-T over a period of 20 minutes. The membrane was then incubated for 1 hour at room temperature in a solution containing the secondary antibody in PBS-T 5% Marvel under gentle rotation. This was followed by washing 4 times with PBS-T over 20 minutes. After this washing step the membrane was developed with developing solution for 5 min (ECL PLUS Western blot detection system, Amersham biosciences). Protein bands where visualised by exposing a chemiluminescence film (CL-XPosure™ Film, Perbio Science) to the membrane in a film cassette for 1 min and 3 min. If bands came out either too strong or too pale additional exposures at shorter or longer time periods would be performed.

### 2.2.3.5 Stripping off antibodies from nitrocellulose membrane.

Removal of antibodies from nitrocellulose membranes was carried out by incubating the nitrocellulose membrane for 10 minutes under gentle rotation in a 0.2M NaOH solution. Then the membrane was washed 3 times for 10 minutes in PBS-T (1 x PBS 0.1% tween) and could be blocked with 5% Marvel in PBS-T and reprobed with a different antibody.

## 2.2.4 Protocols for generation of recombinant adenovirus

### 2.2.4.1 Generation of electrocompetent cells

Electrocompetent BJ5183s cells were generated by plating out approximately 30 µl of BJ5183s glycerol stock on an agar plate containing streptomycin as a selective antibiotic. The plate was incubated overnight in a 37˚C incubater. The next day colonies were picked and grown in 3 ml L-broth with streptomycin 50µg/ml overnight in a 37˚C incubator under rotation at 225 rpm. The following day 200µl culture was inoculated into 200 ml L broth with streptomycin and grown until the culture reached an optical density of $OD_{600}$ = 0.5-0.7. The cells were then chilled in an iced water bath for 20 minutes and then centrifuged for 20 minutes at 1000 x g, 2˚C. The supernatant was removed and the cell pellet resuspended in 200µl ice cold $ddH_2O$ and then a further 20 ml of ice cold $ddH_2O$ was added. The centrifugation and resuspension steps were repeated. Then the supernatant was decanted off the cells and the cells were resuspended in the remaining liquid and centrifuged again for 10 minutes at 2000 x g, at 2˚C. The supernatant was removed and a volume of $ddH_2O$ equivalent to the cell pellet volume was added to resuspend the cells. The cells were used immediately for electroporation.

### 2.2.4.2 Homologous recombination

Homologous recombination is a method commonly used to insert, remove or change DNA sequences in viruses (Chartier et al. 1996).

The basic concept of this method is that the recipient DNA is linearised by restriction digest at the site of interest. The DNA insert should contain DNA homologous to the

recipient DNA flanking the linearization site. By mixing the recipient DNA and the DNA insert and electroporating this in BJ5183s cells (which allow for recombination of DNA), the insert should be inserted into the recipient DNA by recombination of the homologous DNA sequences.

In this project cosmid DNA was linearised with the appropriate enzyme and was cleaned by phenol chloroform isoamyl alcohol and ethanol precipitation. The DNA insert was cut out of its cloning vector and was gel purified. Approximately 300ng of cut cosmid DNA was mixed with 100ng of DNA insert in a volume of 10 µl and was added to 50 µl BJ5183s cells. Cells and DNA was then transferred to a cuvette and electroporated using a Gene Pulser Transfection Apparatus (BIO-RAD) with the machine set at 200 Ohms, 25 µF and 2.5 volts. Immediately afterwards the cell-DNA mixture was transferred to a bijou and mixed with 1ml SOC medium and was incubated while shaking at 225 rpm, 37°C for 1 hour. Then 300-400 µl of incubated cell suspension was plated on agar plates containing ampicillin and incubated over night at 37°C. Colonies were selected and grown as for normal transformations.

#### 2.2.4.3 Small scale preparation of cosmid DNA

Colonies on agar plates from homologous recombination were picked and grown overnight in 3 ml L-Broth with 100 µg/ml ampicillin at 225 rpm 37°C. DNA was following prepared using the QIAprep® Spin Miniprep Kit according to the manufacturer's instructions.

#### 2.2.4.4 Large and medium scale preparations of cosmid DNA

Colonies from cosmid DNA transformed into Stbl 2 cells were picked and grown up in 2 ml L-Broth with 100 µg/ml ampicillin for 6 hours. For large scale DNA preparation 0.5ml of this culture was inoculated into 400 ml L-broth with ampicillin. Cultures were grown over night at 225 rpm 37°C. DNA was prepared using Qiagen Large Construct Kit or NucleoBond® Xtra Maxi Plus Kit. For medium scale preparation, 0.5ml of this culture was inoculated into 200 ml L-broth with ampicillin. Cultures were grown overnight at 225 rpm 37°C. DNA was prepared using NucleoBond® Xtra Midi Kit according to the manufacturer's manual.

### 2.2.4.5 Transfection of MDCK cells with virus DNA to generate live virus.

This was done using the Nucleofector L-kit according to manufacturer's cell type specific manual for MDCK cells (Amaxa Biosystems).

### 2.2.4.6 Infection of cells with virus

Cells were plated out and either left to adhere and grow for 24 hours or infected instantly. Virus was recovered from storage at -80˚C and thawed in a 37˚C water bath. Virus was diluted to the appropriate concentration using DMEM media and added drop wise to the cells.

### 2.2.4.7 Harvest of live virus for virus stock

Virus was harvested from cells when significant cytopathic effect was observed but before 100% cell death. Cell media were collected in 15 ml falcon tubes. Cells were scraped from the cell plate and collected in the same tube. The tube and contents were then centrifuged for 10 minutes at 170 x g, the supernatant was removed from the cell pellet and collected in a separate tube. The pellet was then freeze-thawed 3 times between -80˚C and 37˚C using dry ice and a 37˚C water bath. The pellet was then resuspended in the supernatant and centrifuged for 10 minutes 170 x g at room temperature. The supernatant containing the virus particles was then stored as aliquots at -80˚C. The pellet containing cellular debris was discarded.

### 2.2.4.8 Tissue Culture Infectious Dose where 50 % cells show cytopathic effect assay ($TCID_{50}$ assay).

The $TCID_{50}$ assay is an assay used to determine concentrations of infectious particles in viral stocks. The concept of the assay is that multiple cell containing wells in a 96 well plate are infected with a serial dilution of the viral stock and monitored for cytopathic effect (CPE) until no further changes are observed. At this stage the lowest concentration of virus that causes CPE in 100% of the infected wells is noted together with the concentrations and number of wells at which CPE is observed in less than 100% of the infected wells.

The $TCID_{50}$ assay that has been used in this project is a modified assay in which half log dilutions are used to determine the $TCID_{50}$. This should allow for a more accurate estimate of virus concentration. In this assay 4 wells in a 96 well plate are infected with half log dilutions of the virus stock starting from $10^{-1}$ - $10^{-12.5}$.

Cells were plated in a 96 well plate at a density of 1.5 x $10^3$ cells per well in a volume of 150 µl media. Virus dilutions were made by preparing 12 bijou tubes with 1.8 ml media and 12 bijou tubes with 0.8 ml media. Whole log dilutions were made by transferring 200 µl of virus stock into a bijou with 1.8 ml media to make up the $10^{-1}$ dilution and then transferring 200 µl of this dilution into the next bijou containing 1.8 ml media to make up the $10^{-2}$ dilution. This was repeated until the $10^{-12}$ dilution was reached. The half log dilutions were following made by transferring 200 µl of each dilution into a bijou containing 0.8ml media. 4 wells in the prepared 96 well plate were infected with 100 µl of each dilution as shown in figure 2.1. Cells were observed on a daily basis and CPE was noted. When no further CPE was observed all the wells showing CPE and the concentration of virus in these were noted.

The $TCID_{50}$ was following calculated using the Kärber formula.

$\log TCID_{50} = L - d\,(S - 0.5)$, where:

L = log of highest dilution in which 100% of wells showed CPE.

d = difference between log dilution steps

S = sum of proportion of cultures showing cytopathic at dilutions higher than the dilution which showed 100% CPE.

From this result the concentration of virus as reflected by plaque forming units (PFU) can be determined as:

$PFU \text{ per ml} = 1/10^{\log TCID_{50}} \times 10 \times 0.69$

The multiplication with 10 is to get the concentration per ml and the 0.69 constant is applied to obtain a more accurate estimate of PFU by application of the Poisson distribution. For better understanding of the formula a calculated example would be:

| Virus dilution | Number of well showing CPE | Fraction of wells showing CPE |
|---|---|---|
| $10^{-4}$ | 4/4 | 1.00 |
| $10^{-4.5}$ | 2/4 | 0.50 |
| $10^{-5}$ | 1/4 | 0.25 |
| $10^{-5.5}$ | 0/4 | 0.00 |

From this S = 1.00 + 0.50 + 0.25 + 0.00 = 1.75

The calculated log $TCID_{50}$ from this example would then be:

log $TCID_{50}$ = -4 -0.5(1.75-0.5) = -4.625

Pfu would then be: $1/10^{-4.625}$ x 0.69 x10 = $2.9x10^5$

**2.2.3.9 Staining of 96 well plates with crystal violet stain.**

Media was removed from wells by pipetting. The wells were stained with 0.5% crystal violet solution (25% methanol, $ddH_2O$) and incubated for 5 minutes at 80 rpm at room temperature. The crystal violet solution was removed and the cells were washed with $H_2O$ until no excess dye was present. Stained 96 well plates were dried and the cell staining evaluated by visualisation on a light box.

**Figure 2.1**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | -1 | -1.5 | -2 | -2.5 | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| B | -1 | -1.5 | -2 | -2.5 | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| C | -1 | -1.5 | -2 | -2.5 | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| D | -1 | -1.5 | -2 | -2.5 | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| E | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| F | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| G | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| H | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |

**Figure 2.1 Schematic presentation of half log dilutions used in $TCID_{50}$ assay.**

Model of 96 well plate in which cells were plated and infected with half log dilutions of virus stock to determine the concentration of the virus.

# Chapter 3.

## Results

### 3.1 Development of a gene therapy system.

The two step transcriptional amplification (TSTA) mechanism as described (chapter 1.3.2) has been used by others in conjunction with the human telomerase promoter and the Gal4VP16 transcriptional activator fusion protein. In our laboratory the VP16E2 transcriptional activator fusion protein has been studied. VP16E2 is a fusion protein between the acidic c-terminus of the VP16 protein from the human herpes simplex virus type 1 and the E2 DNA binding domain from the bovine papillomavirus type 1 (BPV-1) (Vance et al. 1999, Vance et al. 2001). Papillomaviruses are viruses that have evolved to recruit the mammalian cell replication and transcription machinery to be able to function efficiently in mammalian cells. We anticipated that incorporating the VP16E2 fusion protein into the TSTA system would allow for a higher transcriptional activity in mammalian cells than the Gal4VP16 due to the fungal origin of the Gal4 domain.

Another improvement we anticipated we could include in this system was the use of a unique minimal promoter. The TSTA system is commonly used with a Gal4 responsive minimal promoter as a secondary promoter. This promoter consists of multiple Gal4 binding sites upstream of a TATA box promoter derived from the adenovirus E1b promoter (Sadowski et al. 1992). Previous work in our laboratory identified a minimal promoter from bovine papillomavirus type 4 (BPV-4) (Vance et al. 2001). This promoter named 3'BpTATA consists of a TATA box and 3 upstream base pairs. 6 upstream E2 binding sites are located immediately upstream to activate the promoter. A strength of this promoter is that it is a very strong promoter when activated in the presence of E2, however in the absence of E2 the 3'BpTATA promoter only shows minimal activity.

#### 3.1.1 Comparison of the VP16E2 and the Gal4VP16 transcriptional activators.

The pCG-VP16E2 plasmid which contains the VP16E2 sequence under transcriptional control of a CMV promoter, was a gift from a collaborator (Dr. Mart Ustav, Estonian Biocenter). For manipulation purposes the VP16E2 fragment was

sequenced using primers as described in Materials and Methods. The full VP16E2 sequence is 729 base pairs, encoding 242 amino acids. The VP16 and the E2 fragments are linked together with a Bsp EI restriction site. The VP16 fragment consists of the terminal 78 amino acids of the acidic C terminal of the VP16 protein from the human Herpes simplex virus type 1. The E2 fragment consists of the c-terminal 161 amino acids of the BPV-1 E2 DNA including the full length DNA binding domain. The full length VP16-E2 fusion protein cDNA is presented in figure 3.1.1.

To be able to compare the activation potential of VP16E2 with Gal4VP16, a Gal4VP16 fusion protein containing the same 78 amino acid domain as in the pCG-VP16E2 was cloned. The VP16 fragment was amplified as a PCR product using the pCG-VP16E2 as a template and primers with overhanging BamH I and Kpn I 5' and 3' restriction sites. The cut PCR product was inserted into the pBIND plasmid which encodes the Gal4 DNA binding domain downstream from a CMV promoter. This was done using the BamH I and Kpn I sites in the multiple cloning region of the plasmid. The new plasmid was entitled pBind-Gal4VP16.

To compare the transcriptional activator potential of the VP16E2 and the Gal4VP16 in conjunction with the two step transcriptional amplification system a series of transcription assays was carried out in 293-T cells. Cells were transfected with titrated concentrations of either pCG-VP16E2 or pBind-Gal4VP16; both transcriptional activators were transcribed by a CMV promoter. 1μg pTK-Gal4-6xE2 reporter plasmid was co-transfected into all samples. Schematic models of the pCG-VP16E2, pBind-Gal4VP16 and pTK-Gal4-6xE2 are illustrated in figure 3.1.2 a). The pTK-Gal4-6xE2 reporter plasmid contains 6 E2 and 5 Gal4 binding sites located upstream of a tyrosine kinase (TK) promoter which is responsible for transcription of a firefly luciferase gene. The ability of the two transcriptional activator fusion proteins to activate the promoter in this plasmid was reflected as relative light units (rlu) from the expressed firefly luciferase gene. The experiment was carried out 3 times in duplicate and results were adjusted for protein content of the harvested cells. In figure 3.1.2 b) the summarised results from the 3 experiments are shown as fold transcriptional increase compared to cells transfected with 1μg pTK-Gal4-6xE2 only. A large increase in transcriptional activity is seen in samples transfected with the VP16E2 coding plasmid compared to the Gal4VP16 coding plasmid. This effect is particularly pronounced at transfection doses of 0.01ng, 0.1ng and 1ng and statistically significant at the 0.1ng dose as seen by the low P-value.

The standard error bars seen in figure 3.1.2 b) are relatively large reducing the statistical significance of the results. This is a general tendency seen throughout the luciferase assays presented in this thesis. There are several reasons for the high variability between repeated experiments. The major reason is the results being presented as fold activity compared to a control plasmid. Due to the great sensitivity of the firefly luciferase detection, variability seen in the low activity control plasmid led to a relatively large difference in the results for the other plasmids.

Other factors that led to variability were the different passage number of the cell lines used leading to variable transfection efficiency. The variation between DNA quality of different batches plasmid preparations and the measurement of DNA concentration in these were other variables which affected results. Also the measurement of protein content in samples and adjustment of results according to these led to variability. Throughout the results section the sample means in the graphs represent the general tendency that was observed in the individual assays before pooling results.

To determine if the effect seen in the transcription assay was related to protein expression of the transcriptional activator, Western blots were performed. 293-T cells were transfected with 1μg or 0.1μg of either pCG-VP16E2 or Gal4-VP16 or with water as a negative control. The membrane was probed with an antibody against VP16 and an antibody against gammatubulin as a loading control. In figure 3.1.3 the Western blot is shown. The estimated size of the two fusion protein calculated on the estimate that 1000 base pairs of DNA equals approximately 37 kilo Dalton protein is 26.8 and 25.6 for VP16E2 and Gal4VP16 respectively. There is a clear difference in the protein detected from cells transfected with pCG-VP16E2 in lane 1 and 2 compared to cells transfected with pBind-Gal4VP16 seen in lane 3 and 4 with the pCG-VP16E2 samples showing considerably stronger bands.

**Figure 3.1.1**

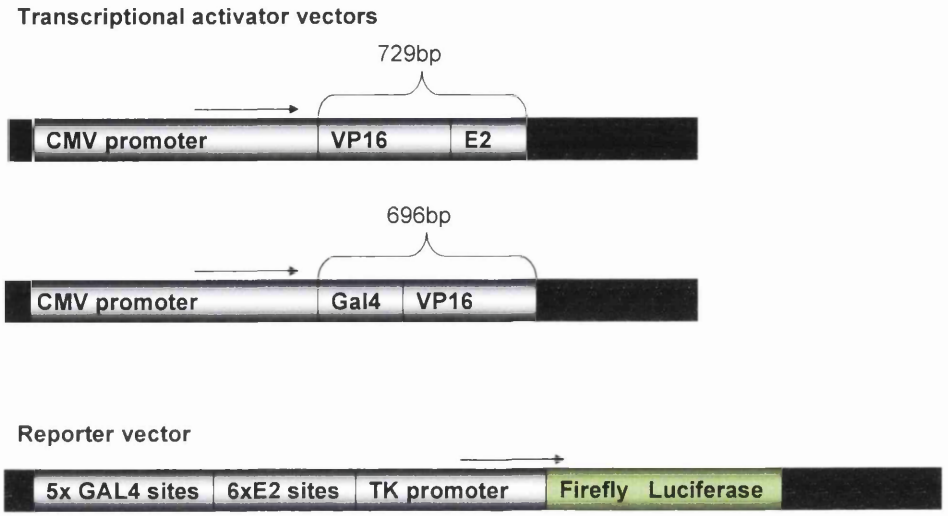
ATGTCGACGGCCCCCCCGACCGATGTCAGCCTGGGGGACGAGCTCCACTTAGACGGCGAGG
ACGTGGCGATGGCGCATGCCGACGCGCTAGACGATTTCGATCTGGACATGTTGGGGGACGG
GGATTCCCCGGGTCCGGGATTTACCCCCCACGACTCCGCCCCCTACGGCGCTCTGGATATGG
CCGACTTCGAGTTTGAGCAGATGTTTACCGATGCCCTTGGAATTGACGAG**TCCGGATCC**CTC
GGTTGGGTACGGGACGGTCCTCGCTCGCACCCCTACAATTTTCCTGCAGGCTCGGGGGGCTC
TATTCTCCGCTCTTCCTCCACCCCGGTGCAGGGCACGGTACCGGTGGACTTGGCATCAAGGC
AGGAAGAAGAGGAGCAGTCGCCCGACTCCACAGAGGAAGAACCAGTGACTCTCCCAAGGC
GCACCACCAATGATGGATTCCACCTGTTAAAGGCAGGAGGGTCATGCTTTGCTCTAATTTCA
GGAACTGCTAACCAGGTAAAGTGCTATCGCTTTCGGGTGAAAAAGAACCATAGACATCGCT
ACGAGAACTGCACCACCACCTGGTTCACAGTTGCTGACAACGGTGCTGAAAGACAAGGACA
AGCACAAATACTGATCACCTTTGGATCGCCAAGTCAAAGGCAAGACTTTCTGAAACATGTAC
CACTACCTCCTGGAATGAACATTTCCGGCTTTACAGCCAGCTTGGACTTC**TGA**

**Figure 3.1.1: Sequence of VP16E2 cDNA.**

Figure shows the full length 729 base pair VP16E2 cDNA. ATG start codon highlighted in green. TGA stop codon highlighted in red. In blue connecting sequence between VP16 and E2 is seen. Sequence codes for 242 amino acids.

**Figure 3.1.2**

a)




b)

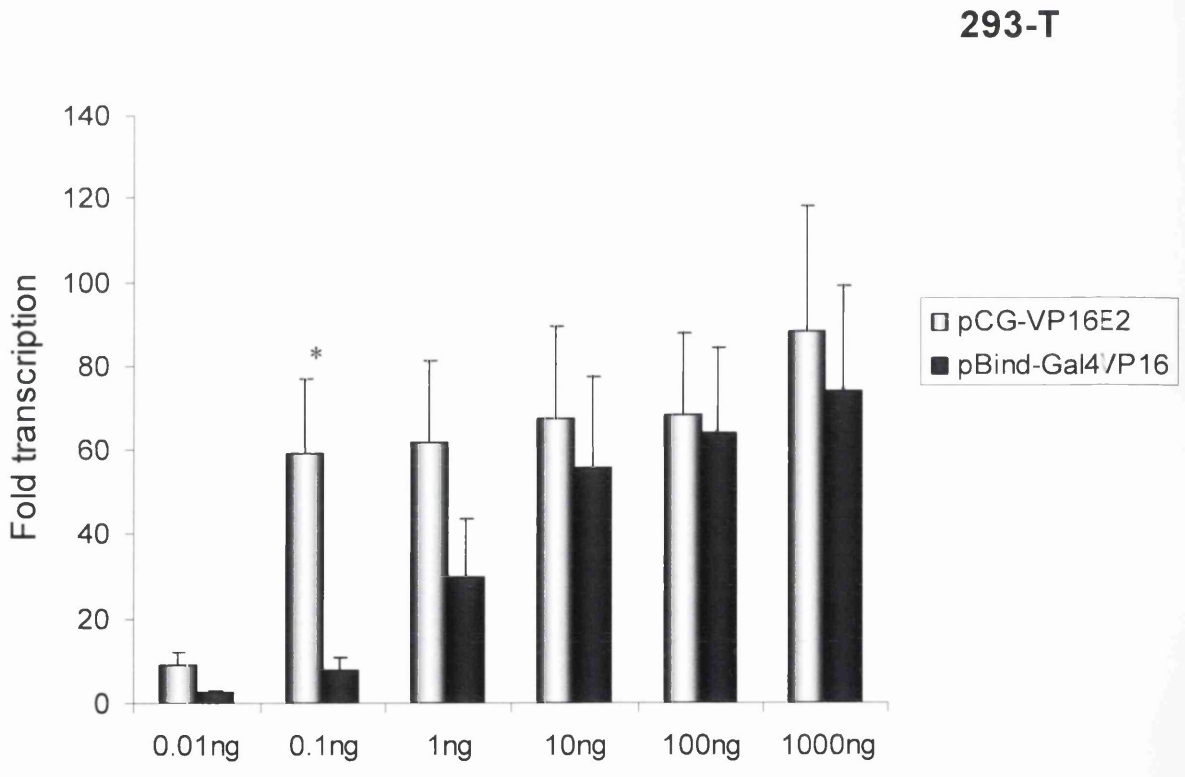


**Figure 3.1.2: VP16E2 transcriptional activator is a stronger activator than Gal4VP16.**

a) Schematic model of the vectors used in the experiments shown in b).

b) Cells were transfected with different amounts of pCG-VP16E2 or pBind-Gal4VP16 as indicated. 1000ng of the reporter plasmid pTK-Gal4-6xE2 was co-transfected into all samples. Results are shown as fold increase in luciferase expression compared to sample transfected with 1000ng pTK-Gal4-6xE2 only. Experiments were carried out 3 times in duplicate. Standard error bars are shown. * = P-value <0.05

**Figure 3.1.3**

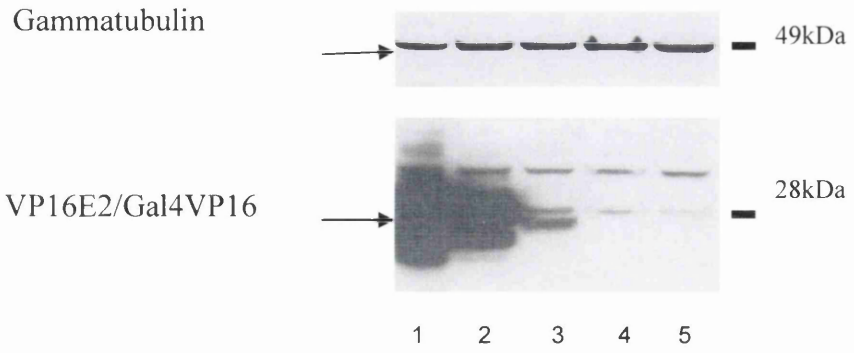



**Figure 3.1.3: Protein expression of transcriptional activators.**

Western blot showing the expression of the VP16E2 and the Gal4VP16 fusion proteins in 293-T cells using antibody against VP16. Arrow to the left of the blot indicates the approximate location of the proteins. Gammatubulin loading control shown above. Cell lysates were harvested as described in Materials and Methods. The location of the kilo Dalton (kDa) molecular weight marker is shown on the right of the blot.

1: pCG-VP16E2 1000ng, 2: pCG-VP16E2 100ng, 3: pBind-Gal4VP16 1000ng, 4: pBind-Gal4VP16 100ng, 5: Negative control. The amount in ng is reffering to the amount of DNA transfected into the cells and not to the amount of protein loaded on the gel.

### 3.1.2 Targeting the TSTA system to cancer cells by incorporating a telomerase promoter.

As described in the introduction a way to target gene expression to certain cell types is by using cell type or disease specific promoters. To target the two step transcriptional amplification system to cancer cells we exchanged the CMV promoter which is active in most mammalian cells with hTR and hTERT telomerase promoters which should be transcriptionally active in telomerase expressing cells. Most cancer cell lines express telomerase as described in chapter 1.3.1.

The hTERT-VP16E2, hTR-VP16E2, hTERT-Gal4VP16 and hTR-Gal4VP16 plasmids were made by digesting the pLh2023 and pGL3hTP19 plasmids (see materials and methods) with the restriction enzymes Xba I and Hind III. The digested plasmids were run on a 1% agarose gel and the upper band was cut out and gel purified. This process removed the firefly luciferase gene in the plasmids. PCR products of the VP16E2 and Gal4VP16 sequences were made using the pCG-VP16E2 and pBind-Gal4VP16 respectively as templates. The primers were designed with a 5' Xba I and a 3' Hind III overhang. The PCR products were digested with Xba I and Hind III and ligated into the pGL3hTP19 (hTERT-LUC) and pLh2023 (hTR-LUC) plasmids. Ligations were transformed into JM-109 cells. Correct inserts were confirmed by restriction digest and sequencing. Schematic illustrations of the cloned constructs are shown in figure 3.1.4.

To compare the transcriptional activity of the two step amplification system with the incorporation of the hTERT or the hTR promoter, a series of transcription assays was performed in telomerase positive cell lines. 293-T, MCF-7 and C33a cells (see materials and methods) were transfected using calcium phosphate with titrations of either hTERT-VP16E2, hTERT-Gal4VP16, hTR-VP16E2 or hTR-Gal4VP16. Cells were co-transfected with 1μg of the luciferase reporter plasmids pTK-Gal4-6xE2 (previously illustrated in figure 3.1.2 a). The transfections were carried out in duplicate 5 times in the 293-T cells and 3 times in the MCF-7 and C33a cell lines. The transcriptional activity was measured as rlu from the firefly luciferase gene expression and all results were adjusted for protein content in the sample.

In figure 3.1.5 the results from the transcription assay in the 293-T cell line are illustrated as fold transcriptional increase compared to cells transfected with 1μg pTKGal4-6xE2 only. It is seen in the figures that the VP16E2 transcriptional activator

elicits a higher level of transcriptional activity than Gal4VP16 up to 100 ng where the effect is lost probably due to saturation. This effect is present in conjunction with the hTERT promoter as seen in figure 3.1.5 a) and in conjunction with the hTR promoter as seen in figure 3.1.5 b). The hTERT and the hTR promoter initiate the two step amplification system to a similar level although the hTR does show an increased activity at the low 1ng and 10ng concentrations indicating that this is a relatively stronger promoter than the hTERT promoter.

In figure 3.1.6 and 3.1.7 the results from the transcription assays in the MCF-7 and the C33a cell lines respectively, are shown as fold transcriptional increase compared to cells transfected with 1μg pTKGal4-6xE2 only. The increase in transcriptional activity from cells transfected with VP16E2 compared to Gal4VP16 is even more pronounced in these cells than the effect seen in the 293-T cell line and the effect is still present at the highest concentration. The hTR promoter shows an increase in transcriptional activity compared to the hTERT promoter in both cell lines with the effect being most prominent in conjunction with the VP16E2 transcriptional activator.

In the MCF-7 and C33a cell lines a squelching effect is observed in cells transfected with pCG-VP16E2 at high concentrations. This is an effect in which the recruitment of cellular transcription factors exceeds the cellular capacity and hence a reduction in gene expression is seen with high concentration of powerful transcriptional activators.

Western blots were performed in 293-T, MCF-7 and C33a cells to examine at the protein expression of VP16E2 and the Gal4VP16 in conjunction with the telomerase promoters. Due to the lower transfection efficiency and telomerase activity in the MCF-7 and C33a, cells the Western blots from these cell lines were inconclusive and are therefore not shown. Cells were transfected with 1μg or 0.1μg either pCG-VP16E2 as a positive control, hTERT-VP16E2, hTR-VP16E2, hTERTGal4VP16 or hTRGal4VP16. The membrane was probed with an antibody against VP16 and an antibody against gammatubulin as a loading control. In figure 3.1.8 the western blot performed in 293-T cells is shown. The VP16E2 fusion protein runs as a double band which could be a degradation product or protein modification. The Gal4VP16 runs as a single band. An increase in VP16 protein detection is seen in the VP16E2 transfected samples lanes 3-6 compared to the Gal4VP16 transfected samples lanes 7-10. Great difference is not observed in protein detection levels of the hTR and the hTERT samples for either VP16E2 or Gal4VP16. High level of protein expression is detected in the pCG-VP16E2 transfected samples lane 1 and 2, whilst no protein expression is detected in the negative

control sample lane 11. The gammatubulin loading control indicates that an equal amount of input has been loaded to all lanes.

**Figure 3.1.4**

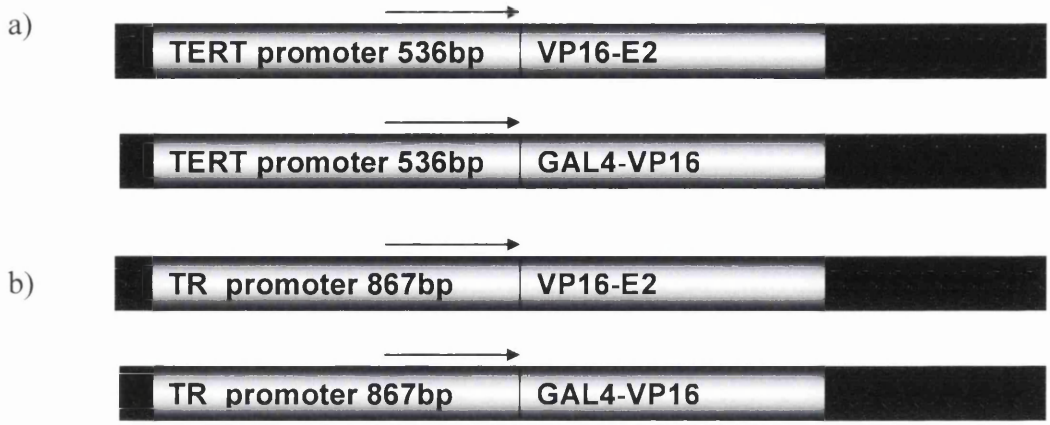


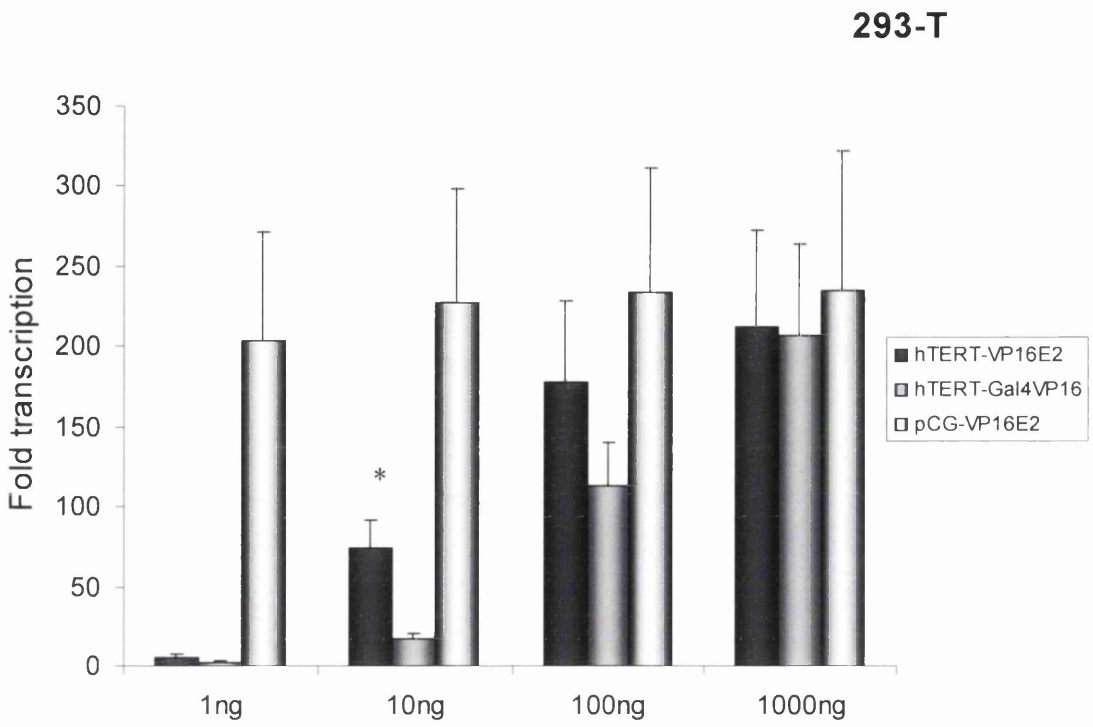

**Figure 3.1.4 : Telomerase constructs.**

a) Schematic reproduction of the hTERT-VP16E2 and the hTERT-Gal4VP16.

b) Schematic reproduction of the hTR-VP16E2 and the hTR-Gal4VP16.

**Figure 3.1.5**

a)




b)

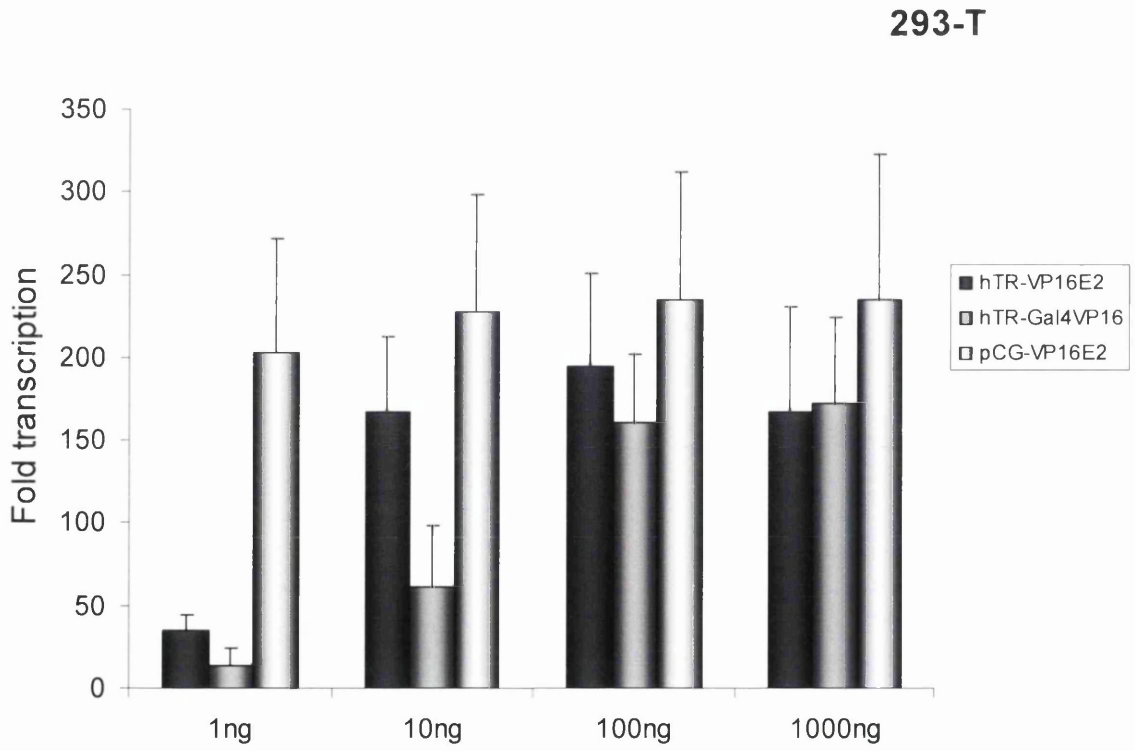


**Figure 3.1.5: VP16E2 induces stronger activation of transcription than Gal4VP16 in telomerase positive 293-T cells.**

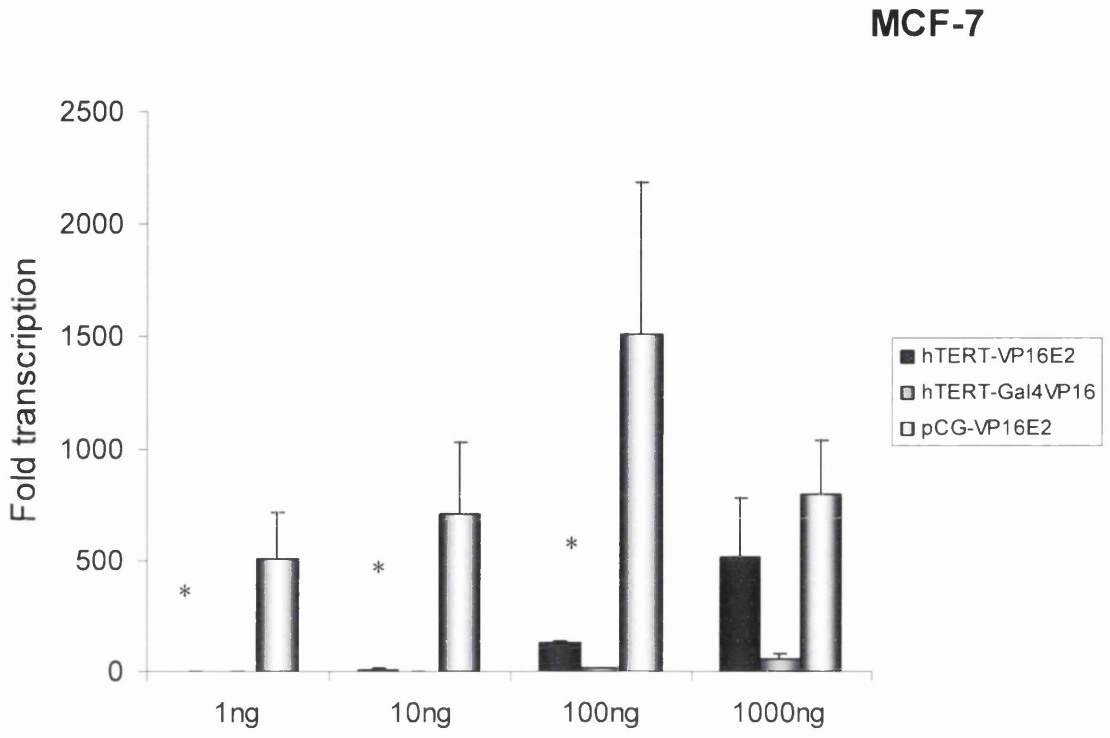
a) Comparison of the VP16E2 and the Gal4VP16 transcriptional activators under control of the hTERT promoter in telomerase positive293-T cells.

b) Comparison of the VP16E2 and the Gal4VP16 transcriptional activators under control of the hTR promoter in telomerase positive 293-T cells.

The pCG-VP16E2 is included in both a) and b) as a positive control independent of telomerase activity. Cells were transfected with different amounts of transcriptional activator plasmid as indicated. In addition all cells were co-transfected with 1000ng pTK-Gal4-6xE2 firefly luciferase reporter plasmid. Bars represent fold increase in luciferase expression compared to cells transfected with 1000ng pTK-Gal4-6xE2 firefly luciferase reporter plasmid alone. Each figure represents summary of 5 experiments carried out in duplicate. Standard error bars are shown. * = P-value <0.05 when comparing hTR-VP16E2 with hTR-GalVP16.

**Figure 3.1.6**

a)




b)

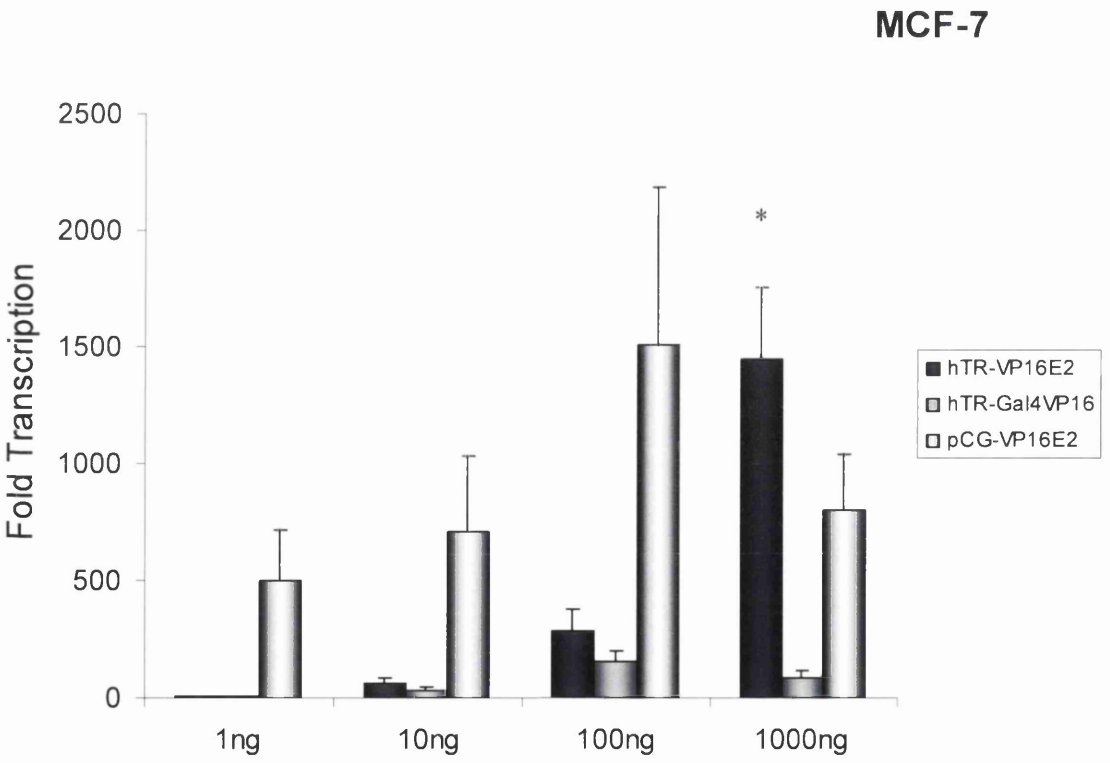


**Figure 3.1.6: VP16E2 induces stronger activation of transcription than Gal4VP16 in telomerase positive MCF-7 cells.**

a) Comparison of the VP16E2 and the Gal4VP16 transcriptional activators under control of the hTERT promoter in telomerase positive MCF-7 cells.

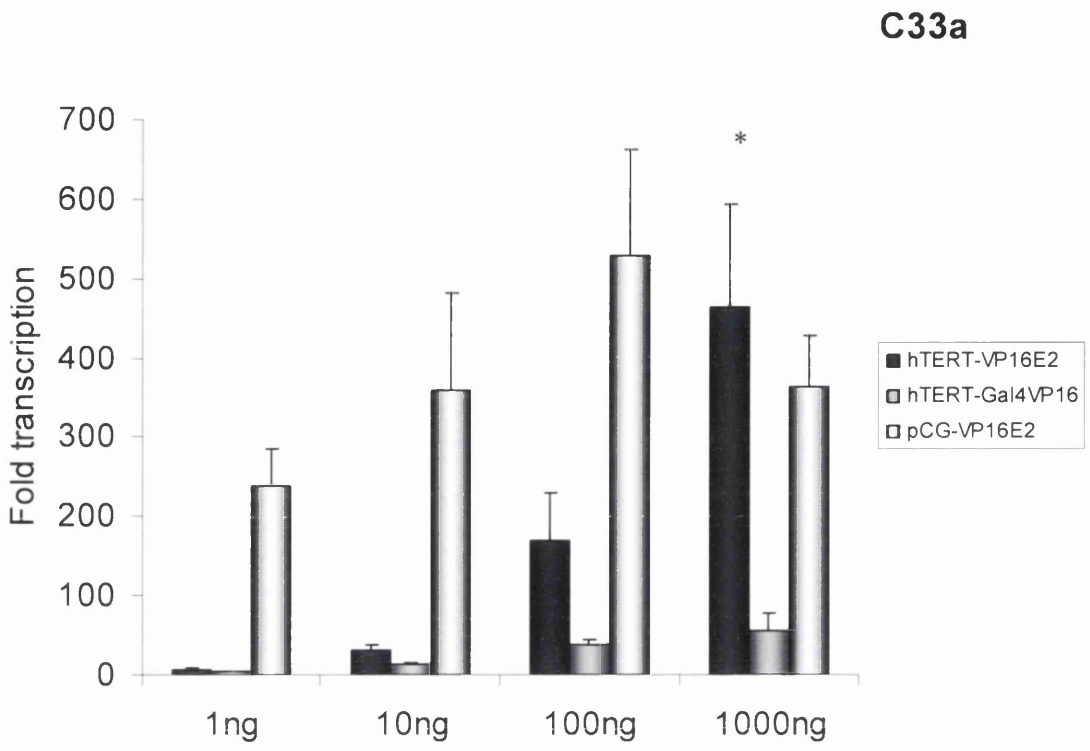
b) Comparison of the VP16E2 and the Gal4VP16 transcriptional activators under control of the hTR promoter in telomerase positive MCF-7 cells.

The pCG-VP16E2 is included in both a) and b) as a positive control independent of telomerase activity.

Cells were transfected with different amounts of transcriptional activator plasmid as indicated. In addition all cells were co-transfected with 1000ng pTK-Gal4-6xE2 firefly luciferase reporter plasmid. Bars represent fold increase in luciferase expression compared to cells transfected with 1000ng pTK-Gal4-6xE2 firefly luciferase reporter plasmid alone. Each figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown. * = P-value <0.05 when comparing hTR/hTERT-VP16E2 with hTR/hTERT-Gal4VP16

**Figure 3.1.7**

a)




b)

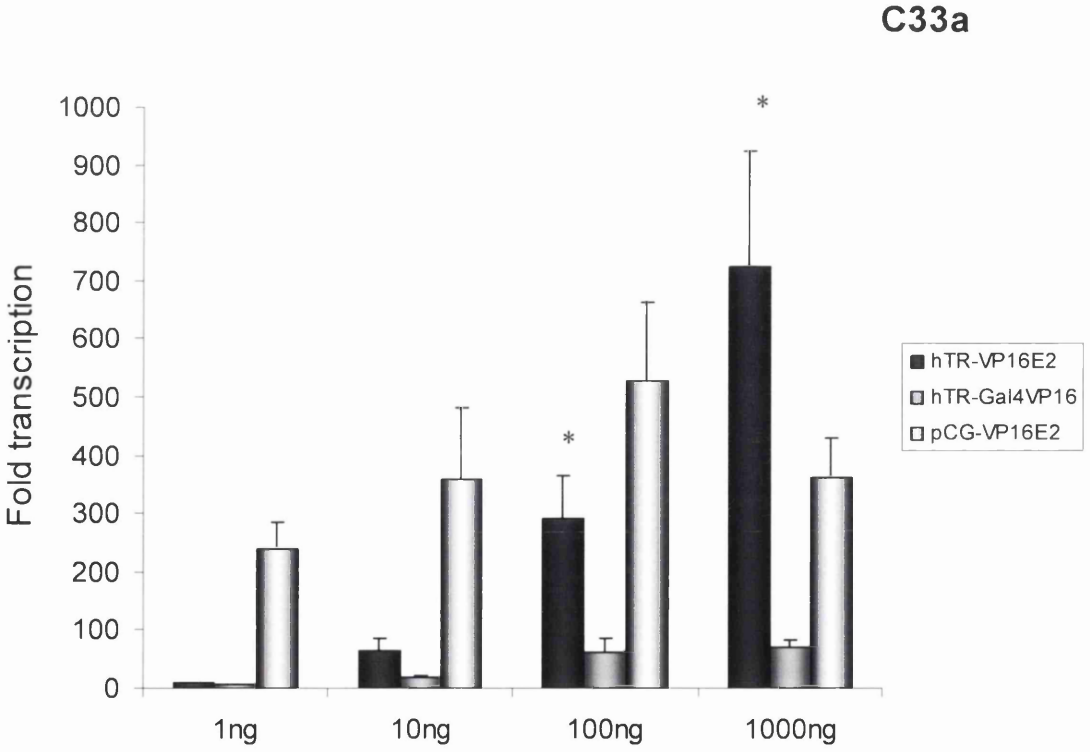


**Figure 3.1.7: VP16E2 induces stronger activation of transcription than Gal4VP16 in telomerase positive C33a cells.**

a) Comparison of the VP16E2 and the Gal4VP16 transcriptional activators under control of the hTERT promoter in telomerase positive C33a cells.

b) Comparison of the VP16E2 and the Gal4VP16 transcriptional activators under control of the hTR promoter in telomerase positive C33a cells.

The pCG-VP16E2 is included in both a) and b) as a positive control independent of telomerase activity.

Cells were transfected with different amounts of initiator plasmids as indicated. In addition all cells were co-transfected with 1000ng pTK-Gal4-6xE2 firefly luciferase reporter plasmid. Bars represent fold increase in luciferase expression compared to cells transfected with 1000ng pTK-Gal4-6xE2 firefly luciferase reporter plasmid alone. Each figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown. * = P-value < 0.05 when comparing hTR/hTERT-VP16E2 with hTR/hTERT-Gal4VP16

**Figure 3.1.8**

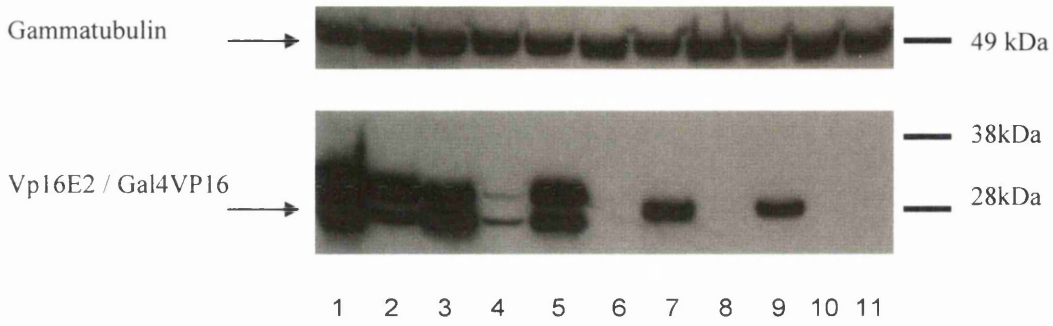



**Figure 3.1.8: Protein expression of transcriptional activators.**

Western blot showing the expression of the VP16E2 and the Gal4VP16 fusion proteins in 293T cells using antibody against VP16. Gammatubulin loading control is shown above. Arrows indicate the approximate location of the proteins. The location of the molecular weight marker (kDa) is shown to the right of the blot. 1: pCG-VP16E2 1000ng, 2: pCG-VP16E2 100ng, 3: hTERT-VP16E2 1000ng, 4: hTERT-VP16E2 100ng, 5: hTR-VP16E2 1000ng, 6: hTR-VP16E2 100ng, 7: hTERT-Gal4VP16 1000ng, 8: hTERT-Gal4VP16 100ng, 9: hTR-Gal4VP16 1000ng, 10: hTR-Gal4VP16 100ng, 11: Negative control. The amount in ng is reffering to the amount of DNA transfected into the cells and not to the amount of protein loaded on the gel.

### 3.1.3 Testing the specificity of the telomerase TSTA system with VP16E2.

To investigate whether the sensitivity of the two step amplification mechanism in conjunction with the telomerase promoters and the VP16E2 fusion protein could coexist with a high level of specificity to the telomerase activity of the transfected cells, transcription assays were carried out in telomerase negative cell lines.

Telomerase negative cell lines GM-847, U2OS and MRC-5 (see materials and methods) were transfected using Trans-IT (GM-847) or calcium phosphate (U2OS, MRC-5) with titrated amounts of either hTERT-VP16E2, hTR-VP16E2 or pCG-VP16E2 as a positive control independent of the cellular telomerase activity. All cells were co-transfected with 1μg of the 6xE2-3'BpTATA reporter vector. This plasmid contains the E2 activated 3'BpTATA promoter which is responsible for transcription of a firefly luciferase gene as seen in figure 3.1.9. Transcription assays were performed 3 times in duplicate and all results were normalised against protein present in each sample. Results are shown as fold increase in transcription compared to cells transfected with 1μg 6xE2-3'BpTATA only. Results from all three telomerase negative cell lines are collected in figure 3.1.10. In all three cell lines the pCG-VP16E2 transfected samples show a higher level of luciferase activity than hTERT-VP16E2 and hTR-VP16E2 at all DNA concentrations. In the GM-847 cell line 3.1.10 a) it is noted that the hTERT promoter shows very little transcriptional activity, however the hTR shows pronounced transcriptional activity at all DNA concentrations. This effect is apparent but less prominent in the U2OS cell line figure 3.1.10 b). In the MRC-5 cell line this effect is not seen and the hTR and hTERT promoters appear to be equally active. The high transcriptional activity of the hTR promoter in the GM-847 cell line signifies that this promoter's activity is less reflective of telomerase activity correlating with existing literature (Weinrich et al. 1997, Bodnar et al. 1998, Yin et al. 2004, Cairney and Keith 2008).

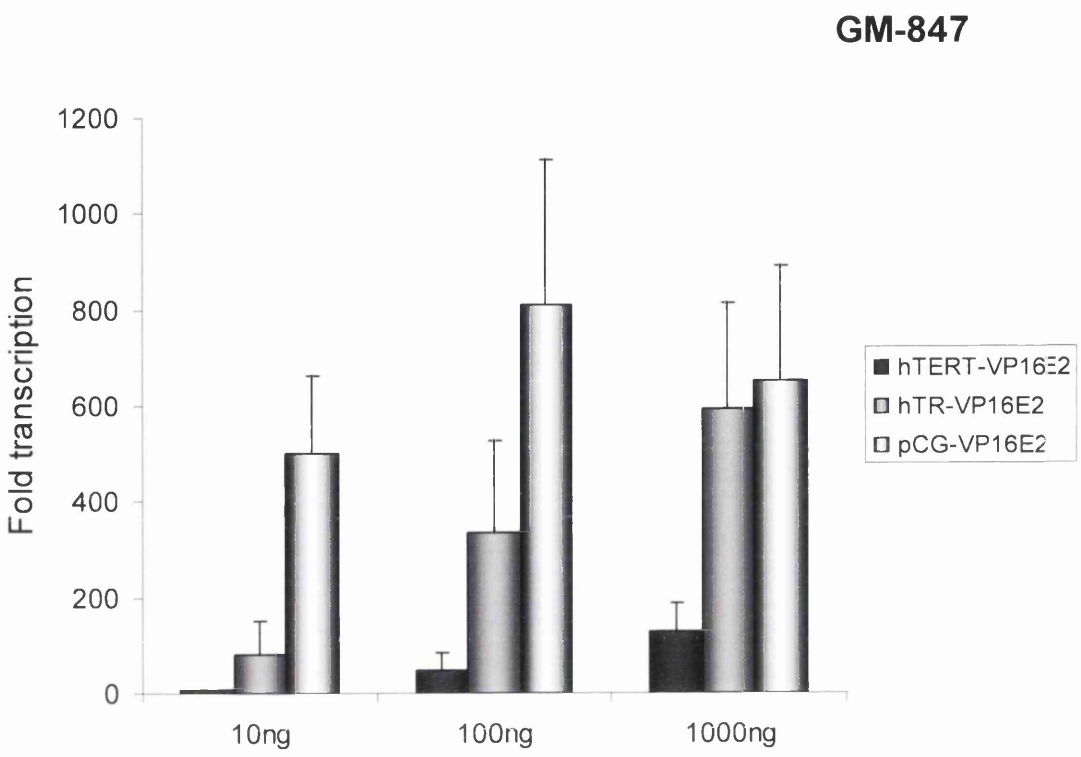
Figure 3.1.9


**Figure 3.1.9: Schematic representation of the 6xE2-3'bpTATA plasmid.**

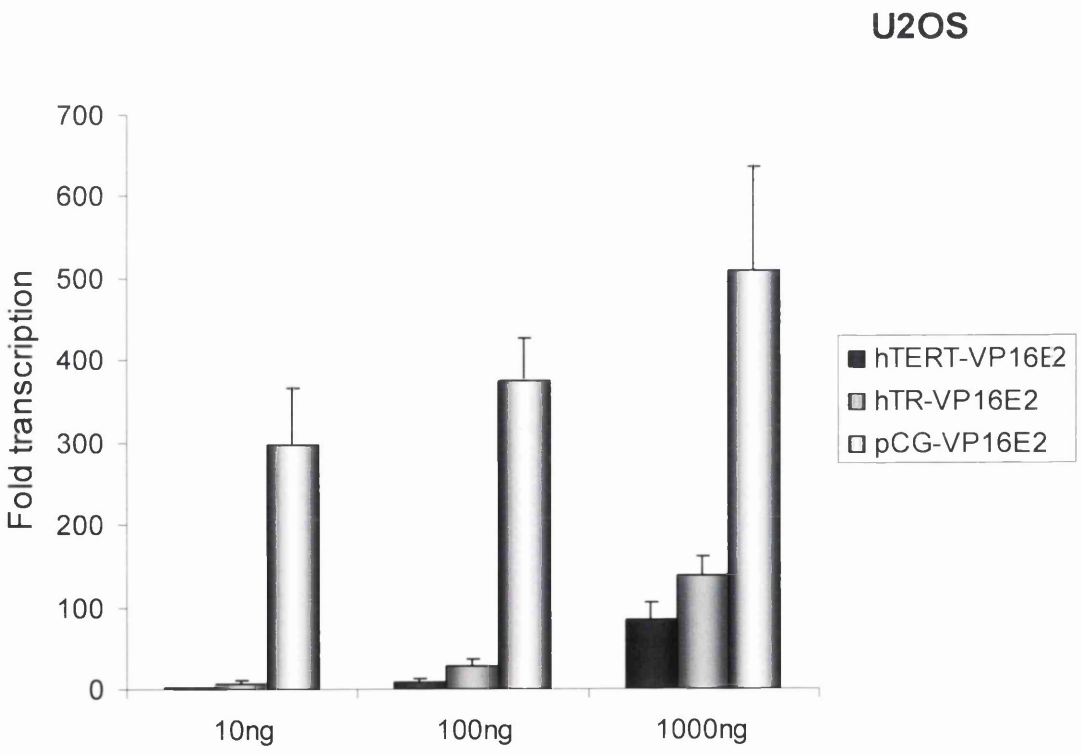
Schematic model of the luciferase reporter plasmid 6xE2-3'BpTATA. Reporter vector contains the short 3'BpTATA promoter, driving the expression of the firefly luciferase gene, activated by six upstream E2 binding sites.

**Figure 3.1.10**

a)




b)



c)

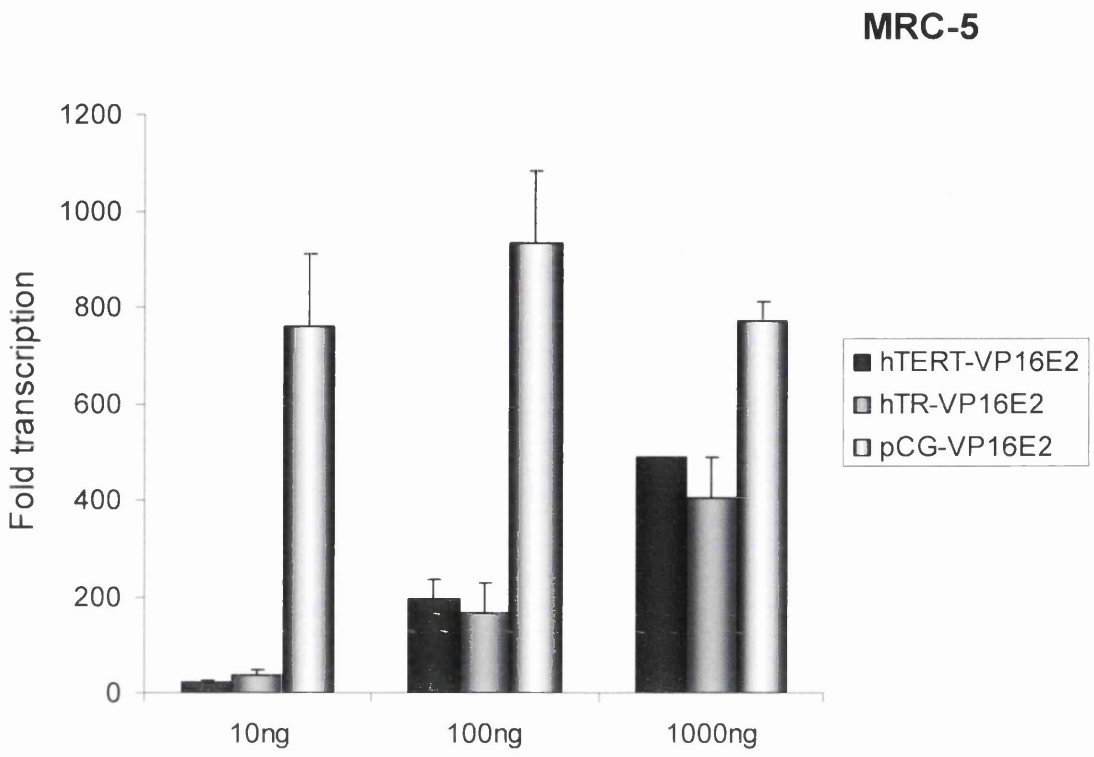



**Figure 3.1.10: Relative activity of the two telomerase promoters and the CMV promoter driving VP16E2 transcription in telomerase negative cells.**

a) GM-847 cells, b) U2OS cells, c) MRC-5 cells. Cells were transfected with different amounts of either hTERT-VP16E2, hTR-VP16E2 or pCG-VP16E2 plasmid as indicated. In addition 1000ng 6xE2-3'BpTATA luciferase reporter plasmid was transfected into each sample. Each bar represents fold increase in luciferase expression compared to cells transfected with 1000ng 6xE2-3'BpTATA alone. Each figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown.

### 3.1.4 Testing of the two step transcriptional amplification system in canine cell lines.

To investigate if the two step transcriptional amplification system in conjunction with the VP16E2 fusion protein and the human telomerase promoter could function in canine cell lines, transcription assays were carried out in the telomerase positive MDCK cell line and a canine primary chondrocyte cell line. The primary chondrocyte cell line was predicted to be telomerase negative due to its origin (see materials and methods). Cells were transfected using Trans-IT with titrated amounts of either hTERT-VP16E2, hTR-VP16E2 or pCG-VP16E2 as a positive control. All cells were co-transfected with 1μg of the 6xE2-3'BpTATA firefly luciferase reporter plasmid as shown in figure 3.1.9. Transcription assays were performed 3 times in duplicate and all results were normalised against protein present in each sample. Result are shown as fold increase in transcription compared to cells transfected with 1μg 6xE2-3'BpTATA only.

In the telomerase positive MDCK cell line seen in figure 3.1.11 transcriptional activity equal to the CMV promoter construct pCG-VP16E2 is seen in cells transfected with hTERT-VP16E2 and hTR-VP16E2 indicating that the human telomerase promoters and the two step amplification mechanism with VP16E2 does function very efficiently in canine cells.

In the primary chondrocyte cell line seen in figure 3.1.12, the hTR promoter is seen to be as transcriptionally active as the CMV promoter at the two highest DNA concentrations. The hTERT promoter shows a very low level of transcriptional activity at the two lower DNA concentrations but this effect is lost at the highest DNA concentration. Here the hTERT promoter shows transcriptional activity comparable to the CMV and hTR promoters. A squelching effect is observed at the highest concentration of the pCG-VP16E2 transfected cells.

This experiment shows that the human derived telomerase promoters and the VP16E2 two step amplification system functions in canine cells. The transcriptional activity seen in the primary canine chondrocyte cell line, suggests that these cells are telomerase positive which was confirmed by later investigations (chapter 3.1.5).

**Figure 3.1.11**

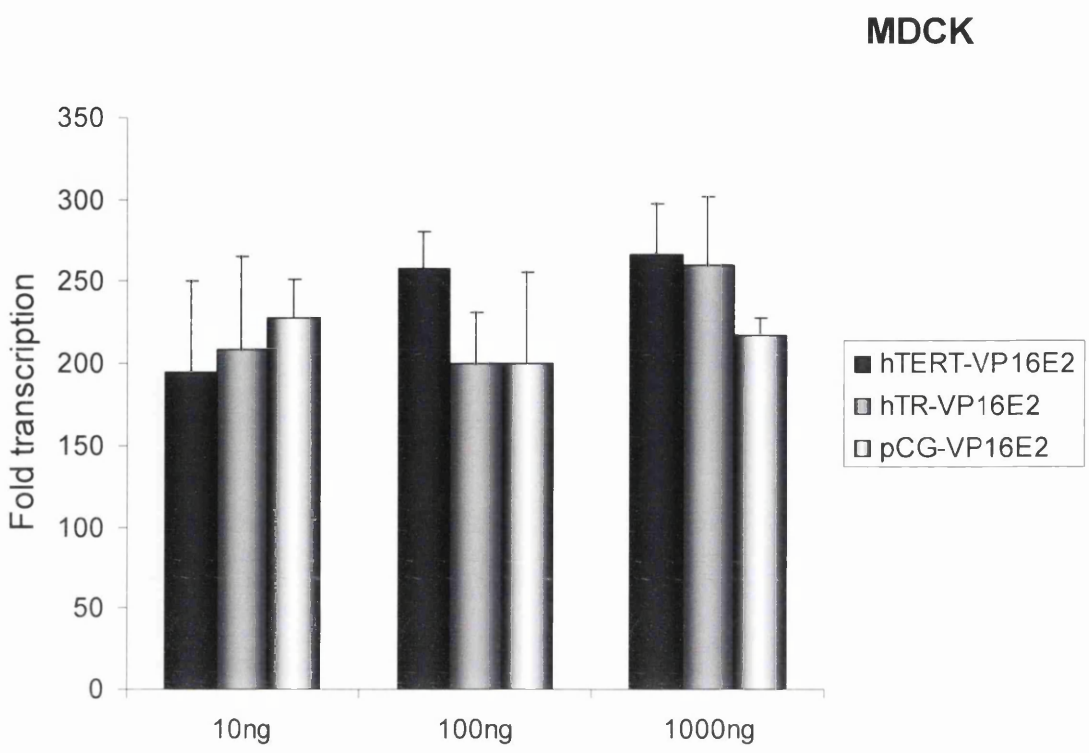



**Figure 3.1.11: Relative activity of the two telomerase promoters and the CMV promoter driving VP16E2 transcription in canine telomerase positive cells.**

Telomerase positive MDCK cells were transfected with different amounts of either hTERT-VP16E2, hTR-VP16E2 or pCG-VP16E2 plasmid as indicated. In addition 1000ng 6xE2-3'BpTATA luciferase reporter plasmid was co-transfected into each sample. Each bar represents fold increase in luciferase expression compared to cells transfected with 1000ng 6xE2-3'BpTATA alone. Figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown.

**Figure 3.1.12**

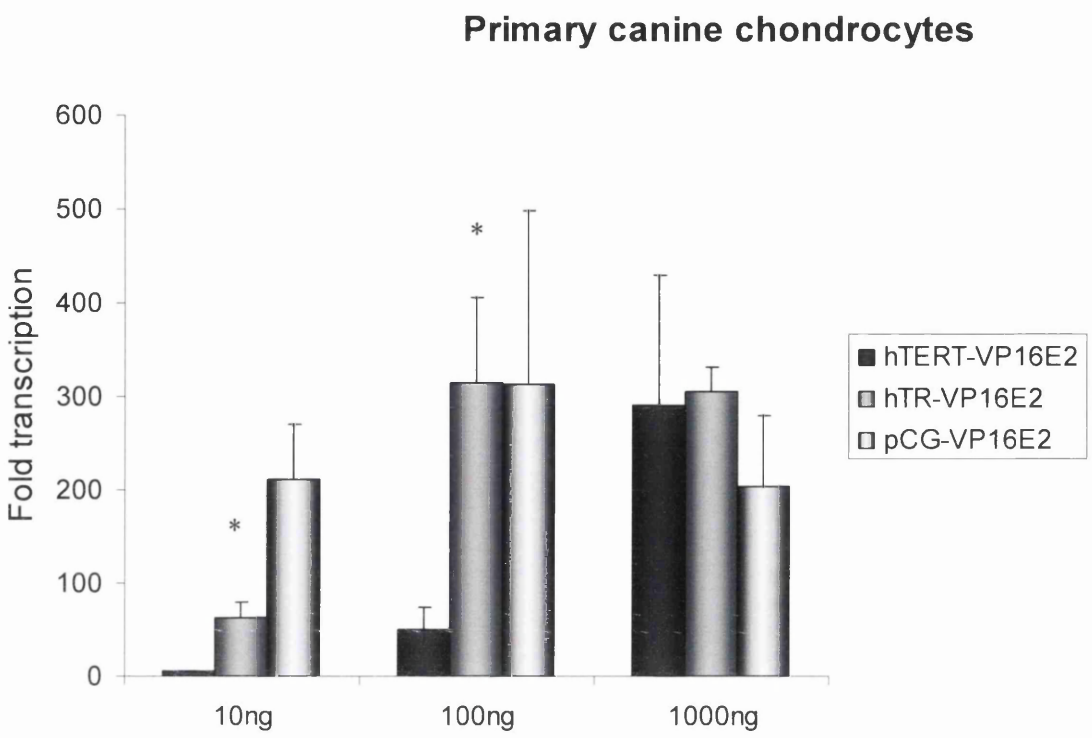



**Figure 3.1.12: Relative activity of the two telomerase promoters and the CMV promoter driving VP16E2 transcription in canine primary cells.**

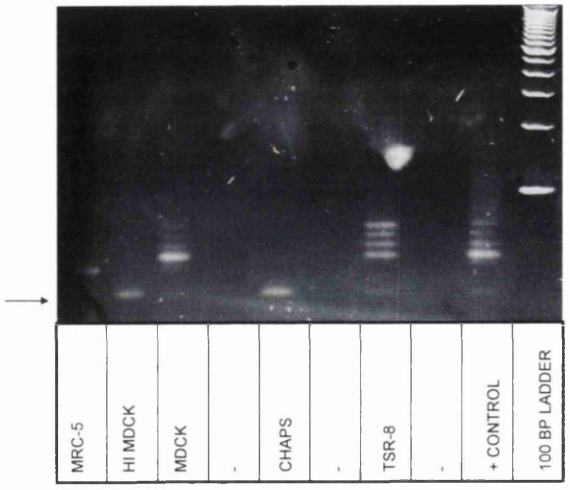
Primary canine chondrocytes were transfected with different amounts of either hTERT-VP16E2, hTR-VP16E2 or pCG-VP16E2 plasmid as indicated. In addition 1000ng 6xE2-3'BpTATA luciferase reporter plasmid was co-transfected into each sample. Each bar represents fold increase in luciferase expression compared to cells transfected with 1000ng 6xE2-3'BpTATA alone. Figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown. * = P-value < 0.05 when comparing hTR-VP16E2 with hTERT-VP16E2.

### 3.1.5 Measurement of telomerase activity in cell lines.

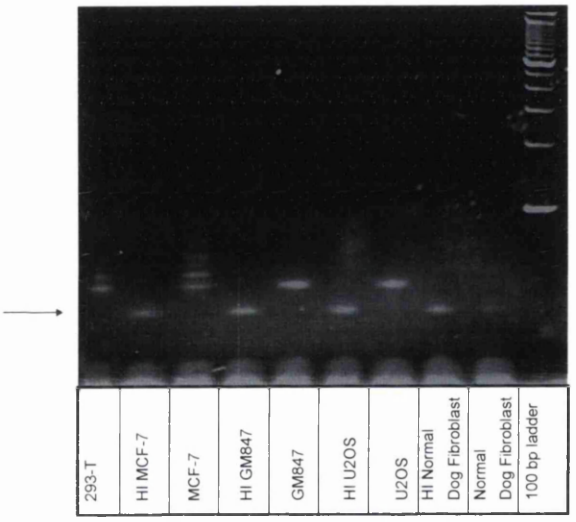
In order to determine the telomerase activity in the cell lines used in sections 3.1.4-3.1.6 telomerase repeat amplification protocol (TRAP) assays were carried out using the TRAPeze kit as described in materials and methods. 293-T, MCF-7, C-33a, GM-847, U2OS, MRC-5, MDCK and primary chondrocytes were tested. In addition a primary canine fibrosarcoma cell line and a primary canine fibroblast cell line which were used in later experiments (chapter 3.2) were tested. A primary canine testes tumour cell line was tested however this cell line was not used for further experiments. Results from the TRAP assay are shown as TRAP ladders run on a SDS polyacrylamide (PAGE) gel as seen in figure 3.1.13. In this figure each sample was run alongside its heat inactivated negative control. The internal band present at arrow should be present in all samples. This is a control indicating that no polymerase inhibiters were present in the tested sample. Lack of an internal band in a sample can result in false negative results due to reduced polymerase activity. The interpretation of the PAGE gel results are shown in table 3.1.1. The intensity of the ladders and the number of bands were used to interpret the telomerase activity. Ladders with several bands of strong intensity were interpreted as having more telomerase activity and samples in which the heat inactivated control was absent were interpreted as being more telomerase active than the ladder indicated. Important results to notice from the TRAP assay are that the canine primary fibrosarcoma cell line, the primary canine fibroblast cell line and the primary canine testes tumour were telomerase negative. Another important result was that low telomerase activity was observed in primary canine chondrocytes cells correlating well with the hTERT promoter activity observed in this cell line in chapter 3.1.4. The quality of the TRAP ladder in the MRC-5 sample shown on figure 3.1.13 a) is very poor and an artefact is disturbing the image of the internal standard. The TRAP ladder in this sample appears to run at a smaller size which could be due to artefacts and the telomerase activity detected in this cell line is therefore questionable.

**Figure 3.1.13**

a)




b)




c)

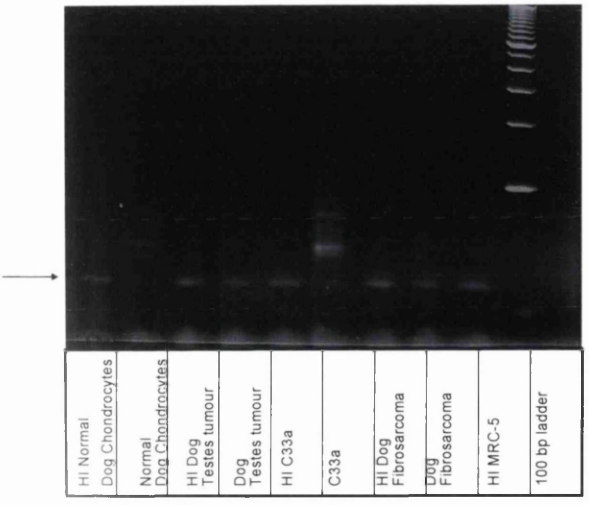


**Figure 3.1.13: Telomerase ladders on PAGE gel .**

10 µl of each TRAP reaction were examined on a on a gel in parallel with a 100 base pair ladder. The gels were stained with ethidium bromide and DNA was visualised under UV light. Arrows indicate the location of the internal control band.

**Table 3.1.1**

| Cell line | HI control | Internal standard | Telomerase status |
|---|---|---|---|
| 293-T | Not tested | + | +++ |
| MCF-7 | - | + | +++ |
| GM-847 | - | + | + |
| U2OS | - | + | + |
| Canine fibroblast | - | +++ | - |
| Chondrocytes | - | ++ | ++ |
| Testes tumour | - | +++ | - |
| C-33a | - | ++ | +++ |
| Fibrosarcoma | - | +++ | - |
| MRC-5 | - | ++ | (+) |
| MDCK | - | + | +++ |

**Table 3.1.1: Results from TRAP assay.**

Interpretation of the page gel trap analysis schematically represented. Plus signs are used to indicate quality of controls or telomerase activity. HI = Heat inactivated control. +=low, ++=moderate, +++=high - = negative.

### 3.1.6 Testing efficiency and overlap of co-transfection with two plasmids.

The two step amplification system used throughout section 3.1.1-3.1.5 is based on a transcriptional activator and a reporter two plasmid system. For this system to function it is necessary that both plasmids are taken up by the transfected cell. As illustrated in figure 3.1.14 a-c) cells have different transfection efficiencies and for cells with a low transfection efficiency the number of cells that take up both plasmids could be as low as the percentage of transfected cells squared unless there is a correlation between cells taking up both plasmids. To test this, co-tranfection experiments were performed in 3 cell lines using green fluorescent protein (GFP) and red fluorescent protein (RFP) expressing plasmids. MDCK, U2OS and 293-T cells were used for this experiment; the latter was transfected with calcium phosphate and the two former were transfected with Trans-IT. Transfection efficiency was determined for each cell line by transfection with 1μg GFP expressing plasmid. 40 hours post transfection the cells were visualised under fluorescent light at a wavelength specific for GFP expression and the GFP versus non GFP expressing cell ratio was determined. The approximate transfection efficiency for the MDCK, U2OS and 293-T cells was 15-20%, 60-70% and 95-99 % respectively. Co-transfection was performed by co-transfecting each of the cell lines with 1μg GFP and 1μg RFP expressing plasmid. 40 hours later cells were visualised using a fluorescent microscope and the co-localisation of GFP and RFP expressing cells was determined. The transfection efficiency of the RFP expressing cells in general seems a bit lower than for GFP however this is due to the weaker expression of RFP in general due to its slow folding and tendency to form tetramers which can reduce its expression (Shrestha & Deo 2006). In general it was estimated that approximately 95 % of cells expressing RFP also expressed GFP suggesting that there may be a correlation between uptakes of both plasmids. In figure 3.1.15, 3.1.16 and 3.1.17 pictures are shown of the MDCK, U2OS and 293-T cells used in the experiments. Cells are shown using a filter specific for GFP expression, a filter specific for RFP expression and phase contrast exposure. A merge of the GFP and RFP images, shows the transfection co-localisation. The figures show that the outcome of the transfection most resembles the model in figure 3.1.14 f) in which both plamids are taken up by transfected cells.

**Figure 3.1.14**

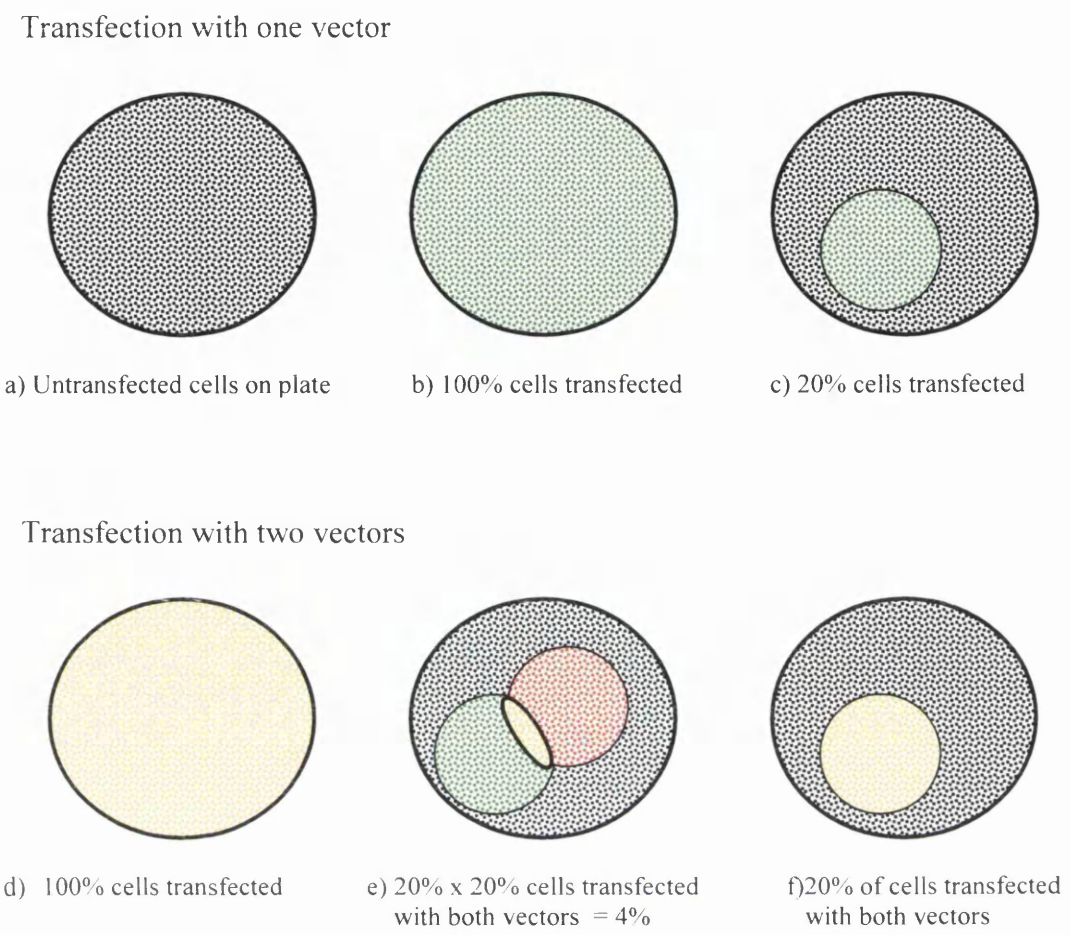



**Figure 3.1.14: Possible transfection efficiency models for co-transfections with two plasmids**

Figure illustrates possible outcomes for cells with either 100% or 20% tranfection efficiency, transfected with either one or two plasmids.

Black represents untransfected cells, green represents cells transfected with GFP expressing plasmid, red represents cells transfected RFP expressing plasmid and yellow represents cells transfected with both GFP and RFP expressing plasmids

**Figure 3.1.15**

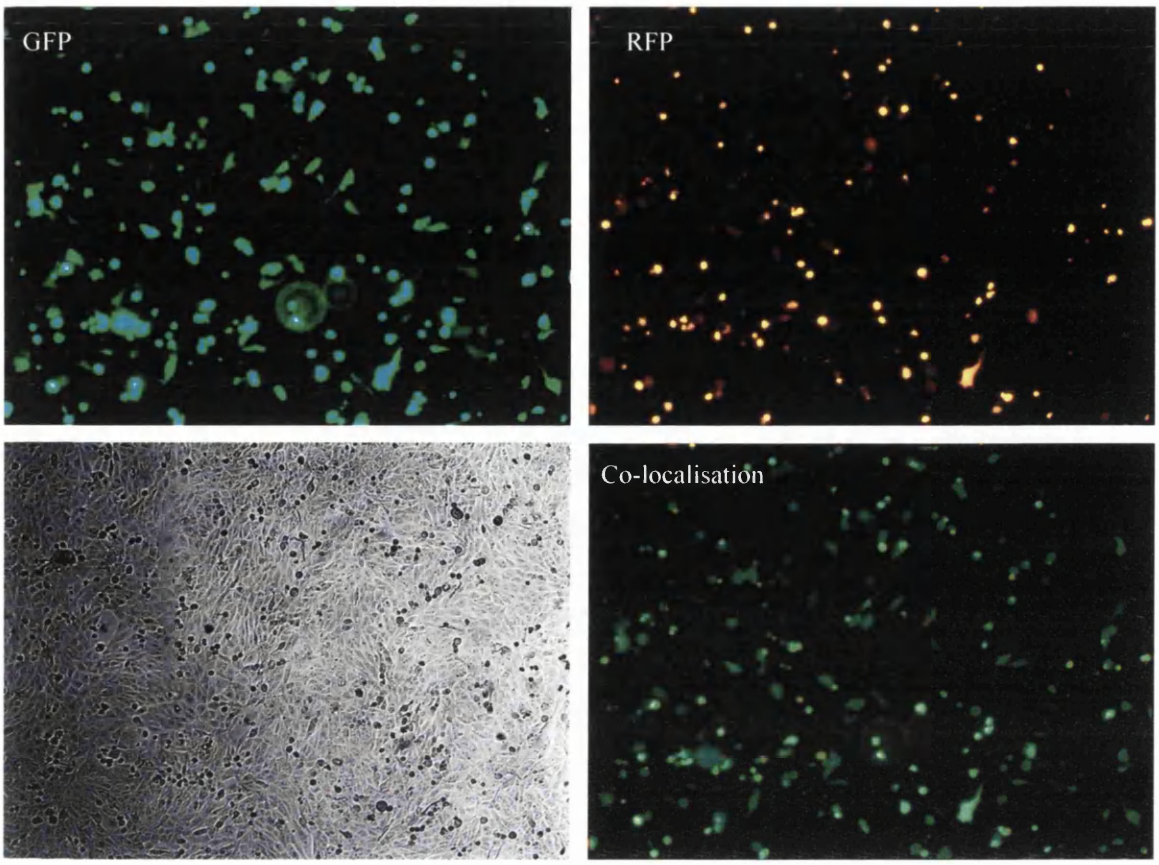



**Figure 3.1.15: Transfection overlap in MDCK cells co-transfected with GFP and RFP expressing plasmids.**

Top left, GFP expressing MDCK cells visualised. Top right, RFP expressing MDCK cells visualised. Bottom left, phase contrast picture of MDCK cells. Bottom right, merged image of pictures top left and top right showing transfection co-localisation.

**Figure 3.1.16**

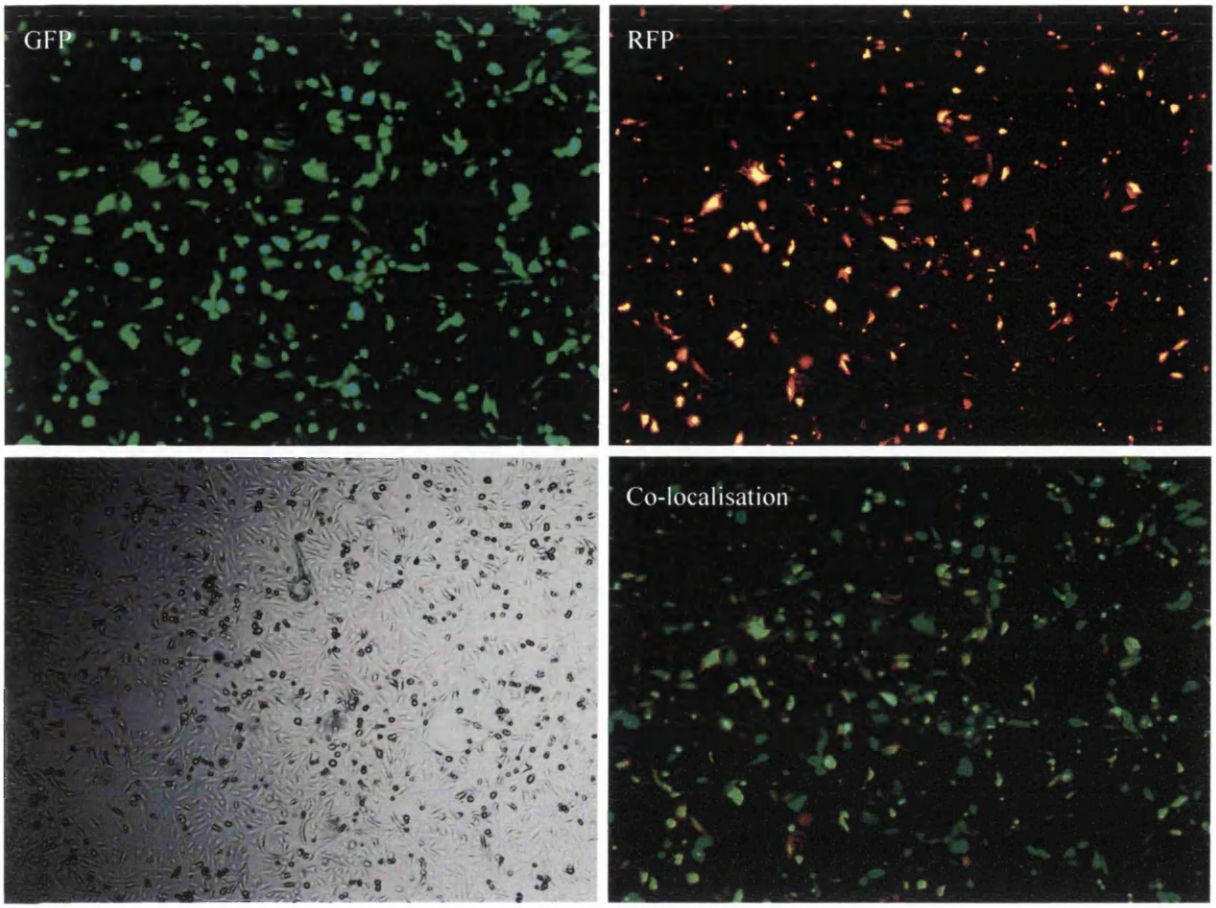



**Figure 3.1.16: Transfection overlap in U2OS cells co-transfected with GFP and RFP expressing plasmids.**

Top left, GFP expressing U2OS cells visualised. Top right, RFP expressing U2OS cells visualised. Bottom left, phase contrast picture of U2OS cells. Bottom right, merged image of pictures top left and top right showing transfection co-localisation.

**Figure 3.1.17**

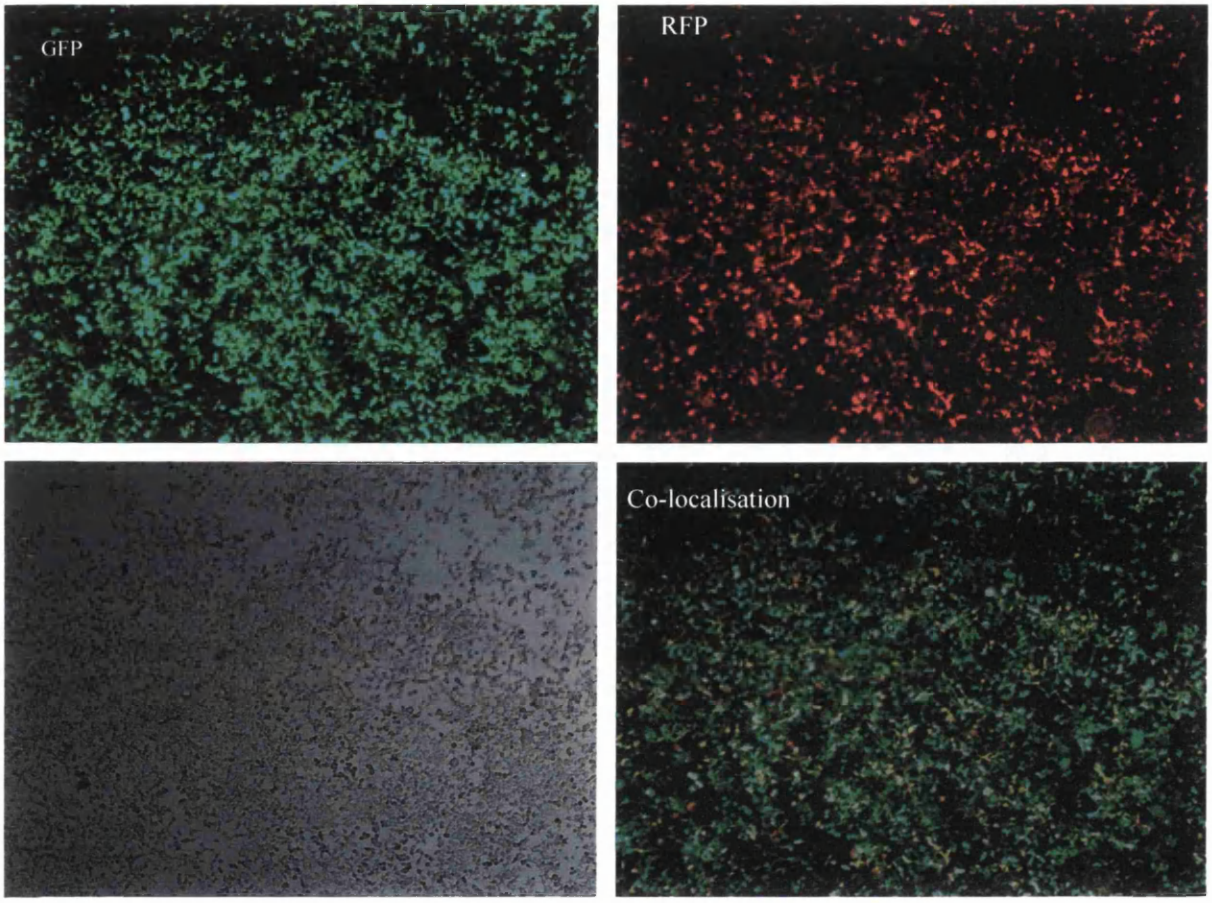



**Figure 3.1.17: Transfection overlap in 293-T cells co-transfected with GFP and RFP expressing plasmids.**

Top left, GFP expressing 293-T cells visualised. Top right, RFP expressing 293-T cells visualised. Bottom left, phase contrast picture of 293-T cells. Bottom right, merged image of pictures top left and top right showing transfection co-localisation.

### 3.1.7 Development of a single plasmid two step vector.

To develop the two step transcriptional amplification system further the two vector system was incorporated into a one vector system by placing the telomerase promoter transcribing the transcriptional activator fusion protein and the 6xE2-3'BpTATA promoter transcribing the reporter gene into one plasmid. The results in section 3.1.3 and the literature indicate that the hTERT promoter is more specific for telomerase activity than the hTR promoter and therefore only the hTERT promoter construct was taken forward into a one vector system.

#### 3.1.7.1 Cloning of the hTERT-VP16E2 one vector system.

To reduce the two plasmid system to one plasmid it was decided to clone the hTERT-VP16E2 sequence into the 6xE2- 3'BpTATA plasmid. This could be done with the 3'BpTATA promoter and the hTERT promoter transcribing in the same direction or the two promoters transcribing in opposite directions. It was decided to clone both constructs to test if there would be a difference between these.

The 6xE2-3'BpTATA vector was digested with BamH I and phosphatase treated. A hTERT-VP16E2 PCR product with 5' and 3' overhanging Bgl II restriction sites was made using primers as described in materials and methods. The PCR product was digested with Bgl II and ligated into the cut 6xE2-3'BpTATA plasmid and transformed into JM-109 cells. The Bgl II and BamH I have compatible ends that at ligation destroy both sites. This method allowed for the insertion of the insert in either direction. Inserts were confirmed and direction determined, by sequencing and restriction digest. The resulting plasmids were named 6xE2-3'BpTATA-hTERT-VP16E2 Forward (FOR) and 6xE2-3'BpTATA-hTERT-VP16E2 Reverse (REV) as shown on figure 3.1.18.

#### 3.1.7.2 Testing the one vector system.

To investigate the ability of the two step amplification system to function in a one plasmid system, transfection assays were performed in telomerase positive 293-T cells and telomerase negative GM-847 cells. Cells were transfected with titrated concentrations of either 6xE2-3'BpTATA-hTERT-VP16E2 FOR or 6xE2-3'BpTATA-hTERT-VP16E2 REV. In parallel cells were transfected with 1μg pCG-VP16E2 and

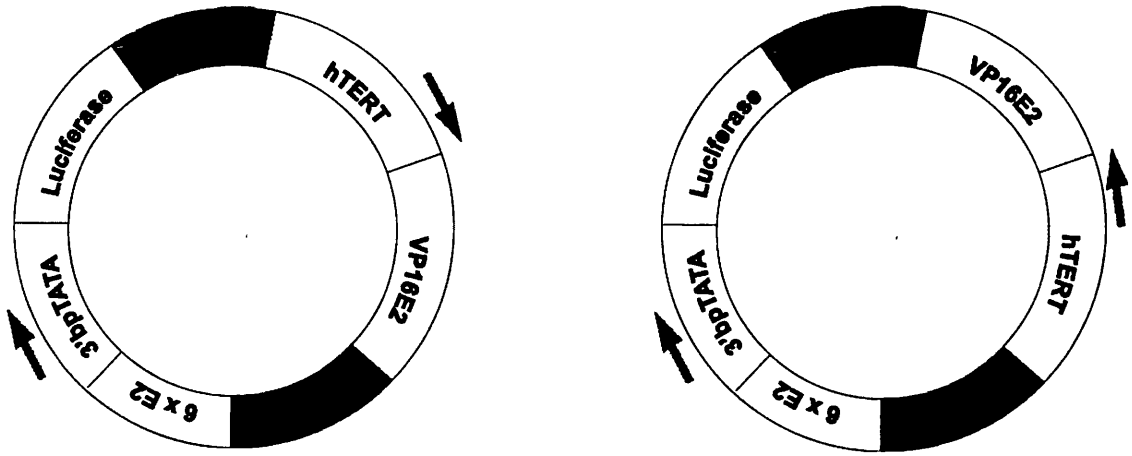
1μg 6xE2-3'BpTATA as a two vector system. Transcription assays were performed 3 times in duplicate and all results were normalised against protein present in each sample. Results are shown as fold increase in transcription compared to cells transfected with 800ng 6xE2-3'BpTATA reporter vector on its own. In figure 3.1.19 the results from the transcription assays are shown. It is seen that sensitivity and specificity to telomerase activity is still conserved in the one plasmid system. This is seen as high transcription activity relative to the pCG-VP16E2 in telomerase positive cells 3.1.19 a) and low transcriptional activity relative to the pCG-VP16E2 in telomerase negative cells 3.1.19 b). A slight difference in transcriptional activity is observed between the 6xE2-3'BpTATA-hTERT-VP16E2 FOR and 6xE2-3'BpTATA-hTERT-VP16E2 REV constructs with the reverse associated with a higher level of activity. The TERT promoter lacks a TATA box which is a common characteristic of bidirectional promoters (Dong et al. 2000, Yang & Elnitski 2008). It is a possibility that the extra activity seen with the 6xE2-3'BpTATA-hTERT-VP16E2 REV could be due to reverse transcription from the TERT promoter. Such activity could compromise the specificity of the system. It is seen that the 6xE2-3'BpTATA-hTERT-VP16E2 FOR shows a low er level of transcriptional activity than the REV construct in telomerase negative cells and therefore it was elected to take the FOR construct forward and the subsequent work using the single plasmid two step system was done with the 6xE2-3'BpTATA-hTERT-VP16E2 FOR.

### 3.1.7.3 Evaluating the effect of the two step amplification system compared to the telomerase promoter on its own.

To look at the advantage of incorporating the telomerase promoter into the two step amplification system in order to target gene expression to telomerase positive cells, transcription assays were performed comparing the transcriptional activity of the hTERT-LUC plasmid and the 3'BpTATA-hTERT-VP16E2 FOR. 293-T cells were transfected with serial dilutions of either plasmid using calcium phosphate. Transcription assays were performed 3 times in duplicate and all results were normalised against protein present in each sample. Results are shown as fold increase in transcription compared to cells transfected with 1μg pGL3-Basic reporter vector on its own. The results from the experiment are summarised in figure 3.1.20 Transcriptional activity for both plasmids is negligible at concentrations below 1000ng. At the 1000ng

concentration a great increase in transcriptional activity is seen and an over 30 fold increase in transcriptional activity is observed in the 3'BpTATA-hTERT-VP16E2 compared to the hTERT-LUC confirming the advantage of including a two step amplification system into targeted gene therapy.

**Figure 3.1.18**




a) b)

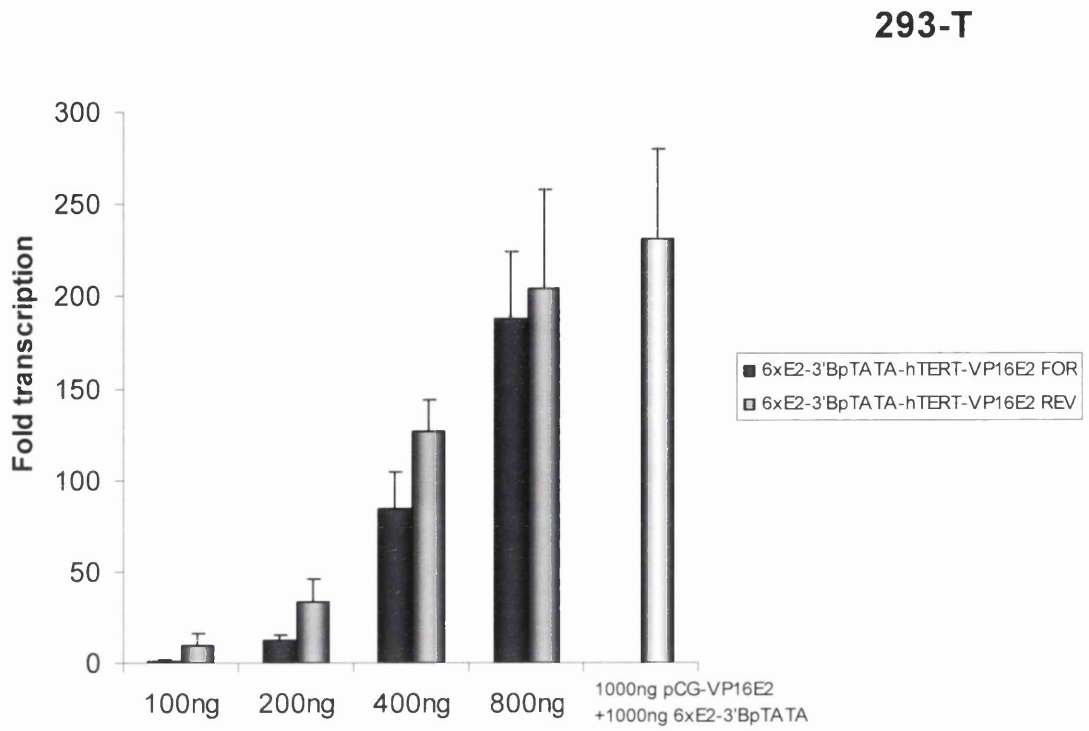
**Figure 3.1.18: Schematic models of one plasmid system.**

a) Schematic representation of one plasmid system 6xE2-3'BpTATA-hTERT-VP16E2 Forward, in which the TERT promoter and the 6xE2-3'BpTATA promoter both transcribe clockwise.

b) Schematic representation of one plasmid system 6xE2-3'BpTATA-hTERT-VP16E2 Reverse, in which the TERT promoter transcribes clockwise and the 6xE2-3'BpTATA promoter transcribes counter clockwise.

**Figure 3.1.19**

a)




b)

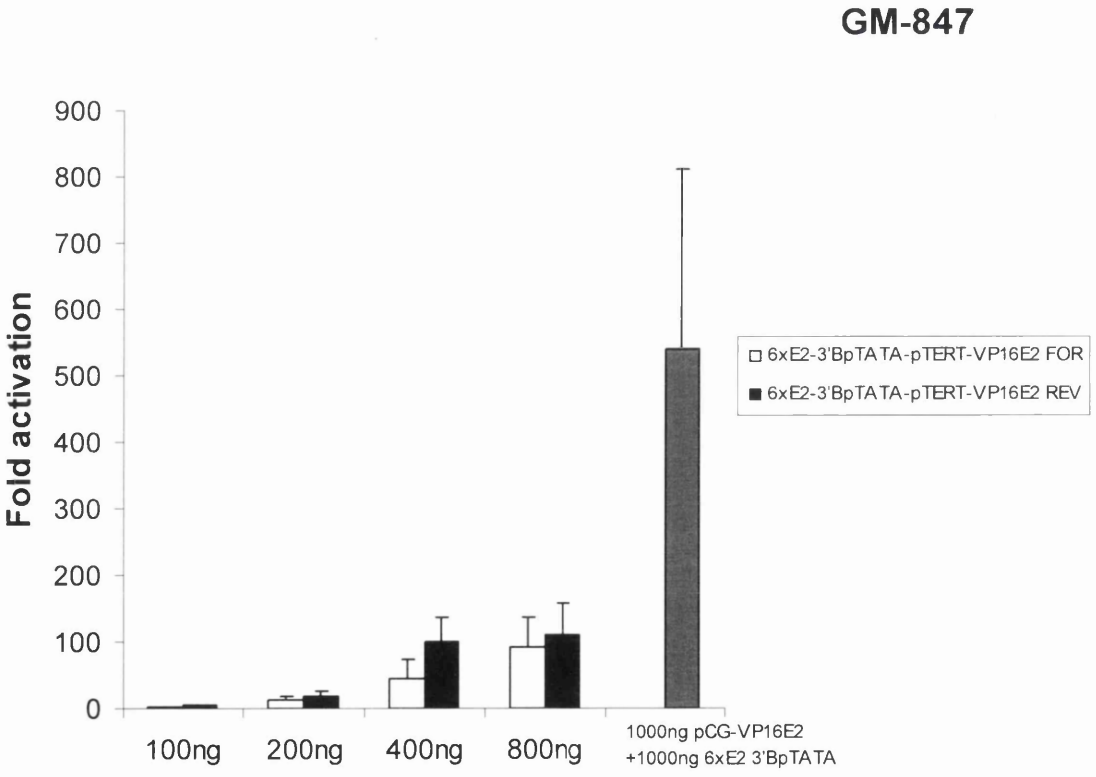


**Figure 3.1.19: One plasmid system in telomerase positive and negative cells**

a) 293-T telomerase positive cells, b) GM-847 telomerase negative cells. Cells were transfected with different amounts of 6xE2-3'BpTATA-hTERT-VP16E2 FOR or REV as indicated. For comparison, cells were co-transfected with 1000ng pCG-VP16 E2 and 1000ng 6xE2-3'BpTATA. Results are shown as fold increase in luciferase expression compared to sample transfected with 800ng 6xE2-3'BpTATA only. Experiments were carried out 3 times in duplicate. Standard error bars are shown.

**Figure 3.1.20**

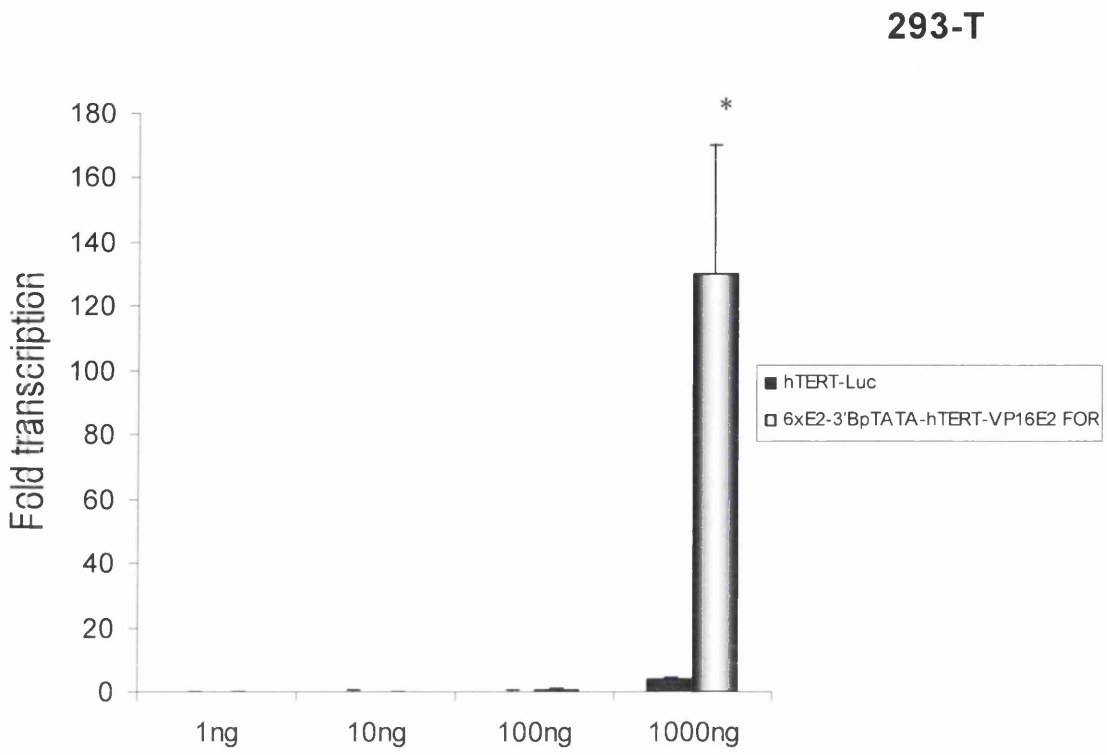



**Figure 3.1.20 TSTA system increases transcription significantly.**

293-T cells were transfected with hTERT-LUC or 3'BpTATA-hTERTVP16E2 FOR as indicated. Each bar represents fold increase in transcription relative to cells transfected with 1µg 6xE2-3'BpTATA. Figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown. *= P-value < 0.05

### 3.1.7.4 Cloning of the hTERT-Gal4VP16 one vector system.

To compare the hTERT-VP16E2 with the hTERT-Gal4VP16E2 in the one plasmid system, a one vector plasmid fusion with the 6xE2-3'BpTATA plasmid and the hTERT-Gal4VP16 insert was made. The 6xE2-3'BpTATA plasmid does not contain Gal4 binding sites so in order for the promoter to be responsive to the Gal4VP16 fusion protein, 5 Gal4 binding sites were cloned in upstream of the 6 E2 binding sites present in the 6xE2-3'BpTATA plasmid. The pTK-Gal4-6xE2 plasmid was digested with Nhe I and KpN I to cut out the 5 Gal4 binding sites in the plasmid. The 5xGal4 fragment was isolated by gel electrophoresis and purification. The 6xE2-3'BpTATA plasmid was digested with KpN I and Nhe I and phosphase treated. The 5xGal4 fragment was ligated with the cut 6xE2-3'BpTATA plasmid and transformed into Dh5α cells. Correct inserts were confirmed by restriction digest. The resulting vector was named 5xGal46xE2-3'BpTATA.

The hTERT-Gal4VP16 fragment was inserted into the 5xGal46xE2-3'BpTATA plasmid in a similar manner as described for the hTERT-VP16E2 3.1.7.1 however only the forward construct was cloned. The resulting vector was named 5xGal46xE2-3'BpTATA-hTERT-Gal4VP16 and is schematically illustrated in figure 3.1.21

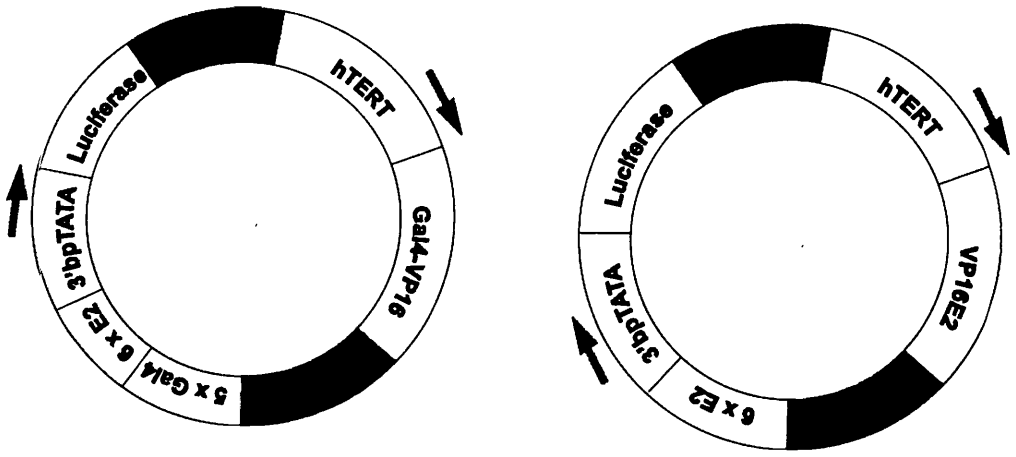
### 3.1.7.5 Comparison of the VP16E2 and the Gal4VP16 one plasmid system.

To compare the transcriptional potential of the hTERT-VP16E2 and hTERT-Gal4VP16 in the one plasmid system, transcription assays were performed in 293-T cells. It was decided to use the forward versions of the one plasmid system for the comparison. 293-T cells were transfected with titrations of either 6xE2-3'BpTATA-hTERT-VP16E2 or 5xGal46xE2-3'BpTATA-hTERT-VP16E2. Transcription assays were performed 3 times in duplicate and all results were normalised against protein present in each sample. Results are shown as fold increase in transcription compared to cells transfected with 1μg 5xGal46xE2-3'BpTATA reporter vector on its own. The higher transcriptional activity seen previously in conjunction with the VP16E2 transcriptional activator compared to the Gal4VP16 is preserved in the one plasmid system as shown in figure 3.1.22.

To look at the protein expression of 6xE2-3'BpTATA-hTERT-VP16E2 or 5xGal46xE2-3'BpTATA-hTERT-VP16E2 293-T cells were transfected with either 1μg

or 0.1 μg of either plasmid and normal protocol was carried out for Western blot. The membrane was probed with an antibody against VP16 and gammatubulin as a loading control. As seen in figure 3.1.23 protein is detected for both transcriptional activators at 1μg concentrations. No bands were detected at 0.1μg concentrations or in the negative control lane. The gammatubulin loading control indicated that equal amounts of protein had been loaded in each lane. No difference was observed in the strength of the bands between the two transcriptional activators. It is noted that the VP16E2 only ran as a single band on this blot, corresponding to the smaller band of the two seen in previous blots (figure 3.1.3 and 3.1.8).

**Figure 3.1.21**




a) b)

**Figure 3.1.21: 5xGal4-6xE2-3'BpTATA-hTERT-VP16E2**

a) Schematic representation of the 5xGal4-6xE2-3'BpTATA-hTERT-Gal4VP16 FOR.

b) Schematic representation of 6xE2-3'BpTATA-hTERT-VP16E2 FOR

**Figure 3.1.22**

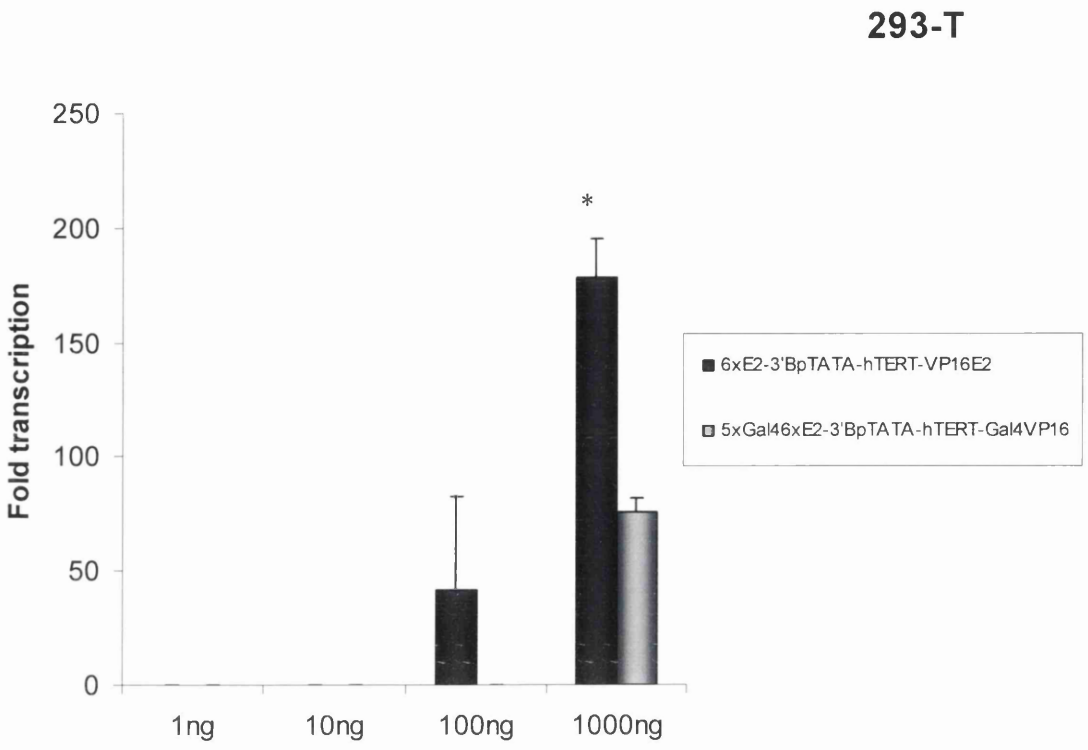



**Figure 3.1.22: VP16E2 is still better than Gal4-VP16 in one plasmid system.**

Cells were transfected with different amounts of 6xE2-3'BpTATA-hTERT-VP16E2 FOR or 5xGal46xE2-3'BpTATA-hTERT-Gal4VP16 FOR as indicated. Results are shown as fold increase in luciferase expression compared to sample transfected with 1000ng 5xGal46xE2-3'BpTATA only. Figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown. * = P-value <0.05

**Figure 3.1.23**

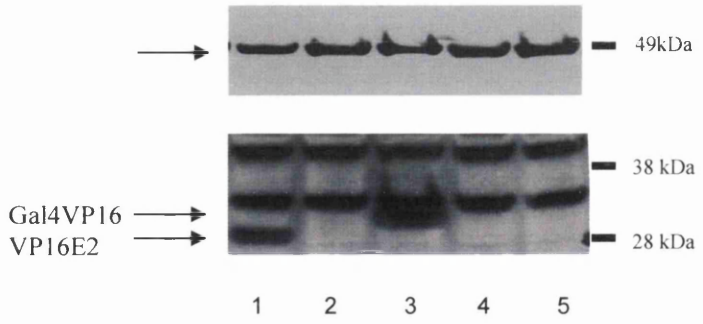



**Figure 3.1.23: Protein expression of transcriptional activators in one plasmid system.**

a) Gammatubulin loading control. b) Western blot showing the expression of the VP16E2 and the Gal4VP16 fusion proteins in 293-T cells, using antibody against VP16. Cell lysates were harvested as described. A non-specific band is seen in all lanes.

The location of the molecular weight marker (kD) is shown on the right of the blot. Arrows indicate location of protein.

1: 6xE2-3'BpTATA-hTERT-VP16E21000ng,

2: 6xE2-3'BpTATA-hTERT-VP16E2 100ng,

3: 5xGal46xE2-3'BpTATA-hTERT-Gal4VP16 1000ng,

4: 5xGal46xE2-3'BpTATA-hTERT-Gal4VP16 100ng,

5: Negative control

The amount in ng is referring to the amount of DNA transfected into the cells and not to the amount of protein loaded on the gel.

### 3.1.8 Applying the two step transcriptional amplification system to cell killing

To develop the two step amplification system towards cancer cell killing and therapeutic use the firefly luciferase gene was exchanged for the full length human TRAIL gene. TRAIL is described in the introduction (chapter 1.4.1.1).

#### 3.1.8.1 Incorporation of the TRAIL gene in the two step transcriptional amplification system.

In order to incorporate the human TRAIL gene into the one vector system the 6xE2-3'BpTATA plasmid was digested with Xba I and Hind III. The digested product the digest was run on a gel and the upper band was cut out and gel purified. This process removed the firefly luciferease gene from the plasmid. A TRAIL PCR product was made using the dogTERT-hTRAIL plasmid (see materials & methods) as a template and primers with a 5' Hind III and a 3' Xba I overhang. The PCR product was digested with the appropriate enzymes and ligated into the cut 6xE2-3'BpTATA plasmid and transformed into JM-109 cells. Correct inserts were confirmed by restriction digest and sequenced. The new plasmid was named 6xE2-3'BpTRAIL. Subsequently this hTERT-VP16E2 was inserted into the plasmid using the same method as in 3.1.7.1 in both forward and reverse directions. The resulting vectors were named 6xE2-3'BpTRAIL-hTERT-VP16 FOR and REV. In figure 3.1.24 the 6xE2-3'BpTRAIL-hTERT-VP16 FOR plasmid is schematically illustrated. As a positive control, a plasmid was cloned containing a CMV promoter driving transcription of the TRAIL gene. This plasmid was made using the TRAIL cloning method as above but inserting the TRAIL insert into the pDNA vector (see materials and methods). This plasmid was named CMV-hTRAIL.

#### 3.1.8.2 Identifying canine TRAIL.

For the comparative aspect of this study the canine TRAIL gene was cloned. A DNA sequence search was performed on the UCSC Genome Browser created by the Genome Bioinformatics Group of UC Santa Cruz. The human full length TRAIL sequence was copied, NCBI accenssion number NM_003810, and was compared with against the canine genome using a basic local alignment search tool. The search identified a sequence which was highly compatible with the human TRAIL sequence

located on the canine chromosome 34, spanning several thousand base pairs including introns. From this sequence primers were designed to isolate the canine TRAIL cDNA.

### 3.1.8.3 Cloning canine TRAIL (cTRAIL)

Normal circulating leukocytes such as neutrophils and monocytes express TRAIL (Ehrlich et al. 2003, Simons et al. 2008). Two canine blood samples in EDTA were therefore obtained from the University of Glasgow Small Animal Hospital. The blood samples were centrifuged and the buffy coat from each sample was isolated and pooled. RNA was isolated from the pooled buffy coat using Quiagen RNAqueous extraction kit. DNA was made by performing a reverse transcriptase reaction using DNA primers designed to bind to cTRAIL with the extracted RNA as a template. The resultant DNA was used as a template for a PCR reaction using the same DNA primers. Products from the PCR reaction were cloned into the TOPO cloning vector and clones were selected by blue-white screening. Inserts were confirmed by restriction digest and correctly sized inserts were sequenced to reveal the full length canine TRAIL cDNA. Several clones were sequenced and compared to the sequence found in the UCSC Genome Browser. The full length canine TRAIL cDNA as seen in figure 3.1.25 is 846 base pairs long and codes for 281 amino acids. An alignment of the translated canine and human TRAIL amino acid sequences is illustrated in figure 3.1.26 and shows 79% amino acid identity between species.
This indicates that the TRAIL gene is conserved between dogs and humans.

### 3.1.8.4 Cloning CMV-cTRAIL

In order to express the canine TRAIL gene in cells and examine its effects, a CMV-cTRAIL construct was cloned. The full length TRAIL was excised from the TOPO vector using the EcoR I restriction enzyme and was gel purified. The pDNA vector was cut with EcoR I and the cTRAIL insert was inserted by ligation. Clones were checked for inserts and insert orientation by restriction digest and sequencing.

**Figure 3.1.24**

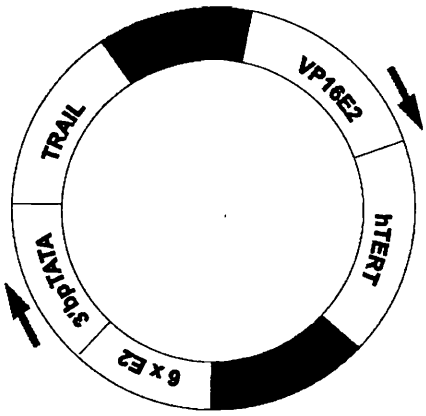



**Figure 3.1.24: Schematic presentation of 6xE2-3'BpTRAIL-hTERT-VP16 FOR**

**Figure 3.1.25**

ATGCAGGCCCCGGGGGGCCCCAGCCTCGGGCTGACGTGCGTGCTGATCCTCATCTTCACTGTGCTGCTCCAGTCCCTCT
GCGTGGCCGTCACCTACATGTACTTCACCAGGGAGCTGAAGCAGATGCAGGACAAGTACTCCCAAAGTGGCATCGCTTGT
TTCTTAAAGGAAGATGATATCCCCTGGGACCCCAGTGATGAAGAGAGTATGAACAACCCCTGCTGGCAAGTGAAGTGGCA
ACTCCGCCAGTTTGTTAGAAAGATGATTTTGAAAACCTATGAGGAAACCATTCCTACAGCTCCAGAAAAGCAGCTAAATATT
CCTTACGTAGTAAGCGACCGAGGTTCTCAGAGAGTAGCTGCTCACATAACTGGAACCAGTCGGAGAAGCATGTTTCCAATT
CCAAGCTCCAAGAATGATAAAGCTTTGGGCCACAAAATAAACTCCTGGGATTCCACAAGAAAAGGACATTCATTCTTGAATA
ATTTGCACTTGAGGAACGGAGAGCTGGTTATCCATCAAAGGGGGTTTTATTACATCTACTCCCAAACATACTTTCGATTTCA
GGAACCTGAGGAAATTCCAACAGGACAGAACAGAAAGAGAAACAAACAAATGGTCCAATATATTTACAAACACACGAGTTA
TCCGGACCCTATACTGCTGATGAAAAGTGCTAGAAATAGTTGTTGGTCTAAAGATTCTGAATATGGACTCTATTCCATCTAT
CAAGGTGGGATATTTGAGCTTAAGGAAAACGATAGAATTTTTGTCTCTGTATCTAACGAGCAATTGATTGACATGGACCAAG
AAGCCAGTTTTTTCGGGGCCTTTTTAATCGGCTAA

**Figure 3.1.25: The full length dog TRAIL cDNA**

cDNA sequence of c TRAIL from TOPO cloned constructs. Total length is 846 bp coding for 281 amino acids and a stop codon. Start and stop codons are denoted in red.

**Figure 3.1.26**

```
Identities = 225/283 (79%), Positives = 248/283 (87%), Gaps = 5/283 (1%)

CTRAIL   1    MQAPGGPSLGLTCVLILIFTVLLQSLCVAVTYMYFTRELKQMQDKYSQSGIACFLKEDDI  60
              M+  GGPSLG TCVLI+IFTVLLQSLCVAVTY+YFT ELKQMQDKYS+SGIACFLKEDD
hTRAIL   4    MEVQGGPSLGQTCVLIVIFTVLLQSLCVAVTYVYFTNELKQMQDKYSKSGIACFLKEDDS  63

CTRAIL   61   PWDPSDEESMNNPCWQVKWQLRQFVRKMILKTYEETIPTAPEKQLNIPYVVSDRGSQRVA  120
               WDP+DEESMN+PCWQVKWQLRQ VRKMIL+T EETI T  EKQ NI  +V +RG QRVA
hTRAIL   64   YWDPNDEESMNSPCWQVKWQLRQLVRKMILRTSEETISTVQEKQQNISPLVRERGPQRVA  123

CTRAIL   121  AHITGTSRRS-MFPIPSSKNDKALGHKINSWDSTRKGHSFLNNLHLRNGELVIHQRGFYY  179
              AHITGT  RS     P+SKN+KALG KINSW+S+R GHSFL+NLHLRNGELVIH++GFYY
hTRAIL   124  AHITGTRGRSNTLSSPNSKNEKALGRKINSWESSRSGHSFLSNLHLRNGELVIHEKGFYY  183

CTRAIL   180  IYSQTYFRFQEPEEIPTGQNRKRNKQMVQYIYKHTSYPDPILLMKSARNSCWSKDSEYGL  239
              IYSQTYFRFQE  +    +N K +KQMVQYIYK+TSYPDPILLMKSARNSCWSKD+EYGL
hTRAIL   184  IYSQTYFRFQEEIK----ENTKNDKQMVQYIYKYTSYPDPILLMKSARNSCWSKDAEYGL  239

CTRAIL   240  YSIYQGGIFELKENDRIFVSVSNEQLIDMDQEASFFGAFLIGX  282
              YSIYQGGIFELKENDRIFVSV+NE LIDMD EASFFGAFL+GX
hTRAIL   240  YSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVGX  282
```

**Figure 3.1.26: Alignment of cTRAIL and hTRAIL amino acid sequence.**

Alignment of the amino acid sequence of full length cTRAIL and full length hTRAIL. In red the shared sequence is shown. There was a 79% sequence identity found between species and 87% sequence homology.

### 3.1.8.5 Effect of canine and human TRAIL in human cells.

The ability of human and canine TRAIL to induce apoptosis in cells was detected by Western blots examining the effect of TRAIL on the activation of apoptosis mediators at different time points after transfection of cells.

293-T cells and U2OS cells were transfected, using calcium phosphate and Trans-It respectively, with 1μg CMV-cTRAIL, CMV-hTRAIL or pGL3-Basic. Cells were harvested at different times between 20 and 40 hours after transfection. Membranes were probed with antibodies recognising Caspase 3 or cleaved Caspase 3 and gammatubulin was identified as a loading control. In 293-T cells shown in figure 3.1.27 caspase cleavage is detected at all time points after transfection with both human and canine TRAIL. No cleaved caspase is detected in the pGL3-Basic transfected cells from the same experiment at any time point.

In the U2OS cells a similar patterns was seen and caspase 3 cleavage was observed in all CMV-cTRAIL3.1.28 b) and CMV-hTRAIL 3.1.28 d) transfected samples. No cleaved caspase was observed in cells transfected with pGL3-Basic 3.1.28 a) and b). It should be noted that the membrane in figure 3.1.28 d) was probed with a Caspase antibody which detects both cleaved and uncleaved caspase 3 and therefore a larger band of uncleaved caspase 3 is detected in all samples including the pGL3-Basic transfected samples.

This experiment indicates that both the human and dog TRAIL can induce apoptosis in human cells and that this effect is present as early as 20 hours post transfection.

### 3.1.8.6 Effect and expression of hTRAIL and cTRAIL in canine cells.

After testing the ability of the human and canine TRAIL to activate the caspase pathway in human cells, the same effect was investigated in canine cells. At this stage the one plasmid two step transcriptional amplification system cloned in 3.1.8.1 was included in the experiment. Canine telomerase positive MDCK cells were transfected using Trans-IT with 1μg CMV-hTRAIL, CMV-cTRAIL, 6xE2-3'BphTRAIL-hTERT-VP16E2 FOR, 6xE2-3'BpTRAIL or pGL3-Basic. Cells were harvested 40 hours after transfection and cell lystates were prepared for Western blot. Membranes were probed with antibodies recognising cleaved caspase 3, cleaved PARP, TRAIL and

gammatubulin as a loading control. The Western blot is shown in figure 3.1.29. It is seen that the human and canine TRAIL and the TRAIL incorporated in the two step amplification system are expressed in the canine cells and that the TRAIL antibody which is raised against human TRAIL cross reacts with canine TRAIL. It is also seen that the level of TRAIL expression from these 3 plasmids correlates with the degree of caspase 3 cleavage, reflecting the initiation of apoptosis. The CMV-hTRAIL and CMV-cTRAIL expression is also correlated with PARP cleavage which is another protein activated prior to apoptosis. There is no detection of caspase 3 cleavage, PARP cleavage or TRAIL in the sample from 6xE2-3'BphTRAIL transfected cells indicating that the 6xE2-3'BphTRAIL is inactive in absence of the VP16E2 fusion transcriptional activator protein. The 6xE2-3'BphTRAIL-hTERT-VP16E2 FOR is detected as a strong band with the TRAIL antibody however this is not reflected with strong expression of apoptosis markers. This diversity in expression between the TRAIL protein and the apoptosis markers in this sample could reflect the delay between the transcription of the TRAIL protein and its expression and effect on the cell surface as a trans-membrane protein. The gammatubulin loading control reflects that an equal amount of protein has been loaded in each well.

**Figure 3.1.27**

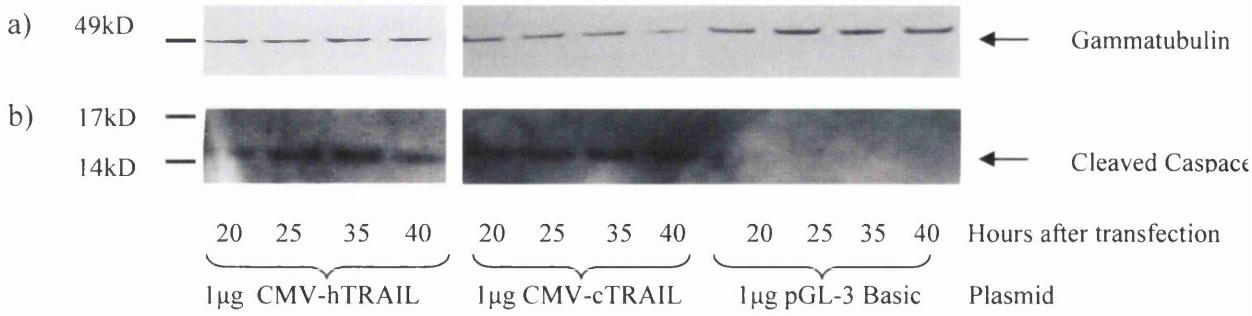



**Figure 3.1.27: Effect of TRAIL expression in 293-T cells at different time points after transfection detected by Western blot.**

Western blot showing Caspase 3 cleavage in 293-Tcells transfected with plasmids as indicated, and harvested at multiple time points after transfection. a) Membrane probed with antibody against gammatubulin as a loading control. b) Membrane probed with antibody against cleaved caspase 3. Arrows indicate the location of the protein of interest. Molecular weight markers are shown to the left.

**Figure 3.1.28**

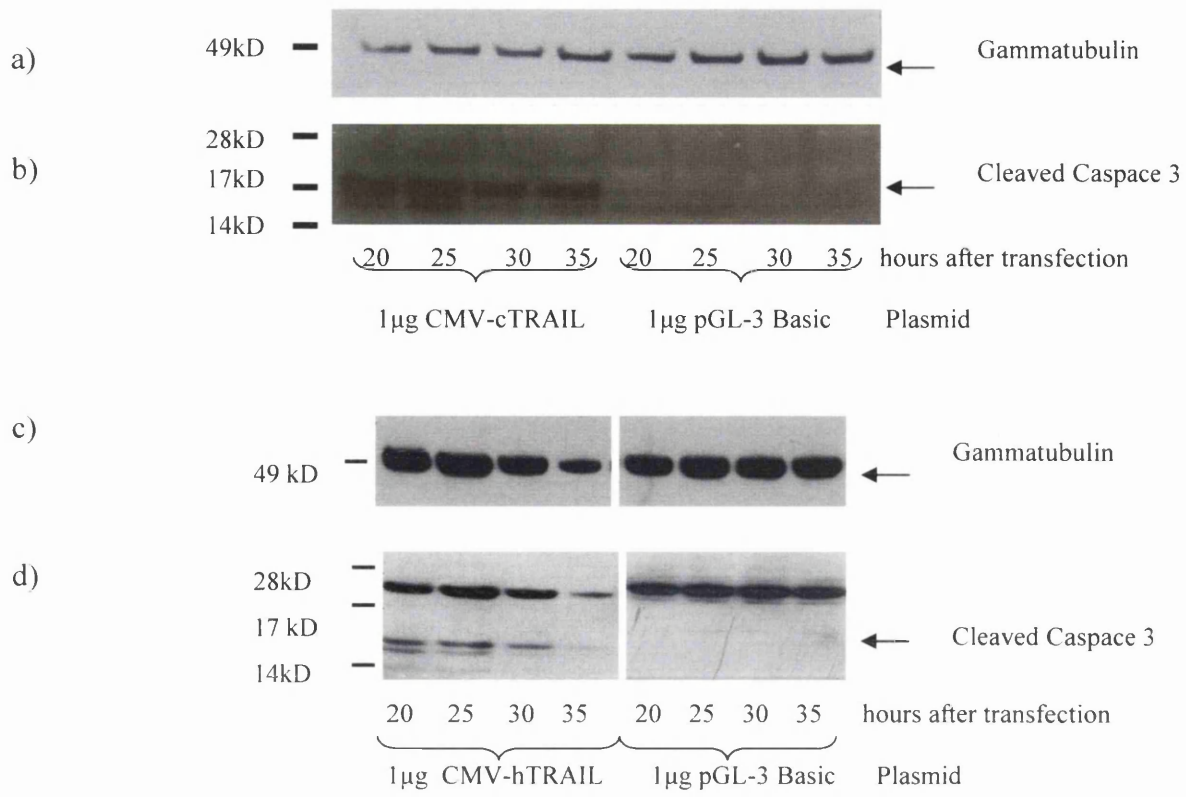



**Figure 3.1.28: CMV-TRAIL effect in human U2OS cells detected by Western blot with a cleaved caspase 3 antibody.**

Western blot showing caspase 3 cleavage in U2OS cells transfected with plasmids as indicated, and harvested at multiple time points after transfection. a) Membrane probed with antibody against gammatubulin as a loading control. b) Membrane probed with antibody against cleaved caspase 3. c) Membrane probed with antibody against gammatubulin as a loading control. d) Membrane probed with antibody against caspase 3. Arrows indicate the location of the protein of interest. Molecular weight markers shown to the left.

**Figure 3.1.29**

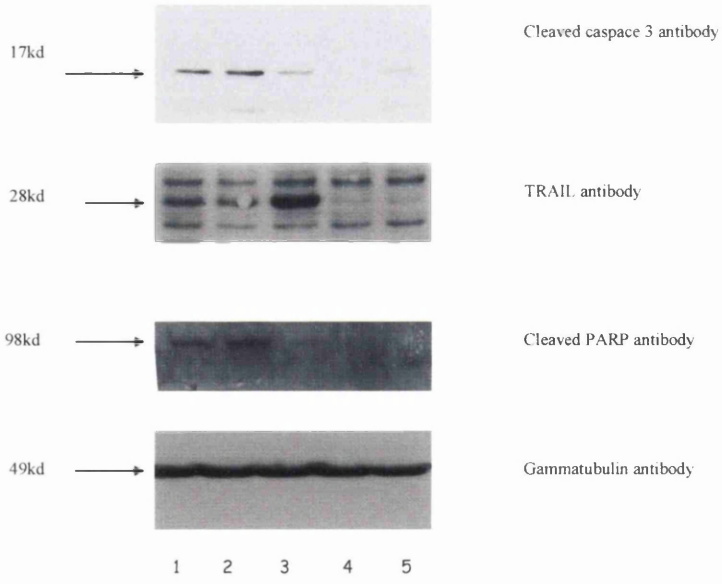



**Figure 3.1.29: TRAIL expression and effect in canine cells detected by Western blot.**

Western blot using antibodies against cleaved caspase 3, TRAIL, cleaved PARP and gammatubulin. MDCK cells were transfected and cells harvested 48 hours later. Molecular marker and arrows on left indicate the location of the protein of interest. 1=CMV-hTRAIL, 2=CMV-cTRAIL, 3= 6xE2-3'BphTRAIL-hTERT-VP16E2 FOR, 4= 6xE2-3'BphTRAIL , 5=pGL3-Basic.

### 3.1.8.7 Specific targeting of TRAIL induced apoptosis to telomerase positive cells detected by TUNEL stain.

With the incorporation of the hTERT promoter into the two step amplification system containing VP16E2 high levels of gene expression are detected in telomerase positive cells whilst not in telomerase negative cells as seen in figures 3.1.5-3.1.10. The TRAIL gene was introduced to the system, and to detect and quantify apoptosis in cells Transferase mediated dUTP Nick End Labelling (TUNEL) assays were performed in transfected telomerase positive and negative cells.

Telomerase positive 293-T and telomerase negative U2OS cells were grown on glass slides and transfected with 1μg CMV-hTRAIL, CMV-cTRAIL, 6xE2-3'BphTRAIL-hTERT-VP16E2 FOR, 6xE2-3'BphTRAIL or pGL3-Basic using calcium phosphate and Trans-IT respectively. 20 hours after transfection, cells were fixed with 4% formaldehyde in PBS and subsequently TUNEL stained. This stain incorporates fluorescein into the broken ends of apoptotic DNA strands. The 20 hour incubation period was chosen due to the early effect of TRAIL and the possibility that late apoptotic cells would float off the slide either before or during fixation. To visualise all cells, the slides were also counterstained with 4',6-diamidino-2-phenylindole (DAPI) stain. Cells were visualised using a fluorescent microscope and pictures from 5 random fields were taken of each slide using filters selective for emission of DAPI and fluorescein stained cells as seen in figure 3.1.30. The number of blue (DAPI) cells and green (fluorescein) cells were counted in each picture and a Yates corrected Chi square test were performed on the counted cells using the pGL3-Basic transfected cells as reference. Transfection and staining was performed 3 times in each cell line.

To determine the amount of apoptosis seen in cells transfected with the different plasmids we tested the null hypothesis.

**$H_0$: There is no difference in cell killing between the pGL3-Basic and the sample of interest.**

Tables 3.1.2 and 3.1.3 summarise the results from the 3 experiments in 293-T and U2OS cells respectively. In the 293-T cells the null hypothesis is confirmed for the samples transfected with 6xE2-3'BphTRAIL. The samples transfected with CMV-hTRAIL, CMV-cTRAIL and 6xE2-3'BphTRAIL-hTERT-VP16E2 all show a relatively

high number of apoptotic cells resulting in Yates corrected p values that discard the null hypothesis.

In the U2OS cells the null hypothesis is confirmed for the samples transfected with 6xE2-3'BpTRAIL and 6xE2-3'BpTRAIL-hTERT-VP16E2. The samples transfected with CMV-hTRAIL and CMV-cTRAIL show a relatively high number of apoptotic cells resulting in Yates corrected p values that discard the null hypothesis.

The results from the tables are summarised in figure 2.1.31 in which the proportion of apoptotic cells in samples is shown relative to the proportion in pGL3-Basic transfected cells which is set as one. The data demonstrate that the 6xE2-3'BpTRAIL-hTERT-VP16E2 specifically induces apoptosis in the telomerase positive cells but not in telomerase negative cells. Also, the 6xE2-3'BpTRAIL is not active in the absence of VP16E2. The ability of both the canine and human TRAIL to induce apoptosis in human cells is again confirmed.

TRAIL protein expression was confirmed in 293-T cells by Western blot. Cells were transfected with the same plasmids as used in the TUNEL stain and cells were harvested 24 hours post transfection. Membranes were probed with a TRAIL antibody and a gammatubulin antibody as a loading control. In figure 3.1.32 the Western blot confirms the TRAIL induced apoptosis pattern observed in the TUNEL stain.

A Western blot was performed in the same way on lysates from the U2OS cell line using the same plasmids and antibodies. The cells were harvested 48 hours after transfection and lysates were examined by Western blotting. The results confirm the TRAIL induced apoptosis observed with the TUNEL stain.

**Table 3.1.2**

| 293-T | | CMV-hTRAIL | CMV-cTRAIL | 6xE2-3'BphTRAIL | 6xE2-3'BphTRAIL-hTERT-VP16E2 |
|---|---|---|---|---|---|
| Exp. 1 | pGL3Basic | | | | |
| p-value | | = 0.0000 | =0.0000 | =0.9288 | =0.0000 |
| Yate's | | = 41.526 | =96.389 | =0.0008 | =209.56 |
| Live/Dead | 5792/24 | 4979/84 | 3551/103 | 5192/20 | 4013/201 |
| Exp. 2 | pGL3Basic | | | | |
| p-value | | =0.0000 | =0.0000 | =0.5363 | =0.0000 |
| Yate's | | =81.998 | =79.81 | =0.382 | =25.508 |
| Live/Dead | 4207/7 | 3513/88 | 4128/100 | 3500/9 | 4347/49 |
| Exp. 3-1 | pGL3Basic | | | | |
| p-value | | | =0.0000 | =0.1335 | |
| Yate's | | | =35.819 | =2.251 | |
| Live/Dead | 4301/10 | | 2761/42 | 4696/4 | |
| Exp. 3-2 | pGL3Basic | | | | |
| p-value | | =0.0000 | | | =0.0000 |
| Yate's | | =188.317 | | | =118.105 |
| Live/Dead | 8291/6 | 6655/170 | | | 7852/131 |

**Table 3.1.2: Cell counts and p-values from TUNEL assays in 293-T cells.**

Table shows number of cells counted and the calculated p-value. Numbers in blue indicate DAPI stained cells. Numbers in green indicate TUNEL stained cells.

**Table 3.1.3**

| **U2OS** | | CMV-hTRAIL | CMV-cTRAIL | 6xE2-3'BphTRAIL | 6xE2-3'BphTRAIL-hTERT-VP16E2 |
|---|---|---|---|---|---|
| Exp 1 | pGL3Basic | | | | |
| p-value | | =0.0000 | | =0.0135 | =0.0670 |
| Yate's | | =83.008 | | =6.1 | =3.364 |
| Live/Dead | 1639/9 | 1012/71 | | 1436/0 | 1277/1 |
| Exp 2 | pGL3Basic | | | | |
| p-value | | =0.0000 | =0.0000 | =0.5522 | =0.9819 |
| Yate's | | =143.661 | =57.462 | =0.353 | =0.001 |
| Live/Dead | 3556/2 | 1771/79 | 3009/56 | 2101/3 | 3651/1 |
| Exp 3 | pGL3Basic | | | | |
| p-value | | =0.0000 | =0.0000 | =0.014 | =0.4599 |
| Yate's | | =434.281 | =97.671 | =0.9068 | =0.546 |
| Live/Dead | 3707/4 | 2451/322 | 2413/77 | 4009/4 | 3346/8 |
| Exp 4 | pGL3Basic | | | | |
| p-value | | | =0.0000 | | |
| Yate's | | | =16.924 | | |
| Live/Dead | 3187/5 | | 2996/29 | | |

**Table 3.1.3: Cell counts and p-values from TUNEL assays in U2OS cells.**

Table shows number of cells counted and the calculated p-value. Numbers in blue indicate DAPI stained cells. Numbers in green indicate TUNEL stained cells.

Figure 3.1.30

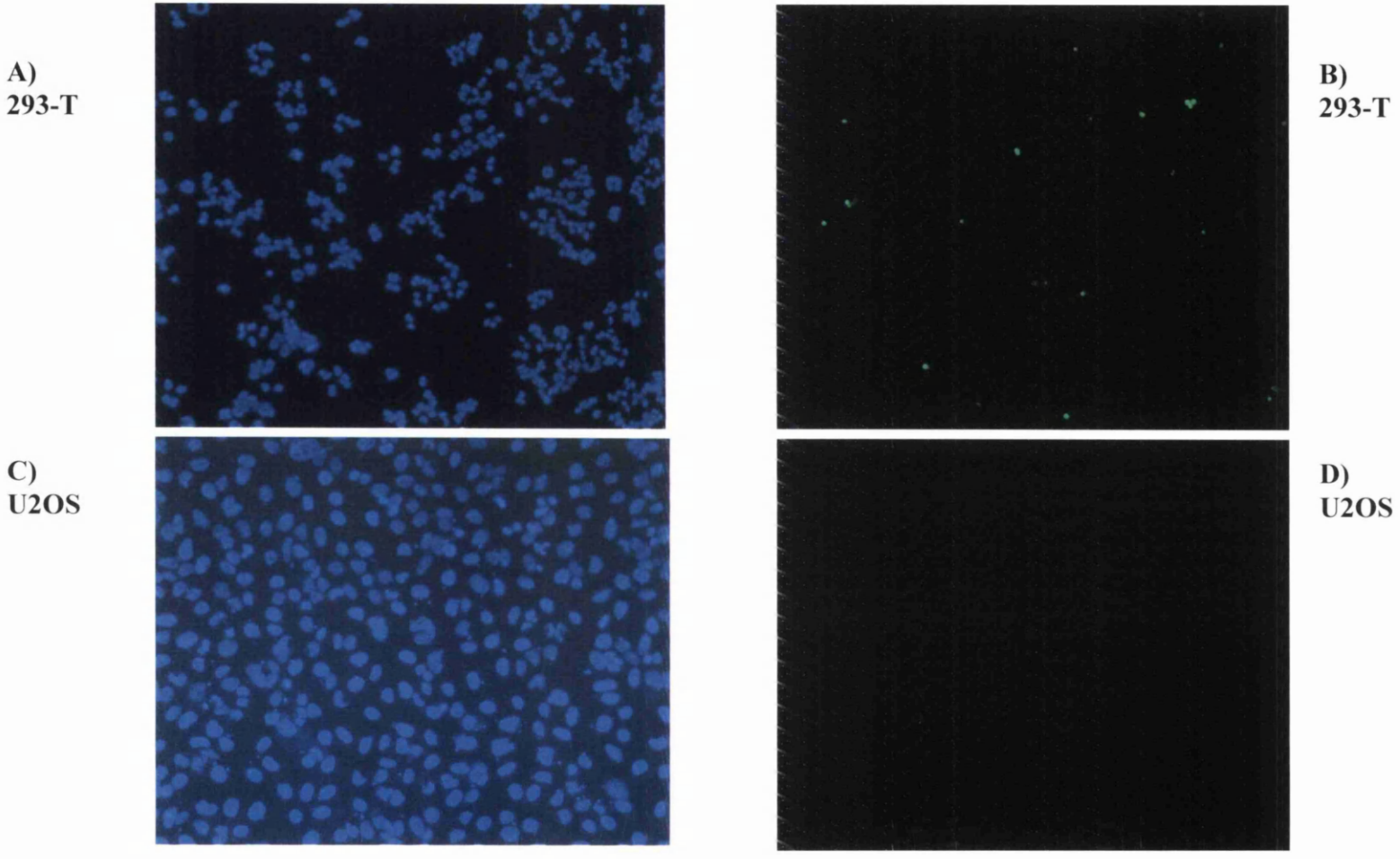


**Figure 3.1.30: DAPI and TUNEL stained cells.**

Pictures showing cells visualised by fluorescence microscope using filters selective for DAPI (panels A and C) and TUNEL (panels B and D) stains.

293-T cells panels A) and B). U2OS cells panels C) and D). Cells transfected with 1μg 6xE2-3'BphTRAIL-hTERT-VP16E2.

**Figure 3.1.31**

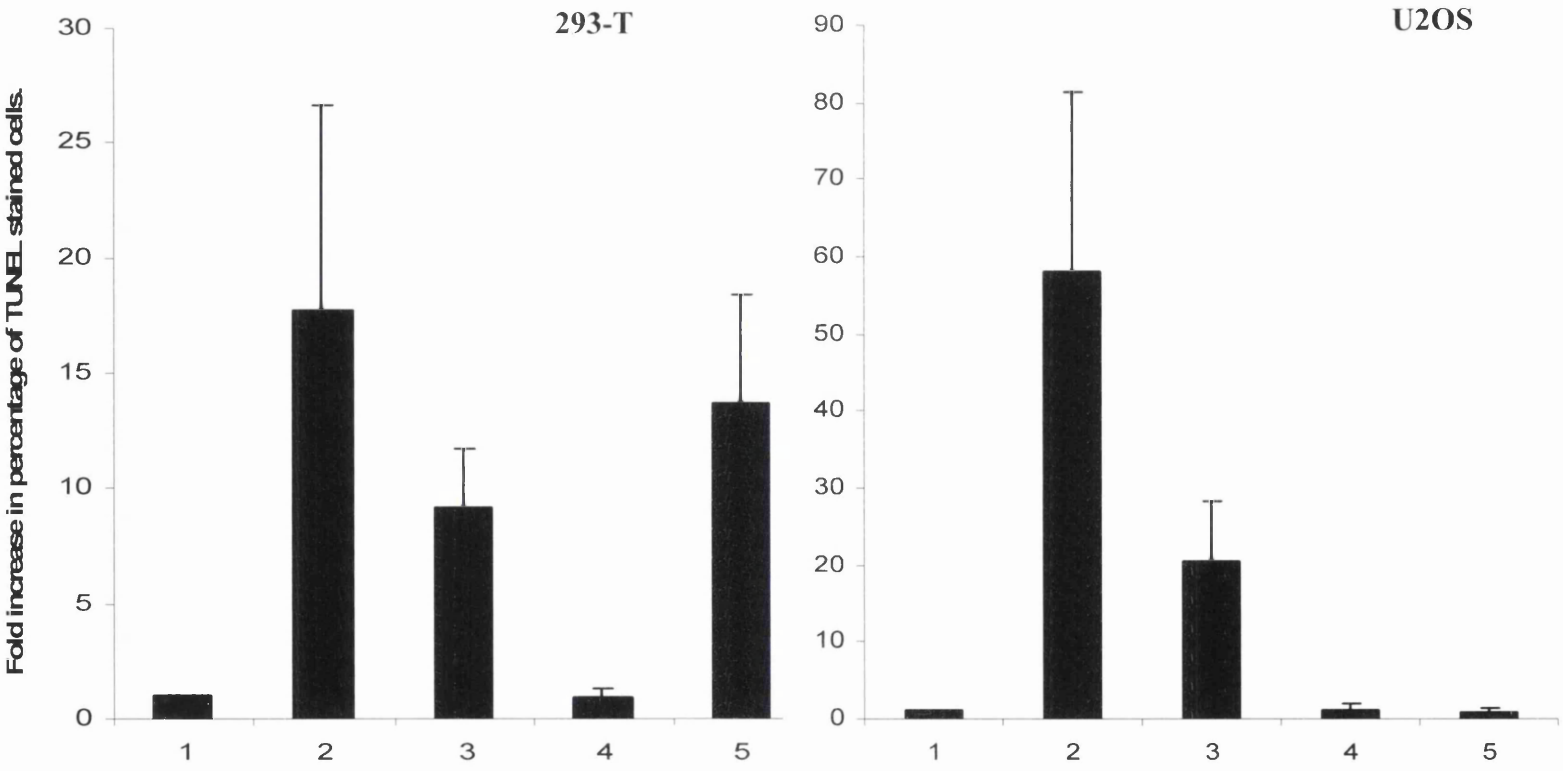



**Figure 3.1.31: Summary of TUNEL stain.**

Figure represents summary of TUNEL assays, derived from data presented in tables 3.1.2 and 3.1.3. For each experiment the percentage of blue cells which were fluorescent was calculated. Bars represent fold increase in percentage of TUNEL stained cells compared to cells transfected with 1μg pGL3-Basic. Standard error bars for the 3 experiments are shown. 1=pGL3-Basic, 2=CMV-hTRAIL, 3=CMV-cTRAIL, 4= 6xE2-3'BphTRAIL, 5= 6xE2-3'BphTRAIL-hTERT-VP16E2.

**Figure 3.1.32**

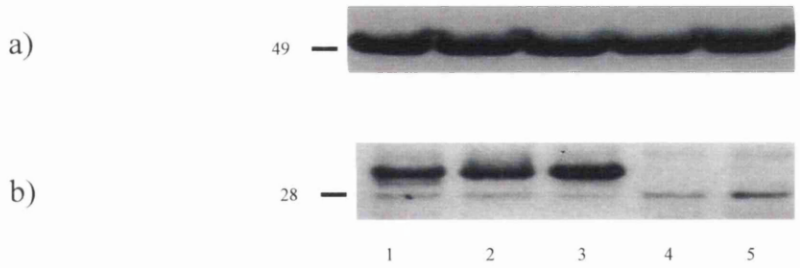



**Figure 3.1.32: TRAIL expression in telomerase positive human 293-T cells detected by Western blot.**

Western blot showing the expression of TRAIL in 293-T cells 24 hours after transfection. a) Membrane probed with gammatubulin antibody as loading control. b) Membrane probed with anti-TRAIL antibody. Molecular weight marker seen on left. 1=CMV-hTRAIL, 2=CMV-cTRAIL, 3= 6xE2-3'BphTRAIL-hTERT-VP16E2, 4= 6xE2-3'BphTRAIL, 5=pGL3-Basic.

**Figure 3.1.33**

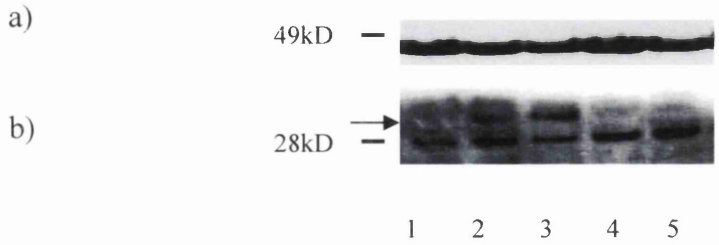



**Figure3.1.33: TRAIL expression in telomerase negative human U2OS cells detected by Western blot.**

Western blot showing the expression of TRAIL in U2OS cells 48 hours after transfection. a) Membrane probed with gammatubulin antibody as loading control. b) Membrane probed with anti-TRAIL antibody. Arrow marks the location of the TRAIL protein. Molecular weight marker seen on left.

1=pGL3-Basic, 2=CMV-hTRAIL, 3=CMV-cTRAIL, 4= 6xE2-3'BphTRAIL-hTERT-VP16E2, 5= 6xE2-3'BphTRAIL.

### 3.1.9 Improving the two step amplification system

Having shown that the VP16E2 is more active transcriptional activator than the Gal4-VP16E2 and that the hTERT promoter allows for higher specificity than the hTR promoter and that these elements combined with the TRAIL gene are able to specifically induce apoptosis in telomerase positive whilst not in telomerase negative cells it was sought to attempt other improvements to the TSTA system to maximise its efficiency.

#### 3.1.9.1 Allogeneic versus xenogeneic TERT promoter.

In chapter 3.1.4 it was seen that the human telomerase promoters are very active in canine telomerase positive cells. Studies have been done (Horikawa et al. 2005) comparing the activity of the mouse and the human TERT promoter in mouse and human cell lines. From these studies it was revealed that the activity of the mouse TERT promoter is not reflected by telomerase activity in the cells due to the lack of a CCCCGCCC sequence upstream of the TERT transcriptional start site. This specific sequence is responsible for down regulating TERT promoter activity in telomerase negative cell lines. It was also shown that by introducing this specific sequence into the mouse promoter the promoter could be tightly regulated in relation to telomerase activity. The mouse TERT promoter was shown to be a stronger promoter than the human TERT promoter in human cells also after introduction of the regulatory sequence (Horikawa et al. 2005). This could suggest that the use of xenogeneic promoters for gene therapy is an advantage and it was therefore investigated if there was a difference between the activity of the core human and canine TERT promoters in human and canine cells. An alignment of the promoter sequence upstream of the TERT transcription start site of the canine and human TERT promoters revealed that the TERT repressive element is conserved in the canine species as shown in figure 3.1.34.

To compare the basic transcriptional activity of the human and canine core telomerase promoters, transcription assays were performed using plasmids containing a human or canine telomerase promoter, responsible for transcription of a firefly luciferase gene. The human 536 base pair core hTERT (hTERT-LUC 536bp) promoter was used and compared with the 314 base pair (dogTERT-LUC 314bp) and the 665 base pair (dogTERT-LUC 665bp) canine core TERT promoters (Long et al. 2005) as

shown schematically in figure 3.1.35. Transcription assays were carried out in human telomerase positive 293-T cells and canine telomerase positive MDCK cells. Cells were transfected with titrations of hTERT-LUC 536 bp, dogTERT-LUC 665 bp or dogTERT-LUC 314 bp. Calcium phosphate was used as a transfection agent for the 293-T cells and the MDCK cells were transfected with Trans-IT. Transcription assays were performed 3 times in duplicate and all results were normalised against protein present in each sample. Results are shown as fold increase in transcription compared to cells transfected with 1μg pGL3-Basic reporter vector on its own.

As seen in figure 3.1.36 all promoters are transcriptionally active in both cell types. The dogTERT-LUC 665 bp promoter is very active compared to the hTERT-LUC 536 bp promoter at 1000ng concentration in the human cell line 3.1.36 a) this effect is though diminished when taking the error bars into account and the effect is lost at the 2000ng plasmid concentration. A similar trend is evident in the canine cell line 3.1.36 b) however, as previously when error bars are taken into account, the effect is diminished and the effect is lost at 2000ng. The experiment shows that the TERT promoters are active at the same level in cells from other species but does not show a direct advantage of using a xenogeneic promoter.

**Figure 3.1.34**

CCCTCCCCCGGCCCGCCCCTTCCTC-CCGCGGA**CCCCGCCC**-CTCCCCG—GC -1 dog TERT

CCCTCCCA-GCCCC TCCCCTTCCTTTCCGCGG- **CCCCGCCC**TCTCCTCGCGGC -11 hTERT

**Figure 3.1.34: Alignment of canine and human TERT promoter repressive element.**

Alignment of the human and canine TERT promoter upstream of the ATG start site for the TERT gene. Alignment shows conservation of the CCCCGCCC TERT repressive element in both species.

**Figure 3.1.35**

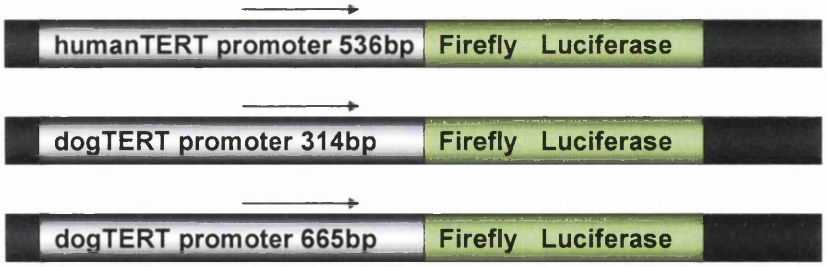
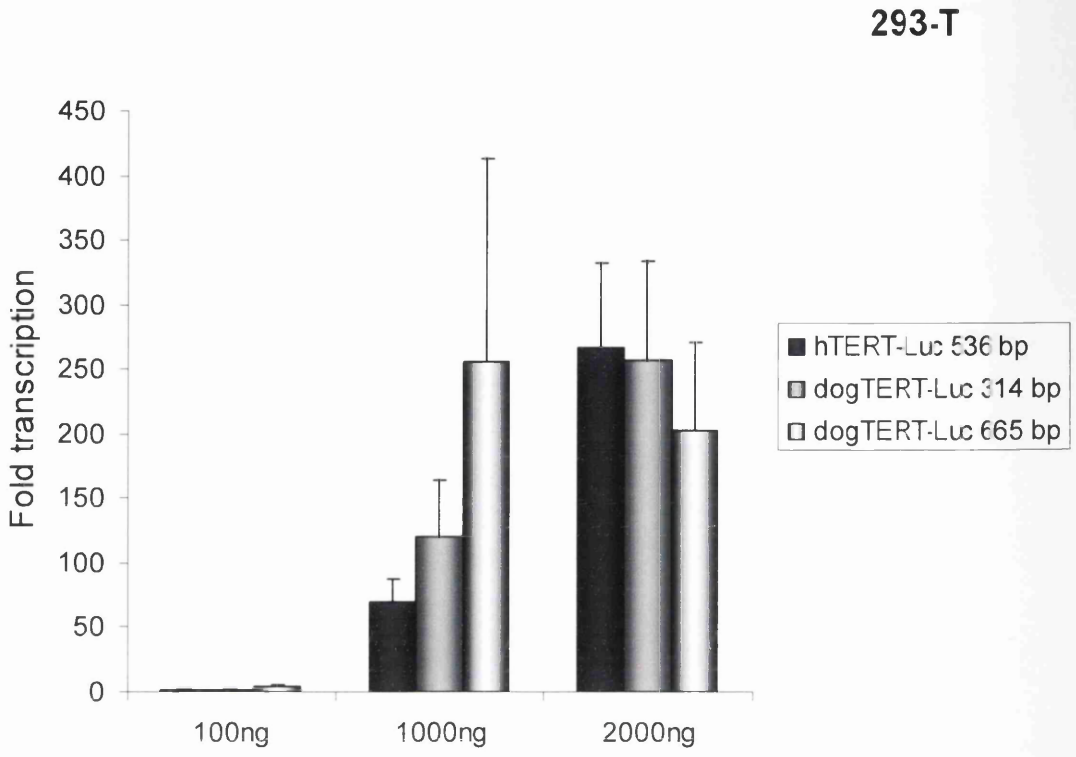



**Figure 3.1.35: Schematic model of human and canine telomerase plasmids**

Figure represents schematic models of the human and the two canine telomerase promoter plasmids.

**Figure 3.1.36**

a)




b)

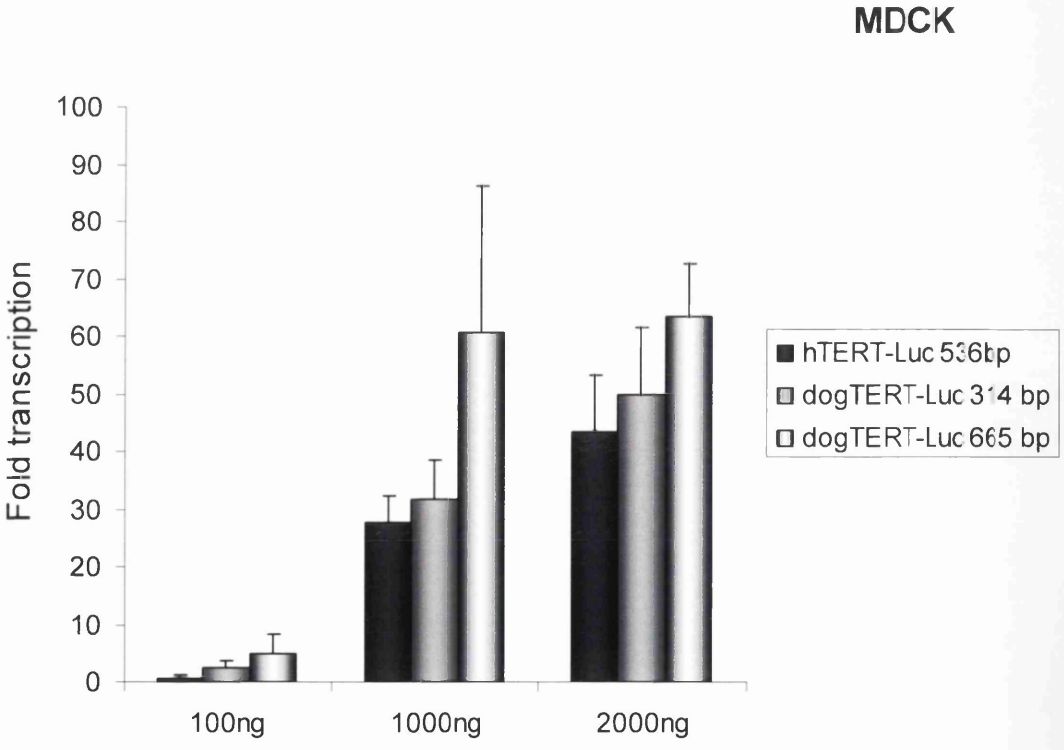


**Figure 3.1.36: Transcriptional activity of human and canine telomerase promoters in human and canine cells.**

a)293-T cells, b)MDCK cells. Cells were transfected with different amounts of hTERT-Luc 536 bp, dogTERT-Luc 314 bp or dogTERT-Luc665 bp as indicated.

Bars represent fold increase in luciferase expression compared to cells transfected with 1000ng pGL-3 Basic plasmid alone. Figures represents summary of 3 experiments carried out in duplicate. Standard error bars are shown.

### 3.1.9.2 Introducing an extra VP16 sequence to the two step amplification system.

An increase in the number of VP16 coding sequences has been shown to increase transcriptional activity significantly in conjunction with the Gal4VP16 transcriptional activator fusion protein. It was shown that a VP16 tandem construct initiated a higher level of transcriptional activity than the single construct however by further increasing the number of VP16 sequences from two to four a reduction in transcriptional activity was seen due to squelching (Zhang et al. 2002). To measure this effect in conjunction with the VP16E2, an extra 78 amino acid VP16 element was cloned upstream from the VP16E2. A schematic representation of the cloning process is illustrated in figure 3.1.37. A 5' VP16 primer was made with a 18 base pair tail coding for the last 18 base pairs of the VP16 sequence. Likewise a 3' primer was made with a tail coding for the 18 first base pairs of the VP16 sequence. A PCR reaction was set up with the tailed 5' primer and a 3' E2 primer with a Xba I tail using the pCG-VP16E2 as template. A second reaction was set up with the 3' tailed primer and a 5' VP16 primer with a Hind III tail using the pCG-VP16E2 as template. Following this a third PCR reaction was set up using the 5' VP16 Hind III tailed primer and the 3' E2 Xba I tailed primer using 1μl of the product from the first and the second PCR mixed as template. The resulting PCR product contained two VP16 sequences and was digested and cloned as previously described into the pGL3hTP19 plasmid containing the TERT promoter as described in section 3.1.2. The resulting plasmid was named hTERT-VP16VP16E2 and is schematically presented in figure 3.1.38.

In order to determine if there was any increase in transcriptional activity with incorporation of an extra VP16 sequence, transcription assays were performed in 293-T cells. Cells were transfected using calcium phosphate with titrated amounts of either hTERT-VP16E2 or hTERT-VP16VP16E2. All cells were co-transfected with 1μg of the 6xE2-3'BpTATA reporter vector. Transcription assays were performed 3 times in duplicate and all results were normalised against protein present in each sample. Results are shown as fold increase in transcription compared to cells transfected with 1μg 6xE2-3'BpTATA only.

The results from the transcription assays are summarised in figure 3.1.39. The hTERT-VP16VP16E2 does show a higher level of transcriptional activity than the hTERT-VP16E2. This effect however is diminished when taking the error bars into account. The small increase in transcriptional activity must also be taken into account of

the additional size of the protein which could be a disadvantage in space limited gene delivery vectors.

**Figure 3.1.37**

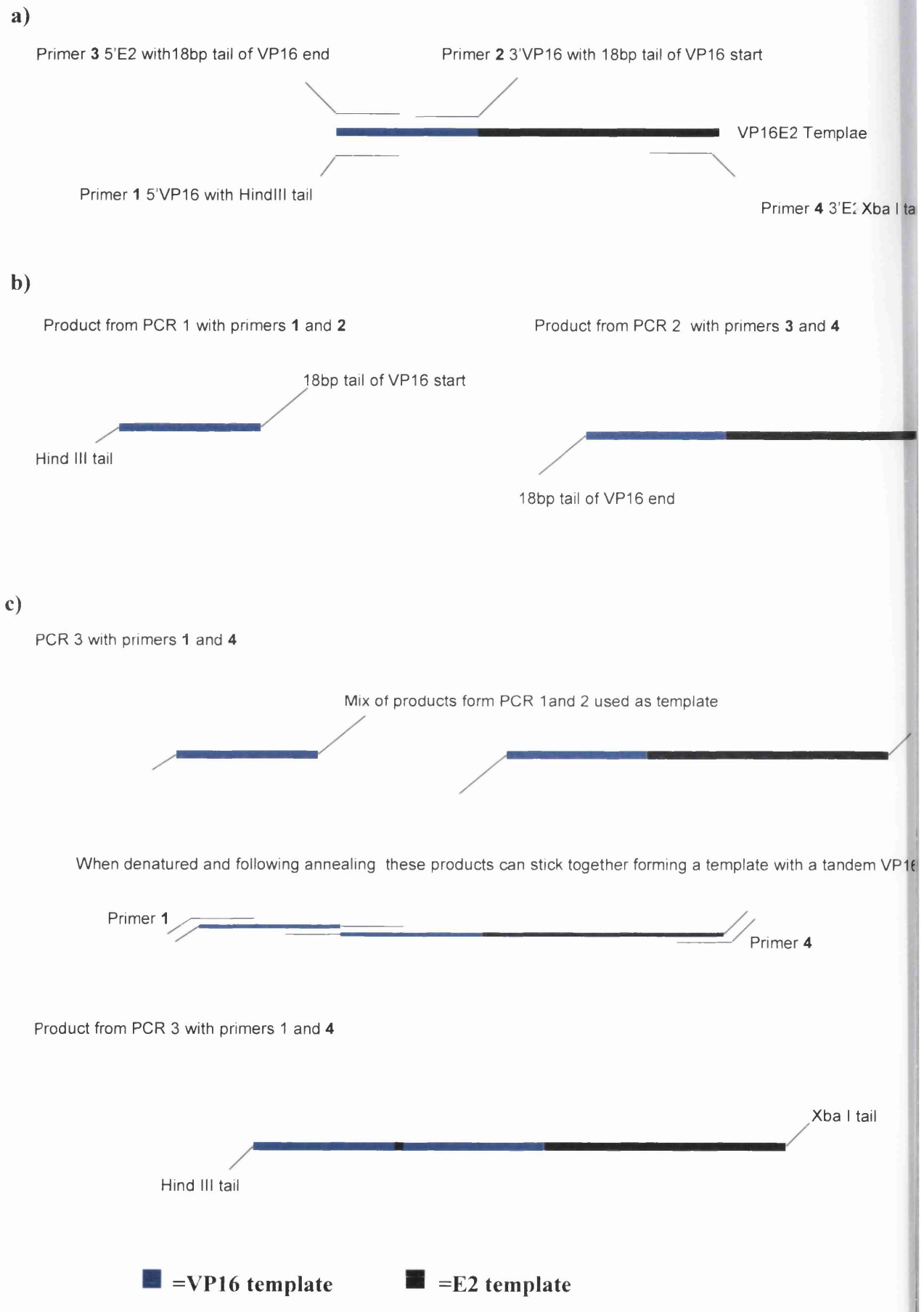

a)
Primer 3 5'E2 with18bp tail of VP16 end
Primer 2 3'VP16 with 18bp tail of VP16 start
VP16E2 Templae
Primer 1 5'VP16 with HindIII tail
Primer 4 3'E2 Xba I ta
b)
Product from PCR 1 with primers 1 and 2
Product from PCR 2 with primers 3 and 4
18bp tail of VP16 start
Hind III tail
18bp tail of VP16 end
c)
PCR 3 with primers 1 and 4
Mix of products form PCR 1and 2 used as template
When denatured and following annealing these products can stick together forming a template with a tandem VP16
Primer 1
Primer 4
Product from PCR 3 with primers 1 and 4
Xba I tail
Hind III tail
=VP16 template
=E2 template

**Figure 3.1.37: Cloning of a tandem VP16 construct.**

a) Model of template and primer binding sites. b) Products from PCR 1 and 2 using primer sets 1+2 and 3+4 respectively. c) Schematic representation of template and products from PCR reaction 3. A mix of the products from PCR 1 and 2 is used as template. When these are mixed they form a tandem VP16 template which when used in reaction with primers 1 and 4 allows for the production of a tandem VP16 PCR product. The resulting tandem VP16 product can be cloned like a normal PCR product.

**Figure 3.1.38**


**Figure 3.1.38: hTERT expression vector with one or two VP16 sequences**

Schematic models of hTERT driven VP16E2 expression plasmids containing either one or two VP16 coding sequences fused to one E2 coding sequence.

**Figure 3.1.39**

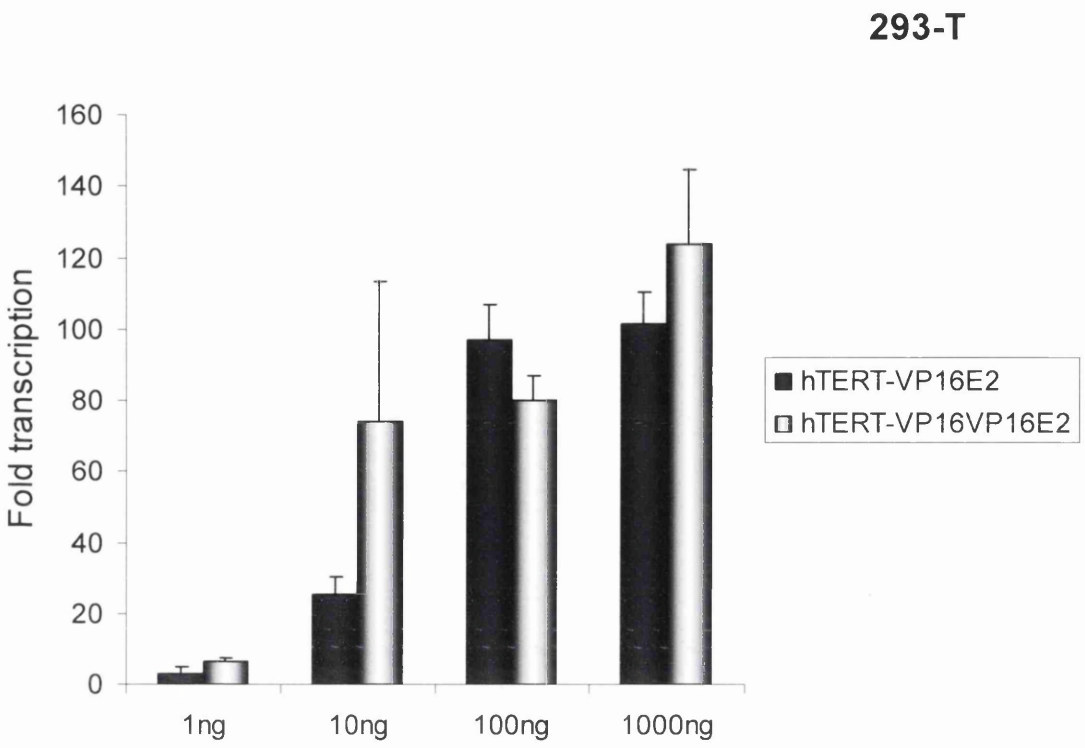



**Figure 3.1.39: Effect of one versus two VP16 coding sequences.**

293-T cells were transfected with different amounts of either hTERT-VP16E2 or hTERT-VP16VP16E2. Cells were co-transfected with 1000ng 6xE2-3'BpTATA reporter plasmid. Results are presented as fold increase in luciferase expression relative to cells transfected with 1000ng 6xE2-3'BpTATA reporter plasmid alone. Figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown.

### 3.1.9.3 The effect of increasing the number of E2 sites.

The effect of increasing the number of E2 binding sites to increase transcriptional activation of the BPV-4 promoter has been studied in detail (Vance et al. 1999). Here it was shown that increasing the number of E2 binding sites increased the transcriptional activity proportionally. It was therefore sought to determine if this effect would be seen in conjunction with the hTERT-VP16E2 two step amplification system. The PV2 x4E2, PV2 x8E2 and PV2 x12E2 plasmids were previously made (Vance et al. 1999). These plasmids contain a mutated minimal promoter derived from the BPV-4 promoter responsible for transcription of a firefly luciferase reporter gene and have 4, 8 and 12 upstream E2 binding sites as illustrated in figure 3.1.40. To determine the effect of the E2 binding sites, transcription assays were performed in 293-T cells. Cells were transfected using calcium phosphate with serial dilutions of PV2 x4E2, PV2 x8E2 or PV2 x12E2. In conjunction all cells were co-transfected with 1μg hTERT-VP16E2. Transcription assays were performed 3 times in duplicate and all results were normalised against protein present in each sample. Results are shown as fold increase in transcription compared to cells transfected with 1μg pGL3-Basic reporter vector on its own. Results from the transcription assays are shown in figure 3.1.41. As seen in the figure, no increase in transcriptional activity is seen in relation to increase in E2 binding sites. The extra E2 binding sites in fact result in a slight decrease in transcription. The BPV 4 promoter has been shown to be more responsive in epithelial cell lines and it was therefore investigated if a response to an increase in E2 binding sites could be elicited in an epithelial cell line. The above experiment was therefore repeated in the C33a cell line which is a keratinised epithelial cell line. The result from the experiment is shown in 3.1.42. It is seen that there is a correlation between increase in transcriptional activity and increasing the number of E2 binding sites. This indicates that the VP16E2 system could be specifically suitable for targeting gene expression in epithelial cells due to the origin of the 6xE2-3'bpTATA promoter from an epitheliotrophic virus.

**Figure 3.1.40**

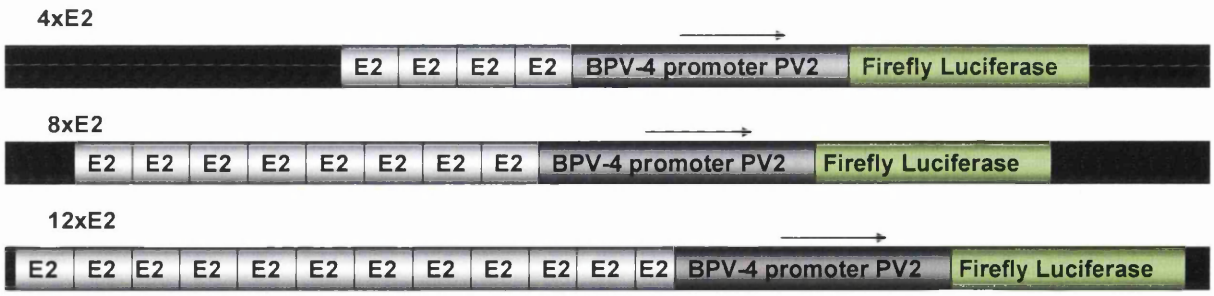



**Figure 3.1.40: E2 responsive reporter plasmids.**

Schematic model of E2 responsive reporter plasmids with 4, 8, and 12 E2 sites. Plasmids contain the BPV-4 promoter PV2 upstream of a firefly luciferase gene.

**Figure 3.1.41**

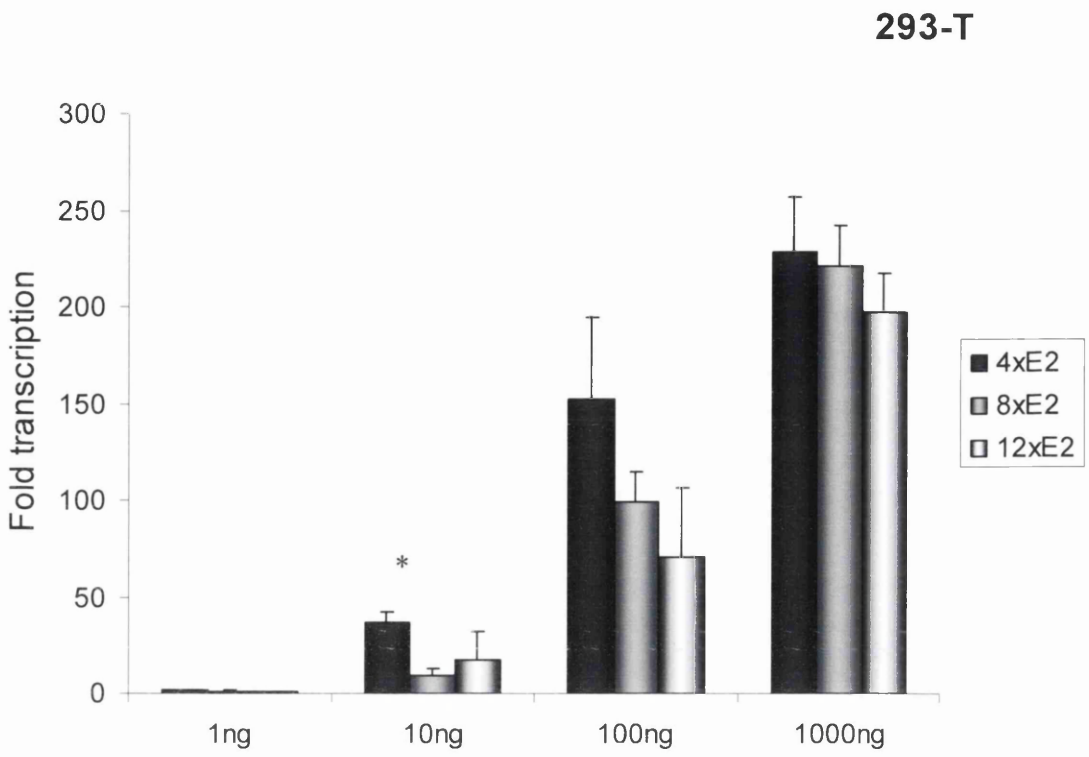



**Figure 3.1.41: Increase in E2 sites has no effect in 293-T cells.**

293-T cells were transfected with different amounts of either 4xE2, 8xE2 or 12xE2 reporter vector as indicated. In addition 1000ng hTERT-VP16E2 expression vector was co-transfected into the cells. Each bar represents fold increase in luciferase expression compared to cells transfected with 1000ng pGL3-Basic alone. Figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown. *= P-value < 0.05 when comparing 4xE2 with 8xE2.

**Figure 3.1.42**

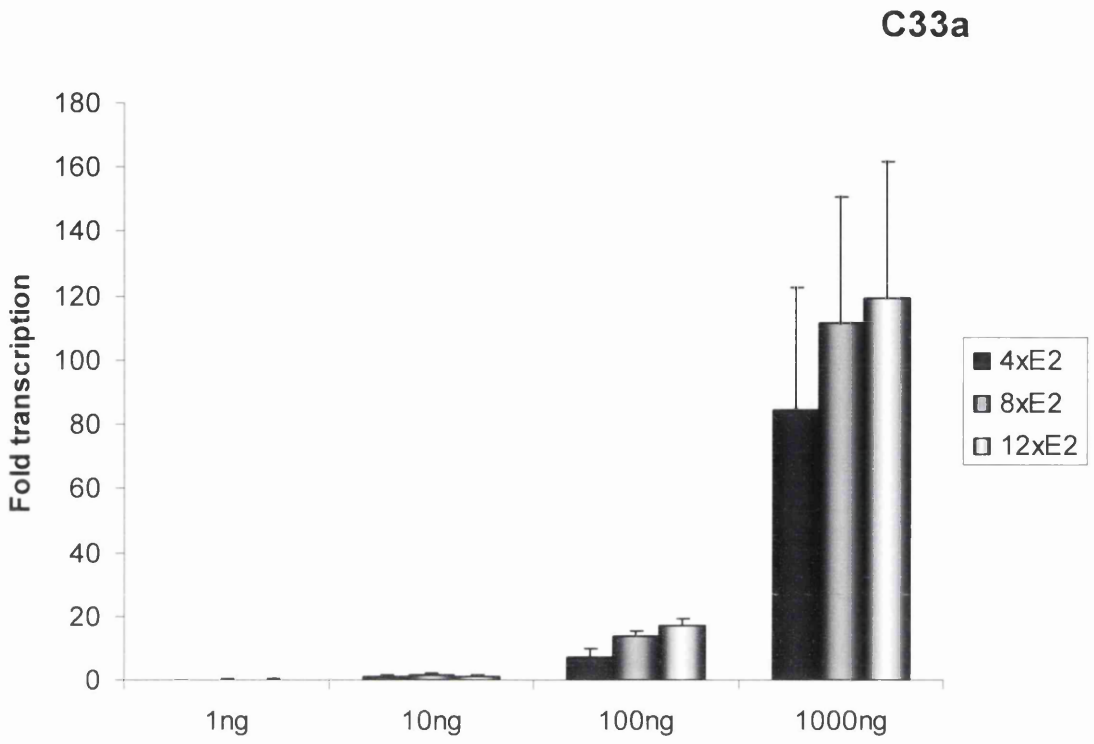



**Figure 3.1.42: Increase in E2 sites has no effect in C33a cells.**

C33a cells were transfected with different amounts of either PV2-4xE2, PV2-8xE2 or PV2-12xE2 reporter vector as indicated. In addition 1000ng hTERT-VP16E2 expression vector was co-transfected into the cells. Each bar represents fold increase in luciferase expression compared to cells transfected with 1000ng pGL3-Basic alone. Figure represents summary of 3 experiments carried out in duplicate. Standard error bars are shown.

### 3.1.9.4 Stabilising E2 by mutation of serine residue 301.

It was shown that mutation of the bovine papilloma virus E2 protein serine residue 301 phophorylation site allowed the virus to replicate at a higher copy number than the wild type virus (McBride & Howley 1991). It was therefore anticipated that such a mutation introduced to the VP16E2 fusion protein would create a more stable protein. The serine 301 in the E2 of the hTERT-VP16E2 plasmid was therefore mutated to an alanine. This was done by site directed mutagenesis (see materials & methods). Mutations were confirmed by sequencing and the mutated plasmid named hTERT-VP16E2 Mut 301. Western blots were then performed in order to look at the stability of the protein. 293-T cells were transfected with either 1μg or 0.1μg hTERT-VP16E2 or hTERT-VP16E2 Mut 301. Normal protocol for western blot was carried out and the membrane was probed with an antibody against VP16 and gammatubulin as a loading control. As seen in figure 3.1.43 an increase in protein is detected in the hTERT-VP16E2 Mut 301 samples. This is especially noticeable in the 0.1μg concentration in which no protein is detected in the hTERT-VP16E2 sample while a clear band is observed in the hTERT-VP16E2 Mut 301 sample. The gammatubulin loading control reflects the amount of protein loaded and indicates that less protein has been loaded in the 1μg hTERT-VP16E2 Mut 301 lane making the effect even more pronounced.

**Figure 3.1.43**

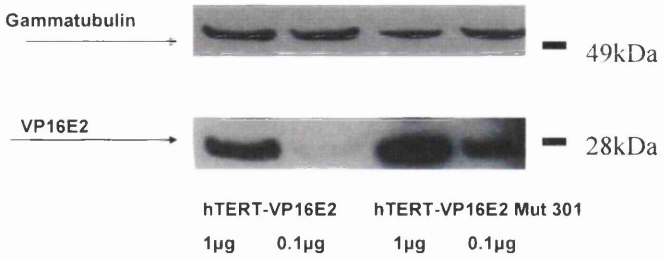



**Figure 3.1.43 Protein expression of VP16E2 and VP16E2 Mut 301.**

Western blot showing the expression of the VP16E2 and the VP16E2 Mut 301 both under transcriptional control by the hTERT promoter in 293-T cells. Membrane probed with antibody against VP16. Gammatubulin antibody used as loading control. Molecular weight marker shown to the right.

### 3.2 Applying the system to a viral system.

The results in chapter 3.1 demonstrate that the two step amplification system in conjunction with the VP16E2 and the hTERT promoter provides a potential means of targeting a strong level of transgene expression to telomerase expressing cells. A major obstacle in cancer gene therapy is finding a reliable *in vivo* transgene delivery mechanism. In order to overcome this obstacle and target cell killing to cancer cells, a conditionally replicative cancer specific virus based on the adenovirus CAV-1 was constructed. The rationale of utilising such a virus was to exhibit tumour selective viral replication and hence cell killing.

To restrict viral replication to cancer cells the two step amplification system in conjunction with the hTERT promoter was incorporated into the virus. To accomplish this, a virus was designed in which the non-essential viral E3 region and the essential E1 promoter region were eliminated. In place of the E3 region the hTERT-VP16E2 was inserted and in place of the E1 promoter the 3'BpTATA with its 6 upstream E2 binding sites was inserted. This rendered viral E1 gene expression dependent on activation of the 6xE2-3'BpTATA promoter by the VP16E2 fusion protein transcribed by the telomerase promoter. The structure of the engineered virus genome and its predicted function is depicted in figure 3.2.1.

The virus construct used as a starting point for the cloning was the fΔE1ΔE3 CAV-1 cosmid. This is a CAV-1 DNA sequence which has been cloned into a cosmid. The full length viral E1 and E3 gene has been removed from this construct such as seen schematically in figure 3.2.1 a).

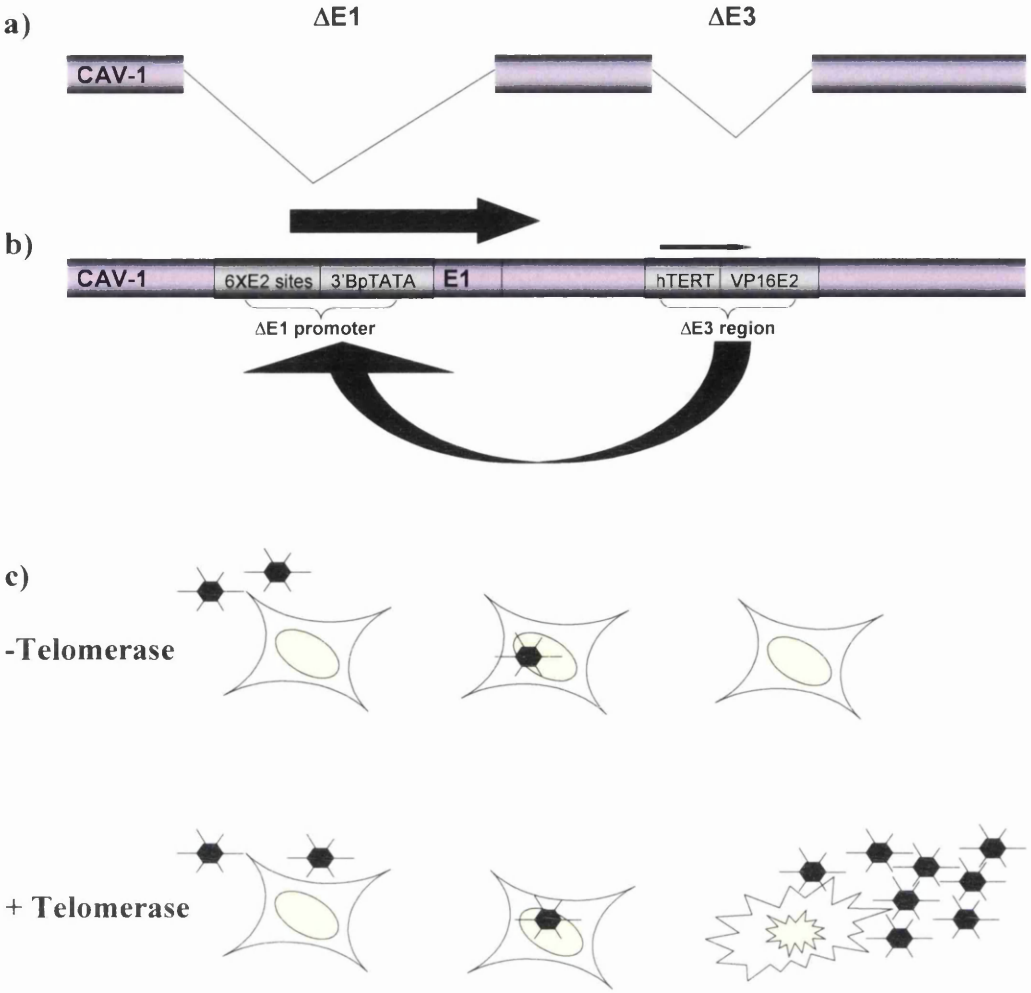



**Figure 3.2.1 Concept of the conditionally replicative canine adenovirus**

a) Schematic representation of the fΔE1ΔE3 CAV-1 cosmid. b) Schematic representation of the two step amplification mechanism in its basic form incorporated into the fΔE1ΔE3 CAV-1 in order to create a conditionally replicative CAV-1 virus. The hTERT promoter transcribes the VP16E2 fusion protein which binds to the E2 binding sites upstream of the minimal promoter. This promoter when active transcribes the E1 gene, allowing the virus to replicate.

c) Schematic illustration of the effect of a telomerase dependent conditionally replicative virus in cells with and without telomerase expression. In the telomerase negative cells the virus is able to infect the cell but no viral replication occurs and the cell survives. In the telomerase positive cell the virus infects the cell and viral replication results in production of viral progeny. These are released from the cell by cell lysis. Viral progeny are then available to infect neighbouring cells.

### 3.2.1 Cloning of the adenovirus ΔE3 region.

The initial cloning of the sequences to be inserted into the ΔE3 region was carried out in plasmids due to their relatively small size making them relatively uncomplicated to work with compared to large cosmids.

#### 3.2.1.1 Preparing the VP16E2 sequence.

To clone the hTERT-VP16E2 into the virus this sequence was first cloned into a modified pBluescript vector due to the convenience of its restriction sites. A hTERT-VP16E2 PCR product was made using a 5' and a 3' primer with a Bgl II tail and the hTERT-VP16E2 plasmid as a template. The resulting PCR product was cleaned and digested with Bgl II. Then the cut PCR product was cleaned by phenol chloroform and ethanol precipitation and the Bgl II sites were blunted using a T4 polymerase. The blunted insert was cleaned again by phenol chloroform and ethanol precipitation and ligated into an EcoR V digested and phosphatase treated pBluescript vector. Ligations were transformed into JM-109 cells. Correct inserts were identified by restriction digestion and the resulting plasmid was named pBluescript-hTERT-VP16E2.

As a positive control a pBluescript-CMV-VP16E2 plasmid was created in parallel. A CMV-VP16E2 PCR product was made using primers with a 5' and 3' Pme I overhang using the pDNA-CMV-VP16E2 plasmid as a template. The PCR product was digested and ligated into a Pme I digested phosphatase treated pBluescript vector and transformed into JM-109 cells. The resulting vector was named pBluescript-CMV-VP16E2. The two pBluescript vectors are illustrated schematically in figure 3.2.2 a).

It was decided to insert the hTERT-VP16E2 and CMV-VP16E2 inserts into a KVlucXX plasmid. This plasmid is derived from the pBluescript plasmid and contains sequence coding for upstream and downstream regions around the E3 gene region from the CAV-1 genome, separated by a multiple cloning site. By using the KVlucXX plasmid the ΔE3 inserts could be inserted into the virus by homologous recombination. The hTERT-VP16E2 and CMV-VP16E2 sequences were cut out of the pBluescript vector by Pme I restriction digestion and the inserts were isolated by gel electrophoresis followed by gel purification. The cleaned inserts were then ligated into a Pme I digested and phosphatase treated KVlucXX vector. Ligations were transformed in JM-109 cells. Correct inserts were confirmed by restriction digestion. The inserts could be inserted with the promoters driving either clockwise or anticlockwise resulting in the

following constructs KVlucXX-hTERT-VP16E2 FOR, KVlucXX-hTERT-VP16E2 REV, KVlucXX-CMV-VP16E2 FOR and KVlucXX-CMV-VP16E2 REV where FOR and REV refers to clockwise and anticlockwise directions. The clockwise constructs are schematically illustrated in figure 3.2.2 b).

### 3.2.1.2 Insertion of the VP16E2 element in the ΔE3 region.

The hTERT-VP16E2 and CMV-VP16E2 were inserted into the viral ΔE3 region by homologous recombination as schematically illustrated in figure 3.2.3. We took forward in the construct with the promoters transcribing in a clockwise direction. The fΔE1ΔE3 CAV-1 cosmid was linearised by restriction digest with Pme I and cleaned by phenol chloroform ethanol precipitation. The Pme I restriction enzyme cuts the fΔE1ΔE3 CAV-1 cosmid at the place were the E3 gene was removed from the sequence. The KVlucXX-hTERT-VP16E2 FOR and KVlucXX-CMV-VP16E2 FOR plasmids were digested with Hpa I which cut out the promoters, the transcriptional activator and a large fragment of the upstream and down stream E3 flanking sequence. The excised fragments were cleaned and isolated by gel electrophoresis and gel purification. Approximately 300ng purified insert was mixed with 100 ng linearised fΔE1ΔE3 CAV-1 cosmid and electroporated into BJ5183s electrocompetent E.coli cells. Inserts were confirmed by restriction digestion with Pme I, diagnostic PCR and sequencing. Due to the poor DNA quality obtained with the BJ5183s cells, constructs with correct inserts were retransformed into chemically competent Stbl 2 cells to be able to obtain larger quantities of better quality DNA. The resulting cosmids were named CAV-1-hTERT-VP16E2 and CAV-1- CMV-VP16E2.

**Figure 3.2.2**

a)


b)

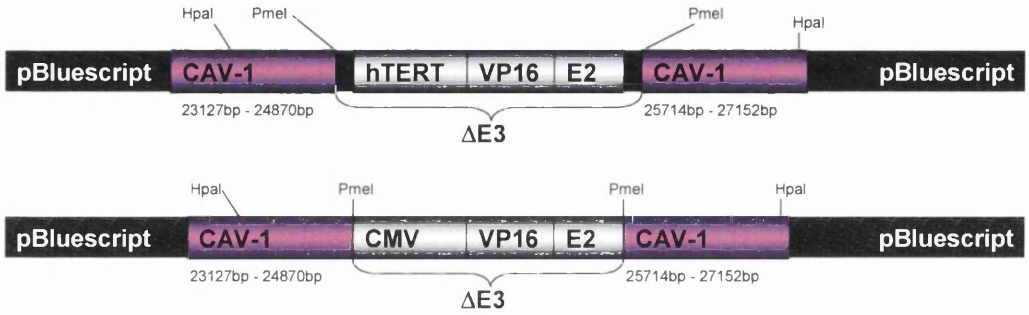



**Figure 3.2.2 Schematic illustration of the vectors cloned to clone the ΔE3 region into the CAV-1 virus.**

a) Schematic representation of the pBluescript-hTERT-VP16E2 and pBluescript-CMV-VP16E2 plasmids.

b) Schematic illustration of the KvlucXX-hTERT-VP16E2 and KvlucXX-CMV-VP16E2 plasmids in which the hTERT-VP16E2 and CMV-VP16E2 inserts are placed in between DNA coding for viral DNA upstream and downstream of the E3 gene region.

**Figure 3.2.3**

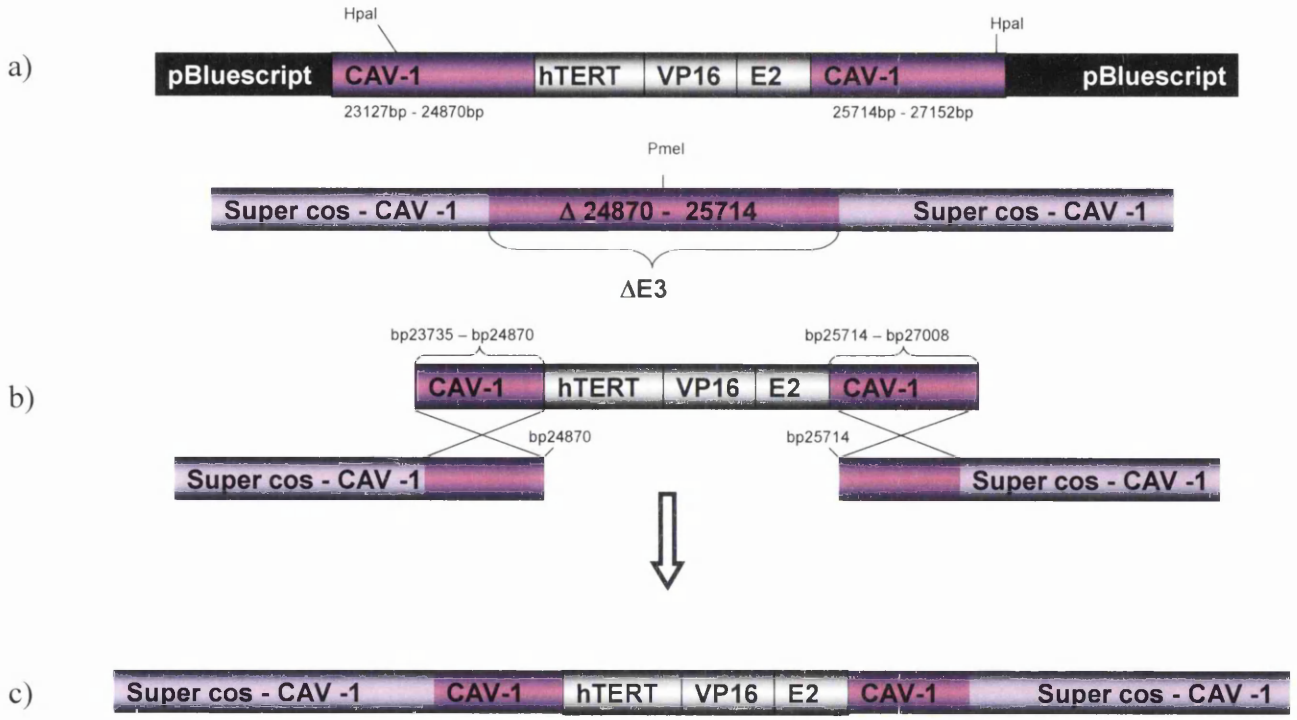



**Figure 3.2.3: Schematic presentation of the homologous recombination step to place insert in the viral ΔE3 region.**

a) Schematic illustration of the KvlucXX-hTERT-VP16E2 plasmid and the fΔE1ΔE3 cosmid with relevant restriction sites.

b) The fΔE1ΔE3 cosmid was linearised by Pme I restriction digest. The KvlucXX-hTERT-VP16E2 was cut with Hpa I. Both constructs were now exposing homologous overlapping DNA sequences at their ends and were then electroporated into BJ5183s cells

c) The resulting cosmid with insertion of hTERT-VP16E2 in the ΔE3 viral region.

Same method was carried out for the CMV-VP16E2 insert.

### 3.2.2.1 Insertion of the E1 coding sequence.

The fΔE1ΔE3 cosmid is lacked the E1 gene however still contained the E1 promoter region. This promoter region was excised by a homologous recombination step in which the 6xE2-3'BpTATA and the E1 coding region would be inserted into the cosmid. The exact extent of the E1 promoter sequence in the CAV-1 virus is not known. The E1 promoter region to be excised from the final virus was therefore estimated by sequence comparison with other adenoviruses. The E1 promoter was estimated to be contained in the sequence between base pairs 281-416 in the CAV-1 genome. The complete E1 insert to be inserted by homologous recombination in the ΔE1 site of the fΔE1ΔE3 cosmid consisted of sequence upstream of the E1 promoter region for homologous recombination, the 6xE2-3'BpTATA promoter, the full length E1 gene and sequence downstream of the E1 gene for homologous recombination. The sequence upstream of the E1 promoter region was named Leftend. The Leftend consisted of viral sequence from base pair 0-281, thereby avoiding the E1 promoter region and having additional base pairs upstream to allow for homologous recombination. It was decided to incorporate 835 base pairs of upstream cosmid sequence due to the location of convenient restriction sites.

As a starting point the E1 insert was cloned in the pGL3-Basic vector. A PCR product was made of the Leftend sequence using primers with a 5' EcoR V restriction site and a 3' Xho I restriction site and using the fΔE1ΔE3 cosmid as template. The pGL3-Basic vector was cut with Kpn I and Xho I and following the Kpn I site was blunted by treatment with T4 DNA polymerase. The vector was phophatase treated and the PCR products were cut with EcoR V and Xho I and ligated into the vector. Ligations were transformed into JM-109 cells and correct clones were confirmed by restriction digestion and sequencing. The resulting vector was named pGL3-Basic-Leftend as shown in figure 3.2.4 a).

Next, the 3'BpTATA promoter and upstream E2 binding sites were inserted downstream of the Leftend sequence. The 6xE2-3'BpTATA insert was constructed as a PCR product using the 6xE2-3'BpTATA vector as template. The 5'primer used in this reaction had a tail encoding a Xho I restriction site while the 3' primer had a tail encoding a Sma I, PmeI and a Xho I restriction site. The PCR product was digested with Xho I and ligated into the pGL3-Basic vector which had been digested with Xho I and Sal I and then phophatase treated. The Xho I and Sal I restriction sites have compatible

ends and the PCR product could be inserted in either direction; however only the clockwise insertion was of interest. Correct inserts were confirmed by restriction digestion and the resulting vector was named pGL3-Basic-Leftend-6xE23'BpTATA as seen in figure 3.2.4 b).

It has been shown that viral sequence can exhibit enhancer activity which can affect and activate downstream promoters. In order to counteract this potential risk it was decided to insert a poly-AAA sequence in between the Leftend sequence and the 6xE2-3'BpTATA sequence. Others have reported a possible positive effect on regulation of non viral promoters by inserting a poly-AAA sequence to isolate the promoter from cis-acting viral sequence (Hoffmann et al. 2005). The poly-AAA insert was made as a PCR product using the pGL3-Basic vector as a template and using primers with a 5' Sal I restriction site and a 3' Xho I restriction site. The PCR product was digested with Xho I and Sal I and ligated into the pGL3-Basic-Leftend-6xE2-3'BpTATA vector which had been cut with Xho I and phosphatase treated. Clones with inserts in the correct orientation were confirmed by restriction digestion and sequencing. The resulting plasmid was named pGL3-Basic-Leftend-PolyAAA-6xE2-3'BpTATA and is shown schematically in figure 3.2.4 c).

To test the effect of the poly-AAA sequence, transcription assays were performed. The pGL3-Basic contains a firefly luciferase reporter gene and therefore luciferease transcription assays could be performed as previously. 293-T cells were transfected with 1μg 6xE2-3'BpTATA, 1μg pGL3-Basic-Leftend1-6xE2-3'BpTATA or 1μg pGL3-Basic-Leftend-PolyAAA-6xE2-3'BpTATA, alone or in conjunction with titrations of pCG-VP16E2. The experiment was repeated 3 times in duplicate and results were normalised against protein present in each sample. Results are shown as fold increase in transcription compared to cells transfected with 1μg pGL3-Basic only. As seen in figure 3.2.5, in absence of VP16E2 the Poly-AAA sequence decreases the activity of the 6xE2-3'BpTATA promoter downstream of the Leftend sequence. A small increase in transcriptional activity is seen in pGL3-Basic-Leftend-PolyAAA-6xE2-3'BpTATA transfected cells compared to 6xE2-3'BpTATA transfected cells in presence of VP16E2, arguing that the Poly-AAA sequence has a positive effect on promoter regulation and should be included in the final construct.

The final element to be included in the E1 insert was the E1 gene and sequence downstream from this gene region. The LASC cosmid (see materials and methods) was digested with Fsp I and a 5080 base pair fragment was isolated and cleaned by gel

electrophoresis and gel purification and named E1++. This fragment consists of the full length CAV-1 E1 gene region which is 2610 base pairs in length flanked by 38 base pairs of upstream E1 sequence and 2432 base pairs of downstream E1 sequence, the latter to be used for homologous recombination. The PGL3-Basic-Leftend1-PolyAAA-6xE2-3'BpTATA vector was digested with Sma I and phophatase treated, and the E1++ fragment was ligated into it. The ligation was transformed in JM-109 cells and correct inserts confirmed by sequencing and restriction digestion. The resulting vector as illustrated in figure 3.2.4 d) was named PGL3-Basic-Leftend1-PolyAAA-6xE2-3'BpTATA-E1++.

**Figure 3.2.4**

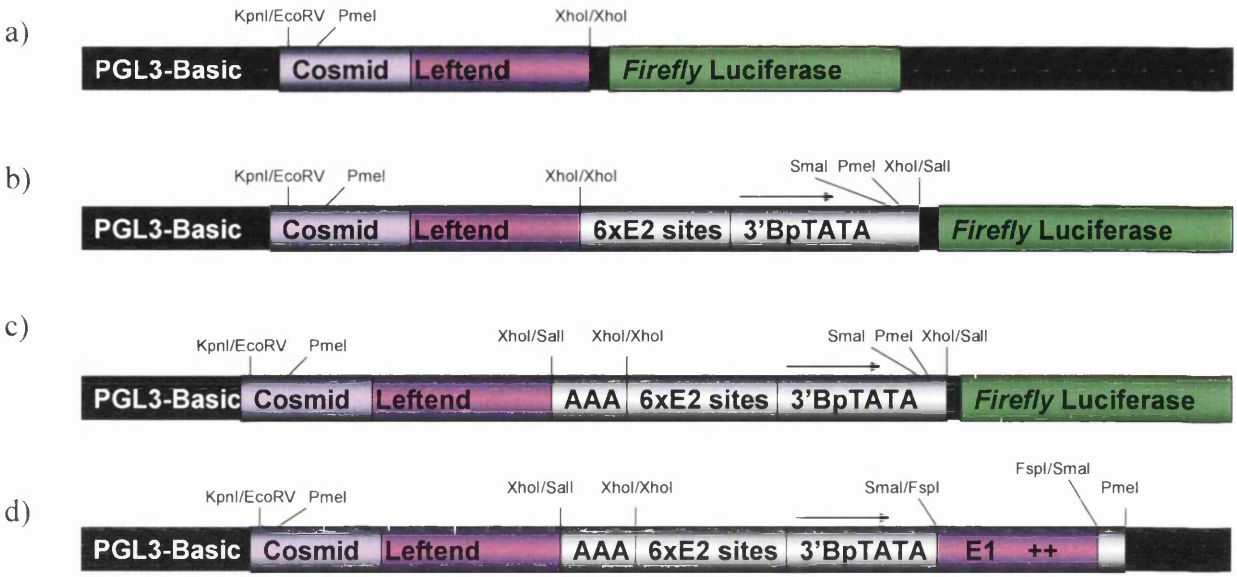



**Figure 3.2.4: Cloning of the ΔE1 insert.**

Schematic representation of the cloned constructs used to make the ΔE1 insert. Models not to scale. Cosmid fragment highlighted in light pink. Virus fragment highlighted in dark pink. Vector backbone highlighted in black. E1 promoter substitute highlighted in grey.

a) pGL3-Basic –Leftend,

b) pGL3-Basic –Leftend-6xE2-3'BpTATA,

c) pGL3-Basic –Leftend-PolyAAA-6xE2-3'BpTATA,

d) pGL3-Basic-Leftend-PolyAAA-6xE2-3'BpTATA-E1++

**Figure 3.2.5**

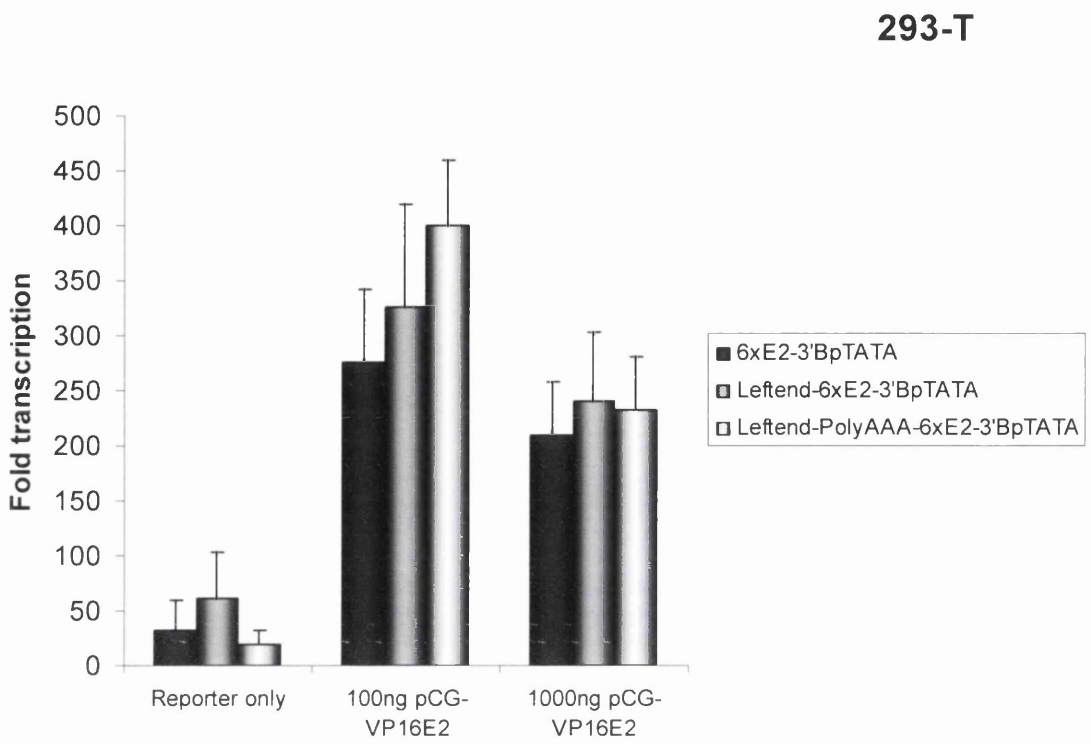



**Figure 3.2.5: Evaluating the effect of the Poly-AAA sequence on promoter activity.** 293-T cells were transfected with 1 μg 6xE2-3'BpTATA, 1 μg Leftend 1-6xE2-3'BpTATA or 1 μg pGL3-Basic –Leftend1-PolyAAA-6xE2-3'BpTATA alone or co-transfected with titrations of pCG-VP16E2 as indicated. Results are shown as fold increase in luciferase expression compared to sample transfected with 1000ng pGL3-Basic only. Experiments carried out 3 times in duplicate. Standard error bars are shown

### 3.2.2.2 Insertion of the E1 region into the CAV-1-hTERT-VP16E2 and CAV-1-CMV-VP16E2.

The E1 sequence was inserted into the CAV-1-hTERT-VP16E2 and CAV-1-CMV-VP16E2 cosmids by homologous recombination. The pGL3-Basic-Leftend1-PolyAAA-6xE2-3'BpTATA-E1++ plasmid was digested with Pme I and the insert for the E1 region was isolated and purified by gel electrophoresis and gel purification. The CAV-1-hTERT-VP16E2 and CAV-1- CMV-VP16E2 cosmids were linearised by I-Ppo I digest which cuts this cosmid at the site were the E1 gene was originally excised. Approximately 300ng purified insert was mixed with 100 ng linearised CAV-1-hTERT-VP16E2 or CAV-1- CMV-VP16E2 cosmid and electroporated into BJ5183s electrocompetent E.coli cells. By this insertion the E1 promoter region from base pair 282-415 in the CAV-1 genome was removed from the cosmids. The homologous recombination is schematically illustrated in figure 3.2.6. Inserts were confirmed by restriction digestion with Hind III and Xho I, diagnostic PCR and sequencing. Due to the poor DNA quality obtained with the BJ5183s cells, constructs with correct inserts were retransformed into Stbl 2cells to be able to obtain larger quantities of better quality DNA. The resulting cosmids were named CAV-1-hTERT+E1 and CAV-1- CMV+E1 and are illustrated schematically in figure 3.2.7. EcoR I restriction digestion of mutant virus and wild type virus cosmids as shown in figure 3.2.8 was conducted to assure DNA quality and correctly sized bands, indicating no unwanted recombination reaction had taken place. The bands on figure 3.2.8 were of predicted size indicating that no gross abnormalities of the mutant viruses were present.

**Figure 3.2.6**

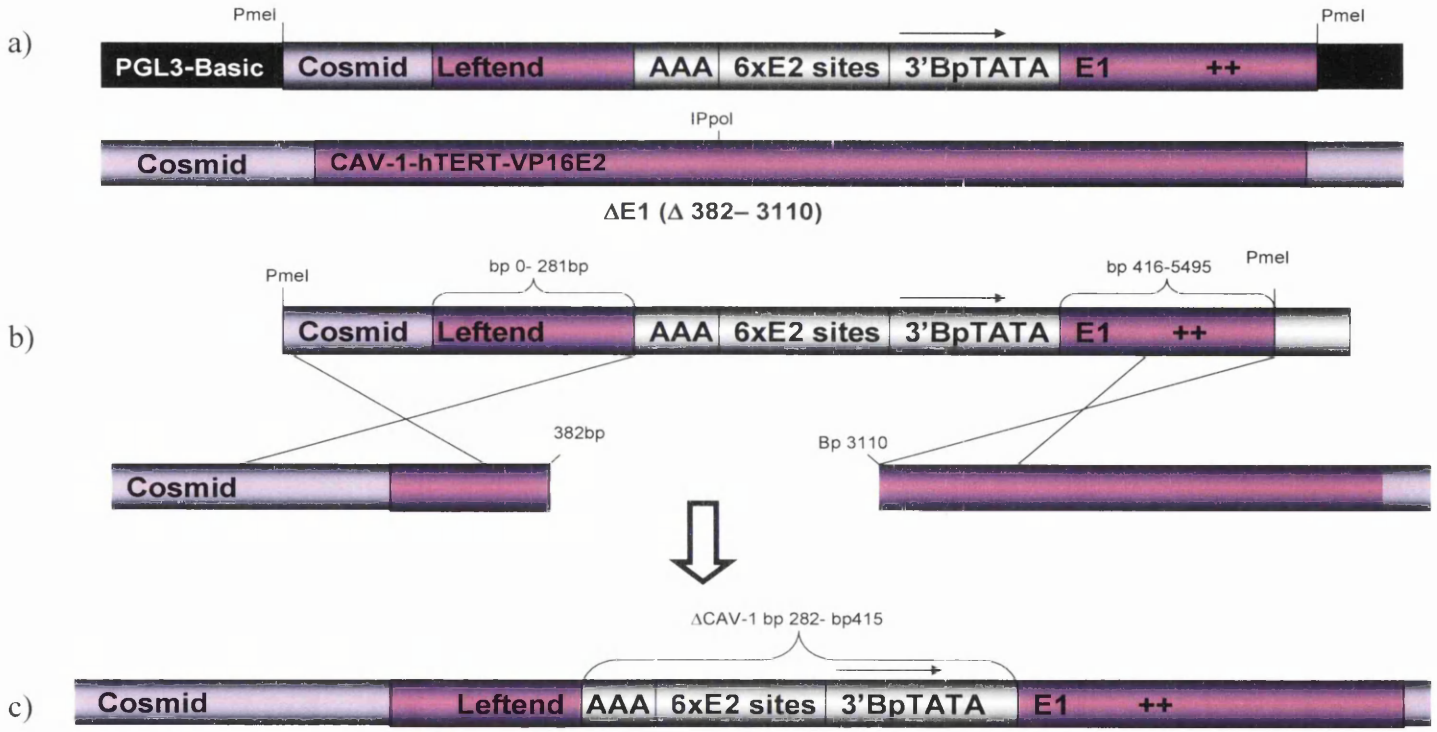



**Figure 3.2.6: Homologous recombination for insertion of the E1 region.**

a) Schematic representation of the pGL3-Basic-Leftend1-PolyAAA-6xE2-3'BpTATA-E1++ plasmid and the CAV-1-hTERT-VP16E2 cosmid. b) The pGL3-Basic-Leftend1-PolyAAA-6xE2-3'BpTATA-E1++ plasmid was cut with Pme I to excise the E1 region insert. The CAV-1-hTERT-VP16E2 was cut with I-Ppo I which cuts the cosmid at the site were the E1 gene was removed. Then the insert and cosmid were electroporated into BJ5183s cells. c) Schematic representation of the incorporated E1 region after the homologous recombination step.

Figure 3.2.7


**Figure 3.2.7: CAV-1-hTERT+E1**

Schematic representation of the CAV-1-hTERT+E1 in the cosmid. Figure not in scale.

**Figure 3.2.8**

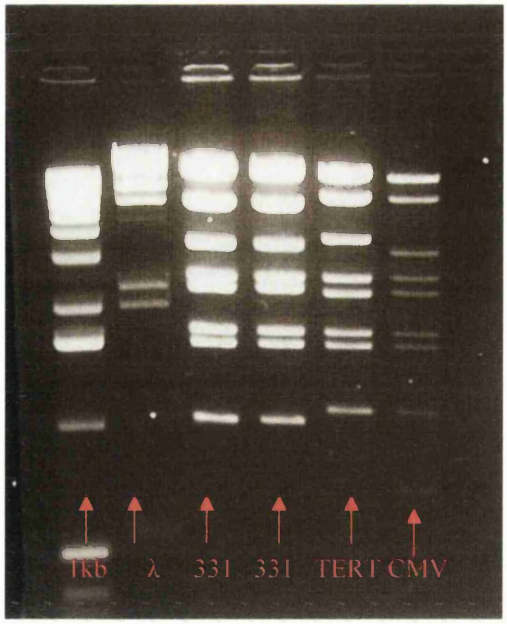



**Figure: 3.2.8 Restriction digest of viral cosmid DNA**

Figure showing restriction digest of wild type viral DNA in cosmid and mutant viruses in cosmids. 1kilo base ladder and λ Hind III phage ladder shown on the left. 331 refers to the wild type virus. TERT and CMV refers to the CAV-1-hTERT+E1 and CAV-1-CMV+E1 viruses respectively.

### 3.2.3 Production of live virus.

To produce live virus, the cosmids containing the viral sequence were digested to separate the cosmid backbone and the virus DNA and was then transfected into canine cells.

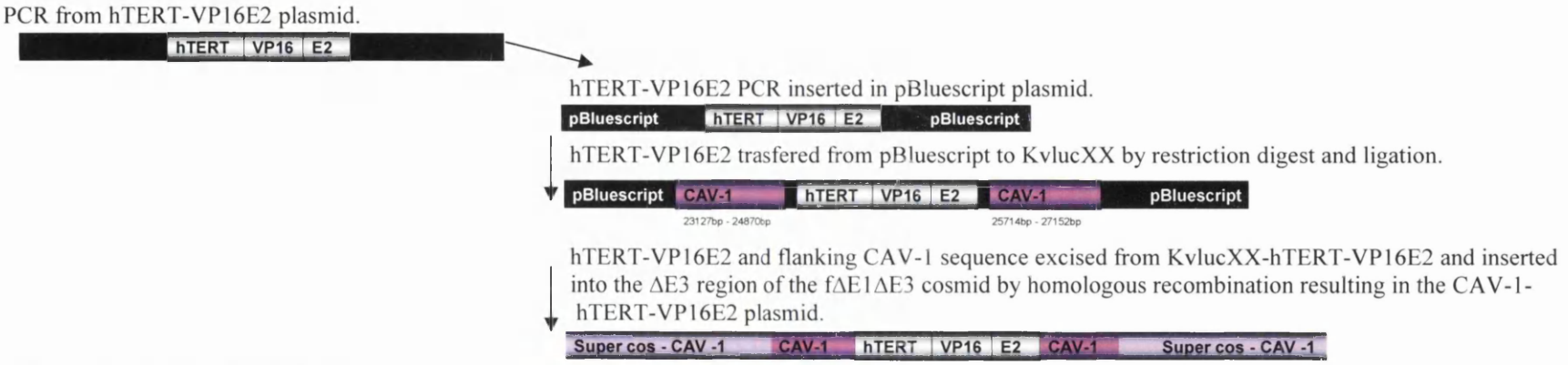
#### 3.2.3.1 Digestion and transfection of virus.

In order to separate the viral DNA sequence from the cosmid backbone, the CAV-1-hTERT+E1 and CAV-1- CMV+E1 cosmids were digested with Fse I. After the digestion the reaction was heat inactivated and cleaned by ethanol precipitation. The digested product was resuspended in a small volume of $ddH_2O$. Approximately 5µg cut cosmid DNA was transfected into MDCK cells using the nucleofector L-Kit as described in materials and methods. Cells were incubated at 37°C in 5% $CO^2$ and every 3-4 days 2 ml fresh medium was added to the plates. 14 days after transfection the cell plates transfected with CAV-1-hTERT+E1 showed almost 100% cell death. Cell death was observed as cells curling up and detaching from the cell plate. No cell death was observed in the CAV-1-CMV+E1 transfected cells. In parallel a positive control wild type CAV-1, named 331, was digested and transfected in the same manner as the mutant viruses (this wild type virus was digested with I-Sce I instead of Fsp I). The wild type virus induced 100% cell death in the nucleofecter transfected cells 10 days after transfection. A summary of the cloning process and the generation of live virus is shown in figure 3.2.9.

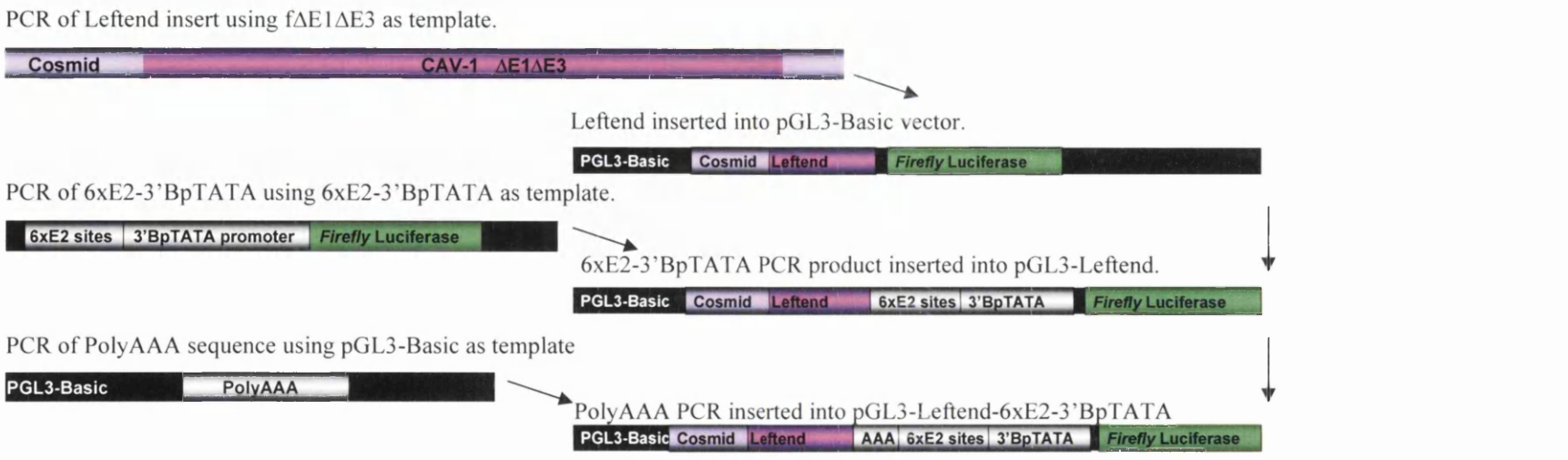
Similar tranfections were repeated in two E1 complementary cell lines MDCK P-3.1 and MDCK Fsp1.9 in order to test whether the lack of cell death observed with the CAV-1- CMV+E1 could be related to a fault in either the E1 gene or the CMV promoter. Cell death was observed in both cell lines transfected with either wild type virus 331 or CAV-1-hTERT+E1. In contrast no cell death was observed in E1 complementing cells transfected with CAV-1- CMV+E1, suggesting that this virus is defective in an area other than the E1 gene.

**Figure 3.2.9 Summary of virus cloning.**

a) Cloning of the ΔE3 region.




b) Cloning of the E1 insert.



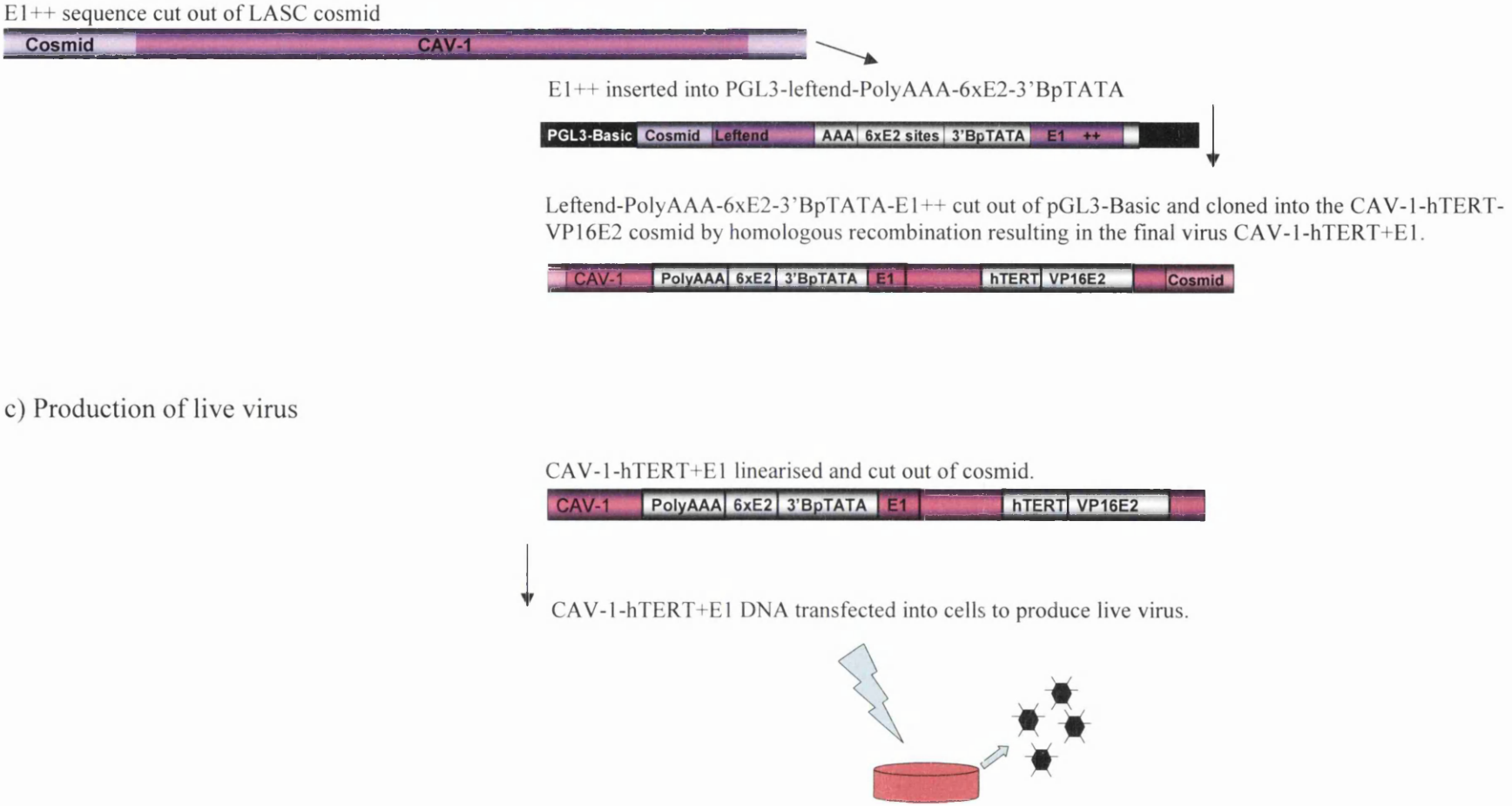



**Figure 3.2.9: Summary of virus cloning.**

Figure showing stepwise summary of the basic cloning and process envolved in producing the live virus CAV-1-hTERT+E1.

### 3.2.3.2 Harvest of virus and generation of virus stock.

At the presence of nearly 100% cell death the above mentioned cell plates transfected with wild type virus 331 and mutant virus CAV-1-hTERT+E1 were harvested as described in materials and methods. This harvested virus was regarded as primary virus stock. Larger volumes of secondary virus stock were made by inoculating a T-75 and T-175 cell flask with MDCK cells and 1ml primary virus stock from wild type virus 331 and mutant virus CAV-1-hTERT+E1 respectively. The wild type virus was left to replicate for 2 days and the mutant virus CAV-1-hTERT+E1 was left to replicate for 4 days before the virus was harvested, aliquoted and frozen at -80°C.

### 3.2.3.3 Determining virus concentration in telomerase positive canine cells.

To determine the concentration and infectivity of the secondary virus stocks $TCID_{50}$ assays were carried out as described in materials and methods. 96-well plates were infected with half log dilutions of wild type virus 331 or mutant virus CAV-1-hTERT+E1 with four replicates for each dilution. The lowest dilution tested was $10^{-1}$ and the highest dilution was $10^{-12.5}$. Cells were examined daily and 8 days after infection the final level of cell killing was observed and the $TCID_{50}$ determined. In figure 3.2.10 the results from the $TCID_{50}$ assay are schematically illustrated. The $TCID_{50}$ was determined using the formula:

**Log $TCID_{50}$ = D-Δ(S-0.5)**

Plaque forming units pr ml was following calculated as:

**Pfu = $(1/10^{\text{Log } TCID_{50}})$ x10x0.69.**

The Log $TCID_{50}$ and Pfu for the wild type virus 331 and mutant virus CAV-1-hTERT+E1 were as follows:

Log $TCID_{50\ 331}$= -7 $Pfu_{331}$=6.89 x$10^{8}$ Pfu $ml^{-1}$

Log $TCID_{50\ CAV\text{-}1\text{-}hTERT+E1}$ =-2.875 $Pfu_{CAV\text{-}1\text{-}hTERT+E1}$ = 5.17x$10^{3}$ Pfu $ml^{-1}$

Based on these calculations MDCK cell plates were infected with 500 Pfu of either virus 331 or CAV-1-hTERT+E1. The cells were observed on a daily basis.

Sporadic cell killing was observed with the CAV-1-hTERT+E1 virus compared to the wild type 331 virus which showed cell killing in plaques as shown (figure 3.2.11). The more evident, rolled up cells on these pictures represent lysed cells.

The virus was harvested from the infected plates as tertiary virus stock 5 days after infection and a new $TCID_{50}$ assay was set up, using the newly harvested viruses. Plates were checked daily and after 8 days a final determination of $TCID_{50}$ was performed and the plates were stained with crystal violet. This stain is taken up by live cells and darker staining is seen in live cells while lighter or complete lack of staining indicates cell death. The results from the crystal violet stain are shown in figure 3.2.12. The pattern seen is very similar to the pattern observed in figure 3.2.10. A schematic presentation of the generation of primary, secondary and tertiary virus stock is presented in figure 3.2.13.

The $TCID_{50}$ and Pfu for the tertiary virus were calculated as following.

$LogTCID_{50\ 331} = -6.65 \quad Pfu_{331} = 3.08x10^{7}\ Pfu\ ml^{-1}$

$LogTCID_{50\ CAV\text{-}1\text{-}hTERT+E1} = -2.5 \quad Pfu_{CAV\text{-}1\text{-}hTERT+E1} = 2.18x10^{3}\ Pfu\ ml^{-1}$

**Figure 3.2.10**

a)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | -1 | -1.5 | -2 | -2.5 | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| B | -1 | -1.5 | -2 | -2.5 | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| C | -1 | -1.5 | -2 | -2.5 | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| D | -1 | -1.5 | -2 | -2.5 | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| E | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| F | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| G | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| H | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |

b)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | x | x | x | x | x | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| B | x | x | x | x | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| C | x | x | x | x | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| D | x | x | x | x | -3 | -3.5 | -4 | -4.5 | -5 | -5.5 | -6 | -6.5 |
| E | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| F | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| G | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| H | -7 | -7.5 | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |

c)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | x | x | x | x | x | x | x | x | x | x | x | x |
| B | x | x | x | x | x | x | x | x | x | x | x | x |
| C | x | x | x | x | x | x | x | x | x | x | x | x |
| D | x | x | x | x | x | x | x | x | x | x | x | x |
| E | x | x | x | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| F | x | x | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| G | x | x | x | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |
| H | x | x | -8 | -8.5 | -9 | -9.5 | -10 | -10.5 | -11 | -11.5 | -12 | -12.5 |

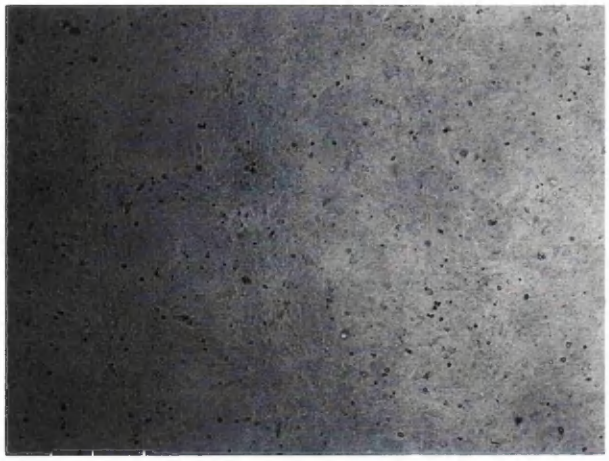
**Figure 3.2.10: Model showing the wells with cell death in the TCID $_{50}$ assay on secondary virus stock.**

a) Negative control plate, b) mutant virus CAV-1-hTERT+E1 infected plate, c) 331 wild type virus infected plate.

Red crosses indicate wells with cytopathic effect. Numbers indicate the log dilution.

**Figure 3.2.11**

a)



b)

c)

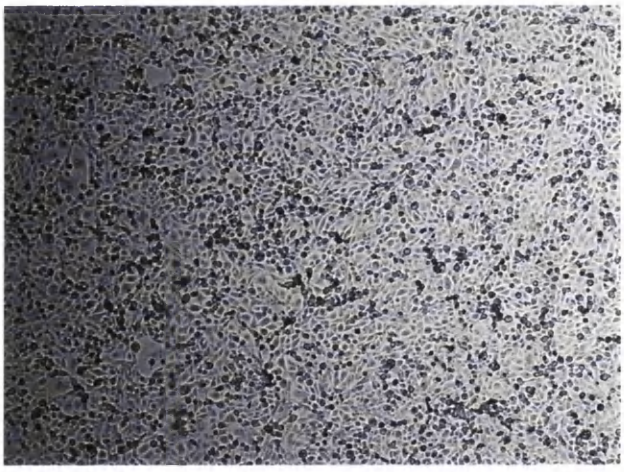


**Figure 3.2.11: MDCK cells infected with 500 Pfu per plate of secondary virus stock 4 days post infection**

a) Negative control uninfected cells, b) cells infected with wild type 331 virus 500 Pfu per plate based on calculations of $TCID_{50}$, c) cells infected with mutant virus CAV-1-hTERT+E1 500 Pfu per plate based on calculations of $TCID_{50}$.

**Figure 3.2.12**

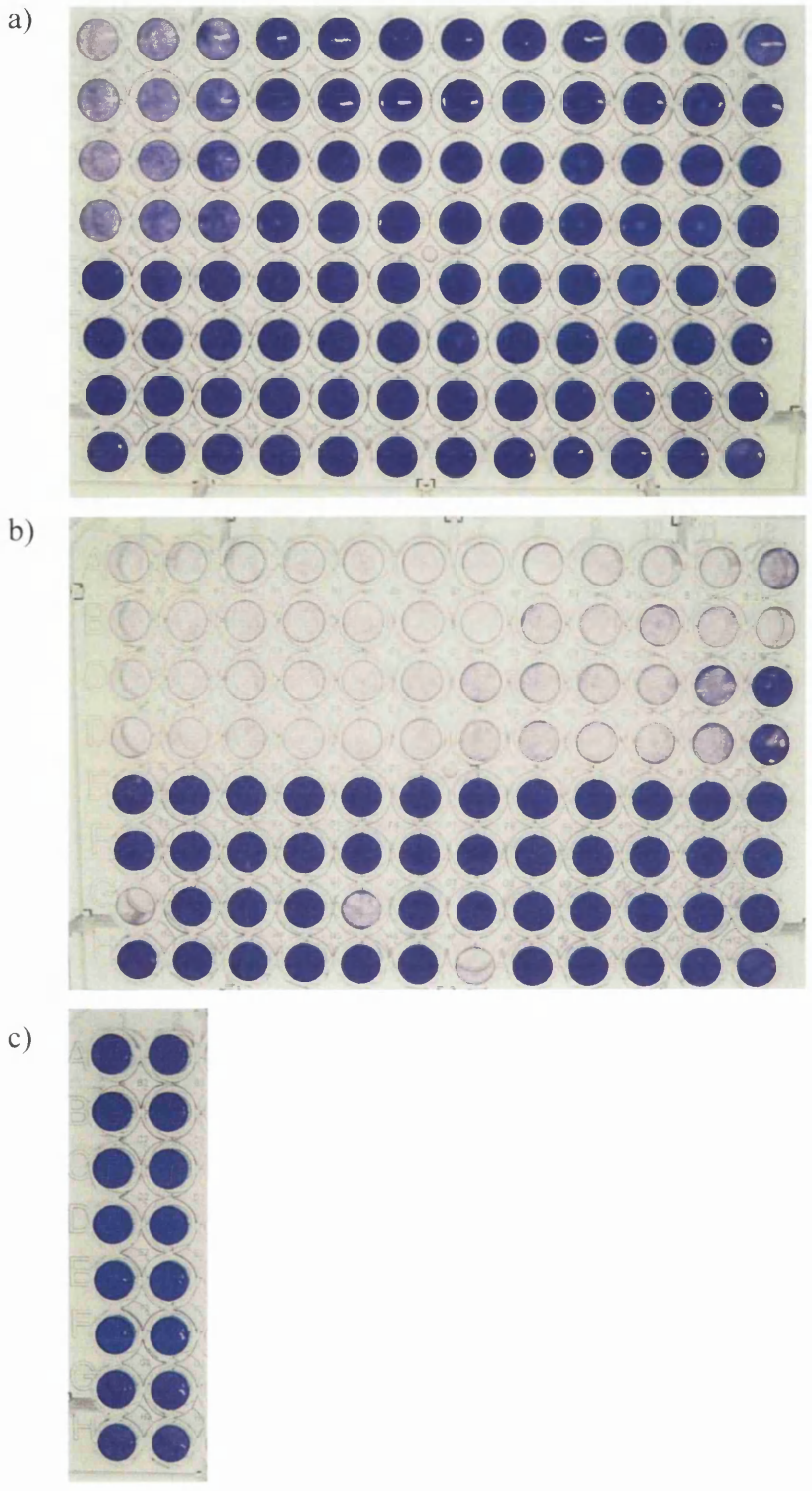



**Figure 3.2.12: Crystal violet stained $TCID_{50}$ plates from tertiary virus**

$TCID_{50}$ assay performed on tertiary virus. Plates stained 8 days after infection. a) mutant virus CAV-1-hTERT+E1 infected plate, b) 331 wild type virus infected plate c) Negative control plate.

**Figure 3.2.13**

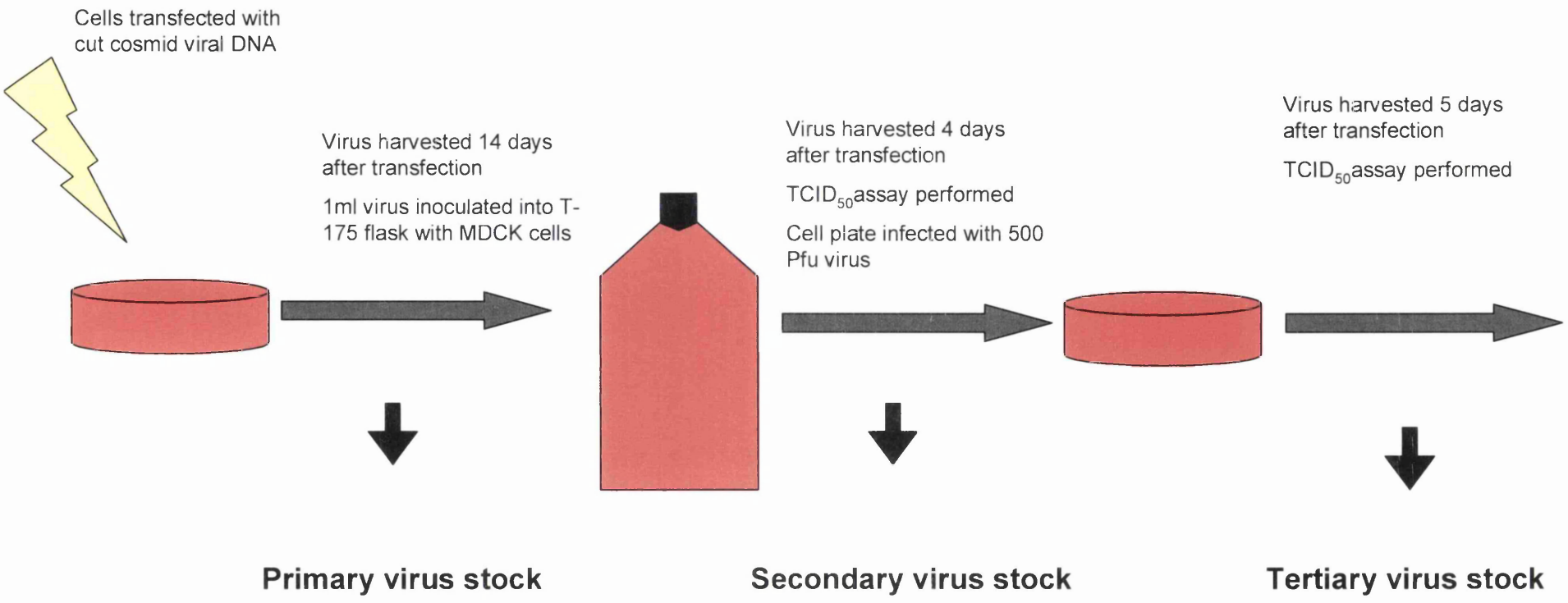



**Figure 3.2.13: Generation of CAV-1-hTERT+E1 viral stock.**

Figure showing a schematic representation of the generation of primary secondary and tertiary viral stock for the CAV-1-hTERT+E1 virus. All stocks were stored as aliquats at -80°C.

### 3.2.3.4 Detecting virus DNA by PCR

To assure that no cross contamination had occurred between the wild type virus 331 and the mutant virus CAV-1-hTERT+E1, a diagnostic PCR was performed on the secondary virus stock. Two PCR reactions were performed using a 5' primer which binds to the beginning of the CAV-1 DNA, and two different 3' primers. The 3' primer used for reaction 1 binds to the sequence upstream of the E1 promoter. The 3' primer used for reaction 2 binds to the presumed E1 promoter sequence. Because the mutant virus CAV-1-hTERT+E1 does not contain the E1 promoter sequence it was expected that only the PCR reaction 1 would result in a PCR product, unless contamination of wild type virus had occurred.

Reactions were set up using either 2µl wild type virus 331 or 2µl mutant virus CAV-1-hTERT+E1 as template. 5 reactions were set up for each virus and each primer set. PCR reactions were allowed to proceed for 10, 15, 20, 25 or 30 cycles. This was to determine whether the clear difference in infectivity from the $TCID_{50}$ assay would be reflected in the amount of virus DNA present in each stock. As seen in figure 3.2.14, reaction 1 results in PCR products after only 15 cycles for the wild type virus whilst only after 20 cycles for the CAV-1-hTERT+E1 virus indicating that there is a difference in the concentration of the virus, however this does not explain the 5 log difference in infectivity seen in the TCID 50 assay. In reaction 2 no PCR product is seen in the reactions using the CAV-1-hTERT+E1 as template indicating that no wild type virus is present in the stock. Clear PCR products are seen after 15 cycles in reaction 2 with the wild type virus as template.

**Figure 3.2.14**

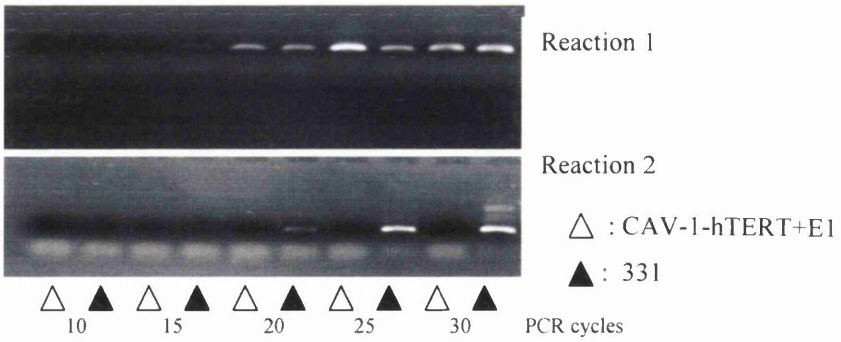



**Figure: 3.2.14: PCR on wild type and mutant virus**

PCR using the secondary virus stock of mutant virus CAV-1-hTERT-VP16E2 and the wild type 331 virus as template. Reaction 1 was performed using primers with binding sites for both viruses. Reaction 2 was performed using primers with binding sites for the wild type virus only. Reactions were taken out after 10, 15, 20, 25 and 30 cycles as indicated.

### 3.2.3.5 Determining cell death in telomerase negative cells

After observing the ability of the mutant virus CAV-1-hTERT+E1 to induce cell lysis in the telomerase positive canine MDCK cell line, the effect was studied in telomerase negative canine cells.

A $TCID_{50}$ assay was set up in two canine telomerase negative cell lines. A primary fibrosarcoma cell line and a primary fibroblast cell line which both tested telomerase negative in the TRAP assay as described in chapter 3.1.5 were used. The $TCID_{50}$ assay was carried out as for the MDCK cells. Due to the slower growth of the cells, primary fibrosarcoma cells were left to grow for 20 days and the primary fibroblast cell line for 14 days before the $TCID_{50}$ was determined and the plates were crystal violet stained. In figure 3.2.16 and 3.2.18 the stained cell plates are shown. It was apparent in both cell lines that significant cell lysis had occurred in the cells infected with virus 331 while cells infected with the CAV-1-hTERT+E1 virus showed no such effect. In figure 3.2.15 and figure 3.2.17 photographs of primary fibroblast and primary fibro sarcoma cells respectively from wells infected with the highest and the lowest concentration of virus illustrate cell death in the 331 virus infected cells whilst no such effect was seen in the CAV-1-hTERT+E1 infected cells. Log $TCID_{50}$ and Pfu were calculated for the wild type virus 331 and the mutant virus CAV-1-hTERT+E1. For both cell lines no cytopathic effect was observed with the CAV-1-hTERT+E1 virus and therefore no Log $TCID_{50}$ and Pfu could be calculated. However as seen below, values were calculated for the wild type virus 331 and were higher than those calculated in MDCK cells.

**Telomerase negative primary fibroblast**

$LogTCID_{50\ 331} = -10.375$ $\quad$ $Pfu_{331} = 1.63 \times 10^{11}$ Pfu $ml^{-1}$

$LogTCID_{50\ CAV\text{-}1\text{-}hTERT+E1} = 0$ $\quad$ $Pfu_{CAV\text{-}1\text{-}hTERT+E1} = 0$

**Telomerase negative fibro sarcoma cells**

$LogTCID_{50\ 331} = -10.625$ $\quad$ $Pfu_{331} = 2.90 \times 10^{11}$ Pfu $ml^{-1}$

$LogTCID_{50\ CAV\text{-}1\text{-}hTERT+E1} = 0$ $\quad$ $Pfu_{CAV\text{-}1\text{-}hTERT+E1} = 0$

**Figure 3.2.15**

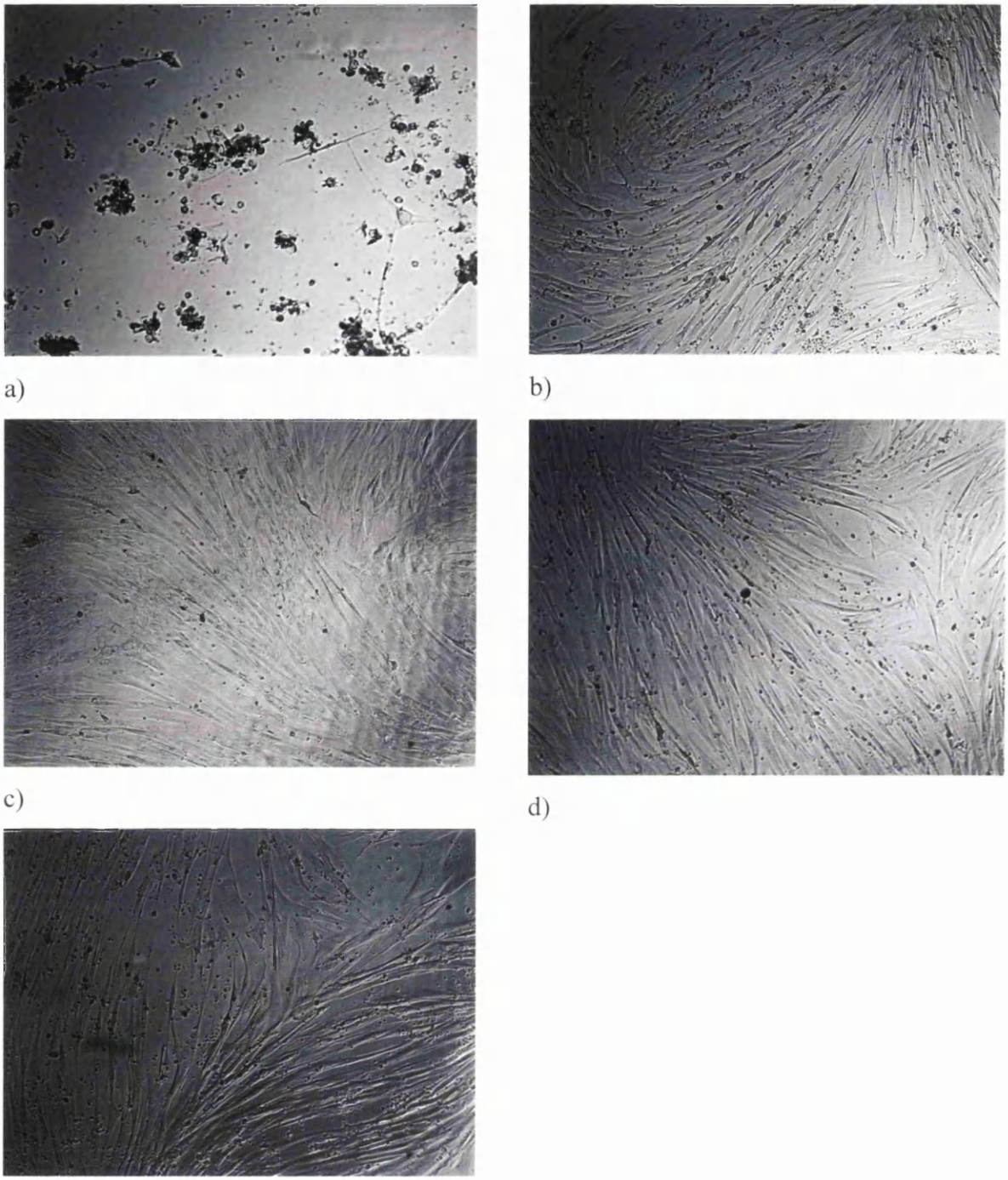
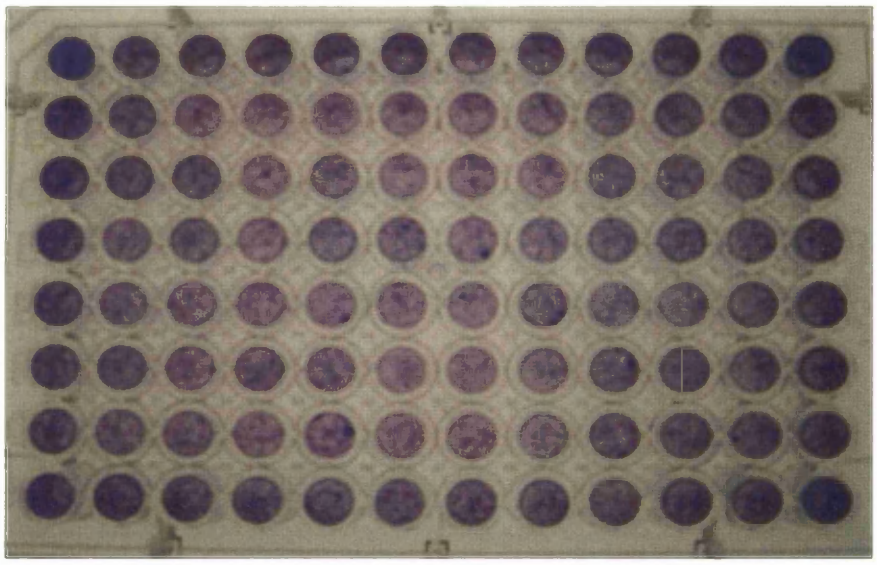



**Figure 3.2.15: Pictures of cells from $TCID_{50}$ assay in primary fibroblasts 14 days after infection.**

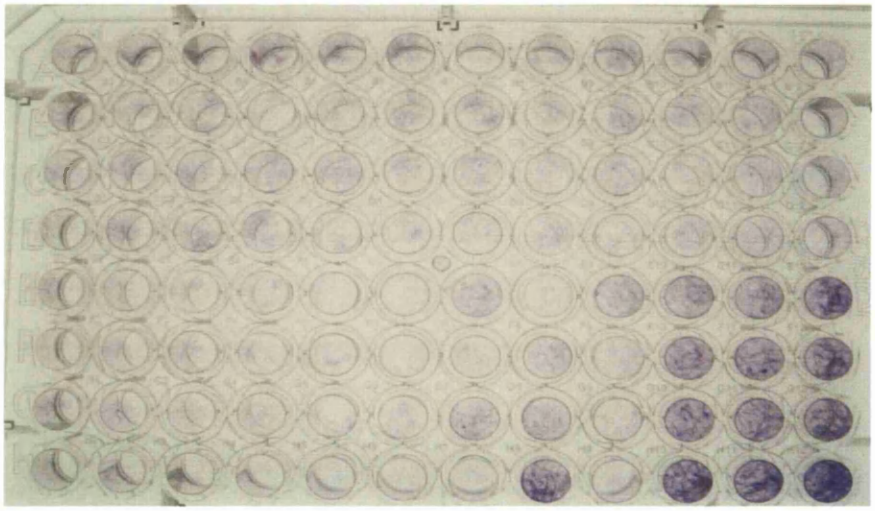
a) $10^{-1}$ dilution of 331 virus, b) $10^{-12.5}$ dilution of 331 virus, c) $10^{-1}$ dilution of CAV-1-hTERT+E1 virus, d) $10^{-12.5}$ dilution of CAV-1-hTERT+E1 virus, e) uninfected control cells.

Figure 3.2.16

a)



b)



c)

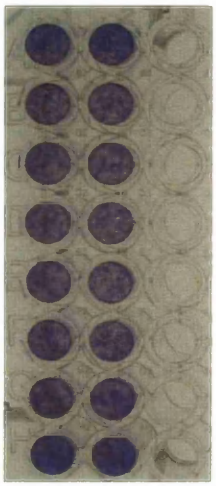


**Figure 3.2.16 Crystal violet stained primary fibroblast $TCID_{50}$ plates.**

a) Plate infected with CAV-1-hTERT+E1 virus, b) Plate infected with 331 wild type virus, c) negative control uninfected cells.

**Figure 3.2.17**

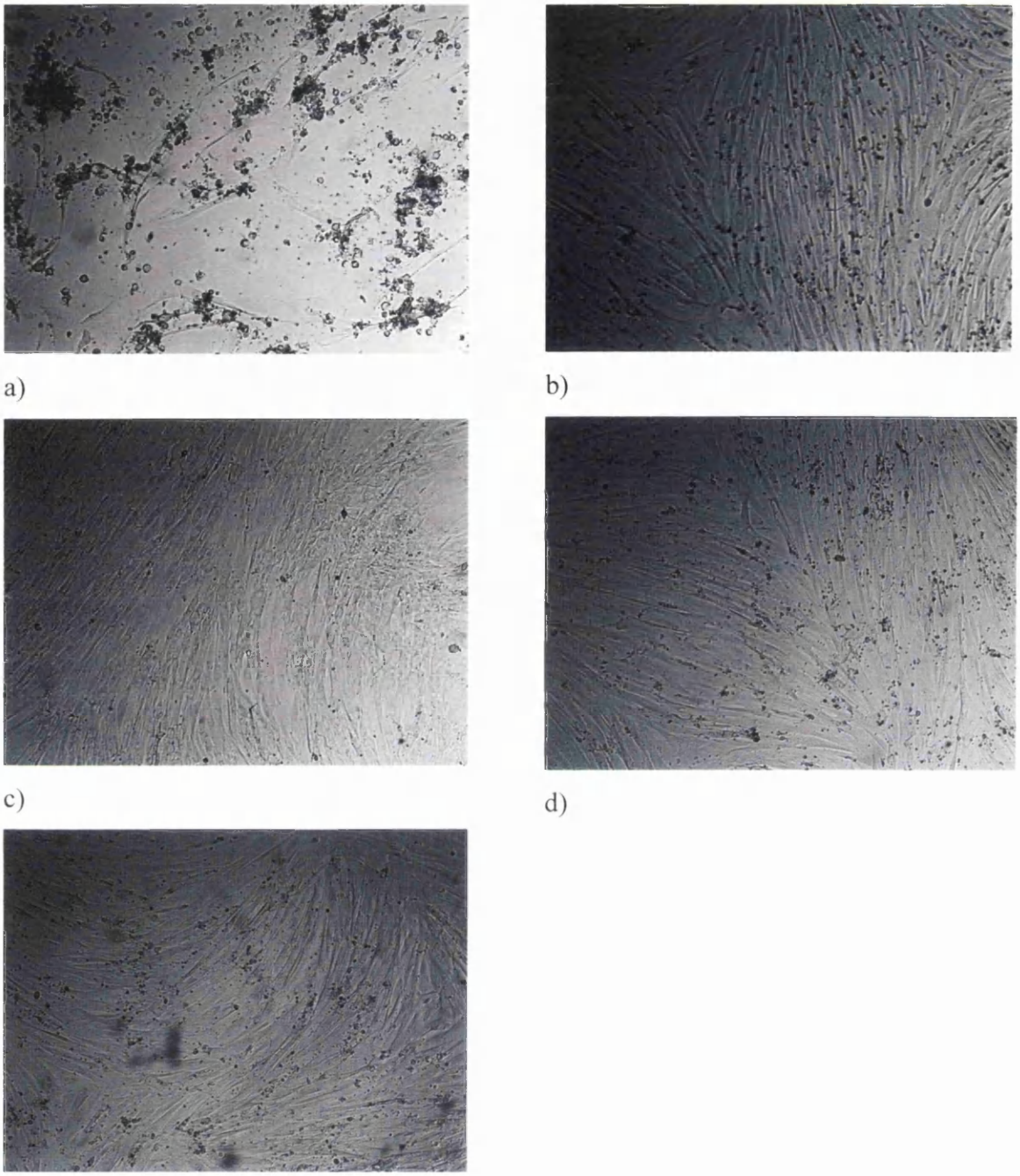
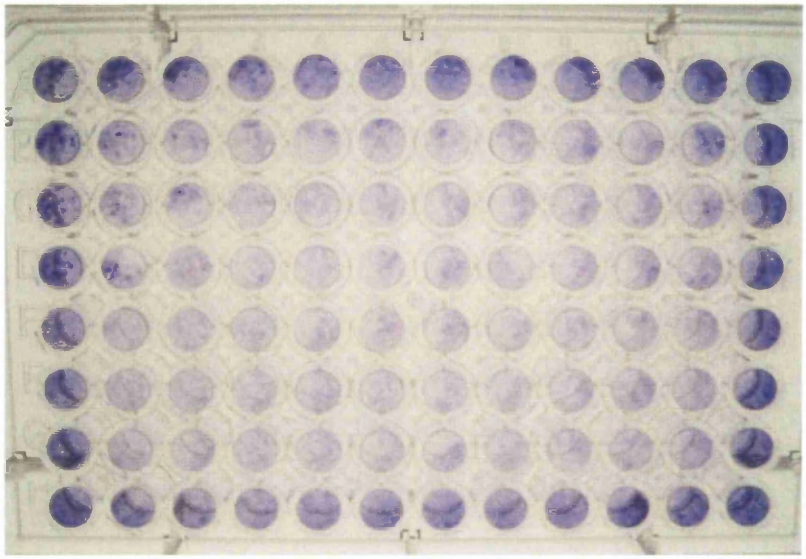



**Figure 3.2.17: Pictures of cells from $TCID_{50}$ assay in primary fibrosarcoma cells 20 days after infection.**

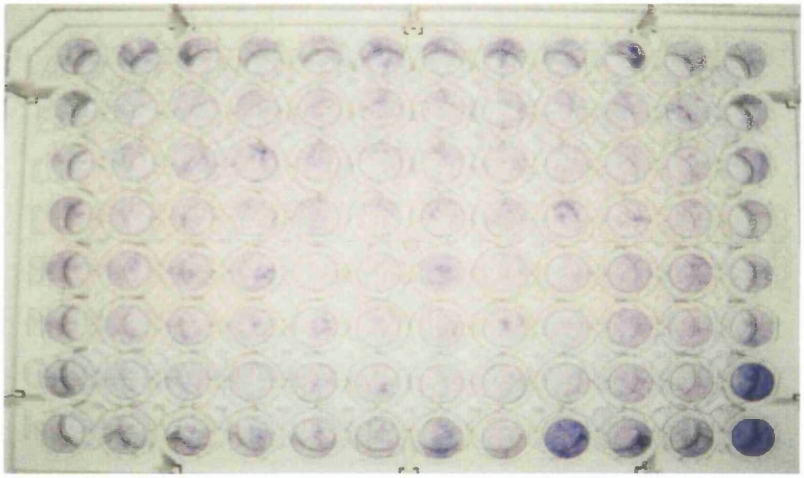
a) $10^{-1}$ dilution of 331 virus, b) $10^{-12.5}$ dilution of 331 virus, c) $10^{-1}$ dilution of CAV-1-hTERT+E1 virus, d) $10^{-12.5}$ dilution of CAV-1-hTERT+E1 virus, e) uninfected control cells.

**Figure 3.2.18**

a)



b)



c)

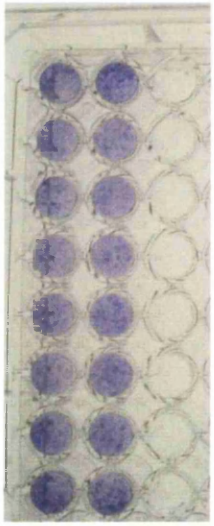


**Figure 3.2.18: Crystal violet stained primary fibrosarcoma $TCID_{50}$ plates.**

a) Plate infected with CAV-1-hTERT+E1 virus, b) Plate infected with 331 wild type virus, c) negative control uninfected cells.

### 3.2.4 Detection of viral protein

In order to test whether viral proteins were expressed during viral infection, Western blots were carried using protein lysates from infected cells.

#### 3.2.4.1 Detection of viral protein using polyclonal antibody against human Ad5

MDCK cells were infected with 500 and 1000 PFU of either wild type virus 331 or mutant virus CAV-1-hTERT+E1. Secondary viral stocks described in chapter 3.2.3.2 were used and dilutions were based on results from $TCID_{50}$ assay 3.2.3.3. Cells were harvested 3 and 5 days after infection and lysates examined by Western blot. 3 days after infection sporadic cell lysis was observed in the CAV-1-hTERT+E1 infected cells while no obvious cell lysis was observed in the 331 infected cells. 5 days after infection an increase in cell lysis was observed in the CAV-1-hTERT+E1 infected cells and in the 331 infected cells clear lytic plaques were observed. It was important to harvest of cells at a time when many cells would be infected and expressing viral proteins but before the virus induced cell lysis.

To our knowledge there are no commercial antibodies against canine adenovirus type 1 available for use in Western blots. The membrane was therefore probed with a polyclonal antibody against human adenovirus type 5. The results from the Western blot are shown in figure 3.2.19. Several bands appear to be specific for the virus infected cells and absent in the negative control lanes. No or low levels of virus specific bands were seen in the 331 infected cells harvested 3 days after infections correlating with the observed lack of cell lysis. Based on the amino acid sequence of the viral surface proteins estimates were made of the protein size, in order to identify the bands on the gel as seen in table 3.2.1 (Morrison et al. 1997). In figure 3.2.19 arrows with the name of the putative viral proteins have been placed at bands thought to be specific for virus infected cells.

#### 3.2.4.2 Detection of viral protein using canine serum as primary antibody.

Dogs are commonly vaccinated against CAV-1 using a vaccine based on attenuated CAV-2 since there is cross reactivity between these two serotypes as described in the introduction. It was therefore anticipated that canine serum could be used as primary antibody for detection of viral proteins in a Western blot. Canine serum samples from the diagnostic laboratory at University of Glasgow Faculty of Veterinary

Medicine were pooled and used for detection of canine adenovirus in conjunction with a secondary HRP conjugated antibody against canine IgG. MDCK cells were infected with 100, 500 or 1000 Pfu of either wild type virus 331 or mutant virus CAV-1-hTERT+E1 or left uninfected as a negative control. Secondary viral stocks described in chapter 3.2.3.2 were used and dilutions were based on results from $TCID_{50}$ assay 3.2.3.3. Cells were harvested 3, 5 and 7 days after infection and lysates examined by Western blot. Prior to harvest pictures were taken of the culture plates to show the presence of cell death. In figure 3.2.20 a, b and c pictures of cell plates taken on day 3, 5 and7 respectively are shown. On day 3, as seen in figure 3.2.20 a), sporadic cell death wasobserved in cells infected with CAV-1-hTERT+E1 at 500 and 1000 Pfu while little or no cell death was observed in the remaining plates. On day 5, as seen in figure 3.2.20 b), increased sporadic cell death was observed on plates infected with CAV-1-hTERT+E1 at 500 and 1000 Pfu. The remaining plates infected with either CAV-1-hTERT+E1 or 331 showed clear cytopathic effect in the form of plaque formation. The number and size of plaques was larger in cells infected with a higher Pfu showing a correlation between the cytopathic effect and the infectious dose. On day 7 as seen on figure 3.2.20 c) sporadic cell death was observed on plates infected with either CAV-1-hTERT+E1 or 331 at 500 and 1000 Pfu. On plates infected with only 100 Pfu of either virus, cell death was still limited to localised plaques. In general at day 7 more cell death was observed with the wild type virus 331 infected cells than the mutant virus. No celldeath was observed in the negative control cell plates.

The harvested cells were prepared for Western blotting and the membrane was incubated with a 1:50000 dilution of pooled canine serum in 5% marvel in PBS-Tween as primary antibody. Subsequently the membrane was incubated with the secondary HRP conjugated antibody against canine IgG.

The results from the Western blot are shown in figure 3.2.21. Two bands on the blots are seen to be specific to virus infected samples and are absent in the negative conrol sample. These two bands are believed to be the viral hexon and penton proteins running on the gel as above 98kDa and above 62 kDa respectively. This size differs from the predicted size according to the calculated protein sizes in table 3.2.1 but correlates with the bands observed in figure 3.2.21. The results from the Western blot reflect the cell death observed in the pictures taken before harvesting the cells. The protein bands are stronger in the samples in which a higher level of cell death was observed and therefore bands increase in density in correlation with the number of days

post transfection reflecting an increased number of infected cells and production of viral proteins.

**Table: 3.2.1**

| Protein | Amino acid size | Estimated kDa size |
|---|---|---|
| Hexon | 905 | 99 |
| Penton | 477 | 52 |
| Protein V | 421 | 46 |
| Protein VI | 238 | 26 |
| Protein VII | 170 | 19 |

**Table 3.2.1: Adenovirus surface protein sizes.**

**Figure 3.2.19**

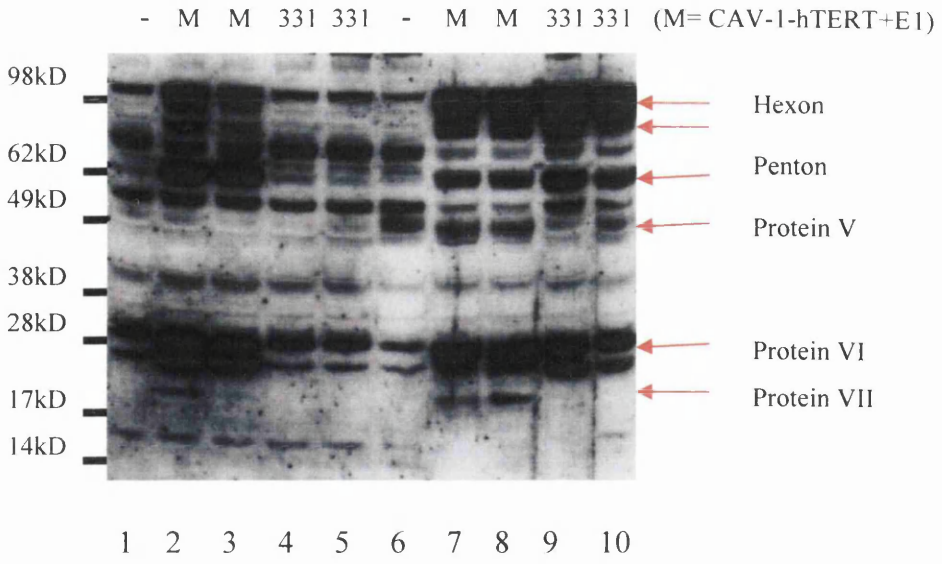
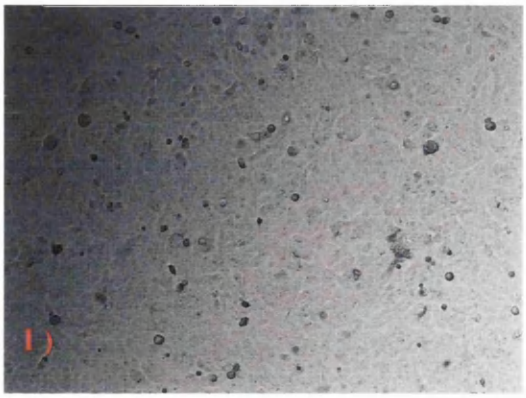



**Figure 3.2.19: Detection of viral proteins using Ad5 antibody**

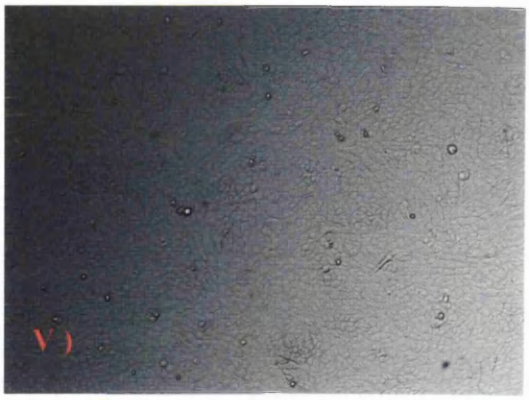
Western blot showing the expression of the viral surface proteins in infected MDCK cells using antibody against human adenovirus type 5. Cell lysates were harvested 3 and 5 days post infection and examined by Western blot as described in materials and methods. The locations of the molecular weight markers (kDa) are shown to the left of the blot. Location of viral proteins and putative proteins are indicated by arrows to the right of the blot.

1: Uninfected cells 3 days post treatment, 2: CAV-1-hTERT+E1 1000Pfu infected cells 3 days post infection, 3: CAV-1-hTERT+E1 500 Pfu 3 days post infection, 4: 331 1000 Pfu infected cells 3 days post infection, 5: 331 500 Pfu infected cells 3 days post infection, 6: Non infected cells 5 days post treatment, 7: CAV-1-hTERT+E1 1000Pfu infected cells 5 days post infection, 8: CAV-1-hTERT+E1 500 Pfu 5 days post infection, 9: 331 1000 Pfu infected cells 5 days post infection, 10: 331 500 Pfu infected cells 5 days post infection.

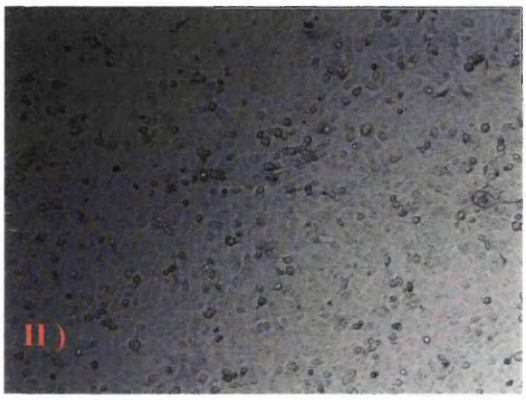
**Figure 3.2.20 a) 3days post infection**




CAV-1-hTERT+E1 100 Pfu

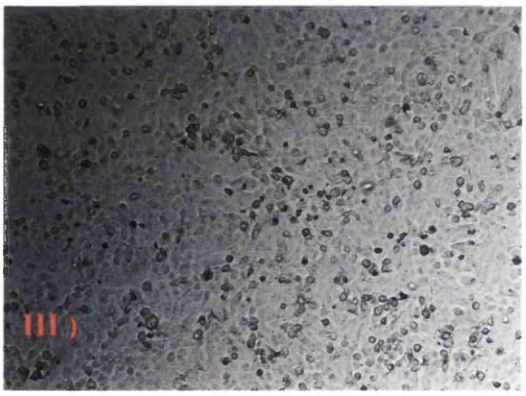



Wild type virus 331 100 Pfu

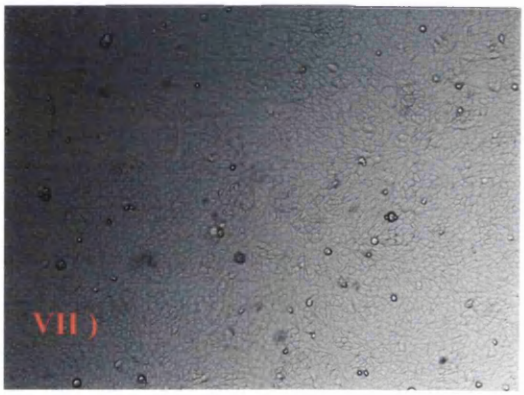



CAV-1-hTERT+E1 500 Pfu

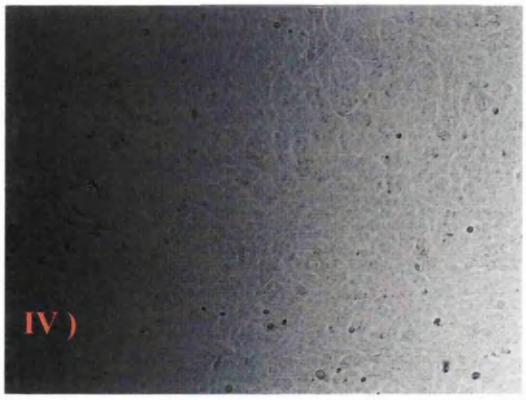

Wild type virus 331 500 Pfu




CAV-1-hTERT+E1 1000 Pfu

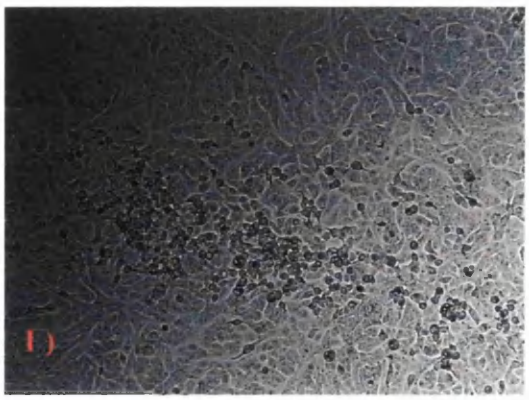



Wild type virus 331 1000 Pfu

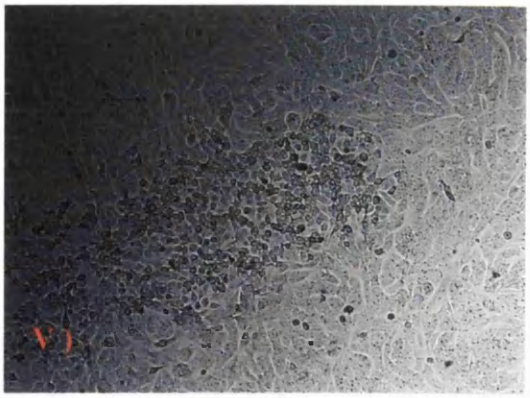



Uninfected cells

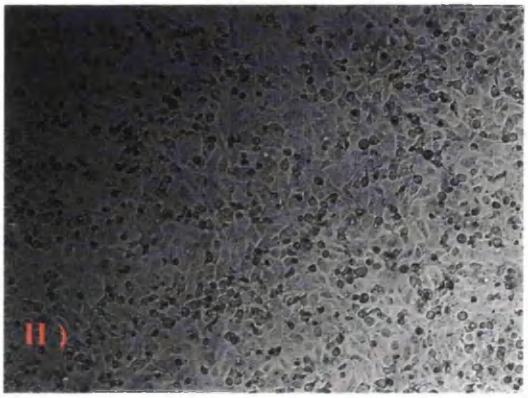
**Figure 3.2.20 b) 5days post infection.**



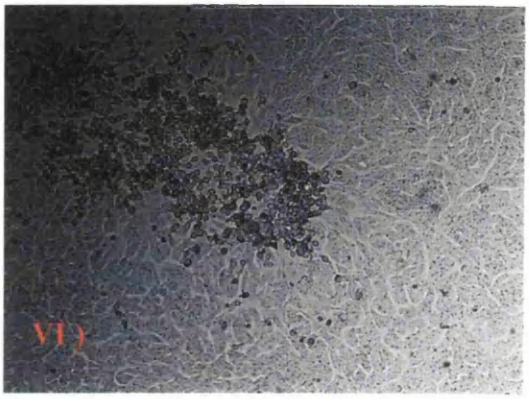

CAV-1-hTERT+E1 100 Pfu



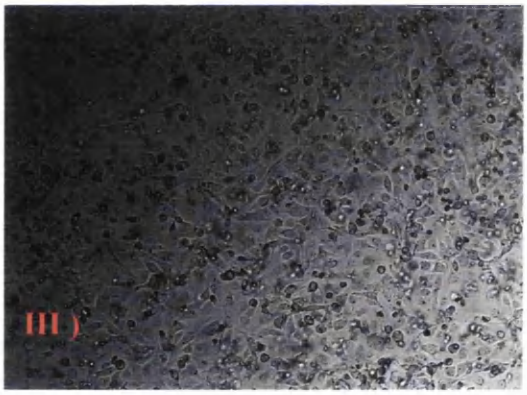

Wild type virus 331 100 Pfu



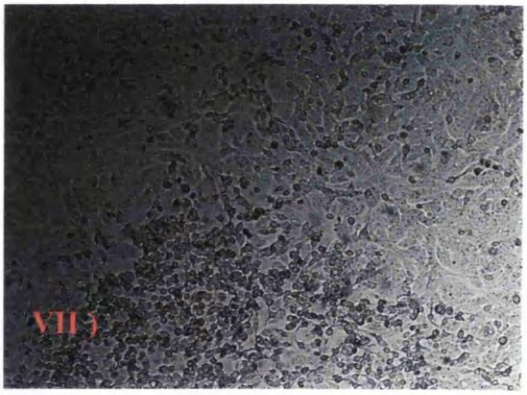

CAV-1-hTERT+E1 500 Pfu



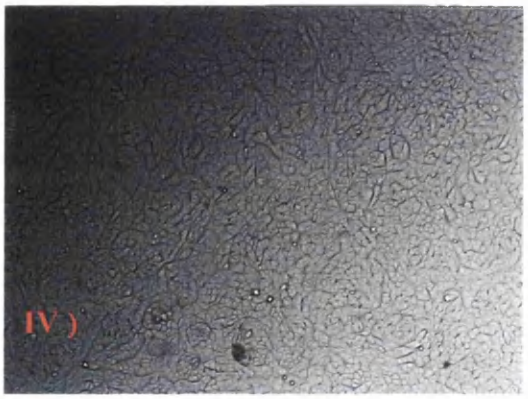

Wild type virus 331 500 Pfu




CAV-1-hTERT+E1 1000 Pfu

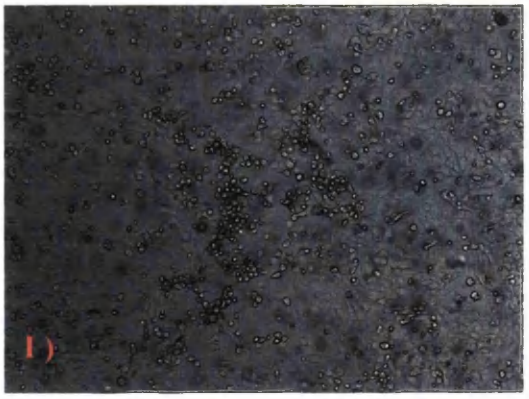



Wild type virus 331 1000 Pfu




Uninfected cells

**Figure 3.2.20 c) 7days post infection.**

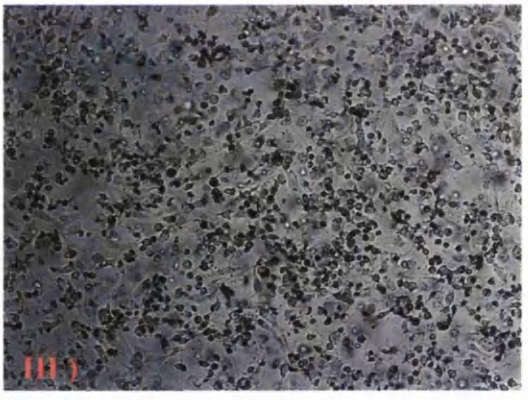



CAV-1-hTERT+E1 100 Pfu

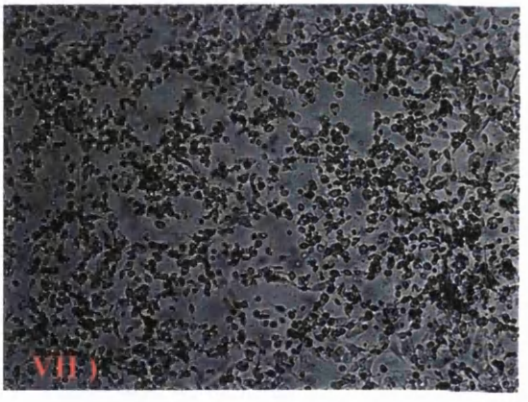

Wild type virus 331 100 Pfu


CAV-1-hTERT+E1 500 Pfu


Wild type virus 331 500 Pfu




CAV-1-hTERT+E1 1000 Pfu




Wild type virus 331 1000 Pfu


Uninfected cells

**Figure 3.2.20: Pictures of cells prior to harvest for western blot.**

Pictures show cell plates after 3, 5 and 7 days after infection with either mutant or wild type virus. Cytopathic effect is seen as dead cells which appear as dense darker clusters on the photos.

a) Pictures taken 3 days after infection, b) pictures taken 5 days after infection, c) pictures taken 7 days after infection.

I) CAV-1-hTERT+E1 100 Pfu, II) CAV-1-hTERT+E1 500 Pfu, III) CAV-1-hTERT+E1 1000 Pfu, IV) Uninfected cells, V) Wild type virus 331 100 Pfu, VI) Wild type virus 331 500 Pfu, VII) Wild type virus 331 1000 Pfu.

**Figure 3.2.21**

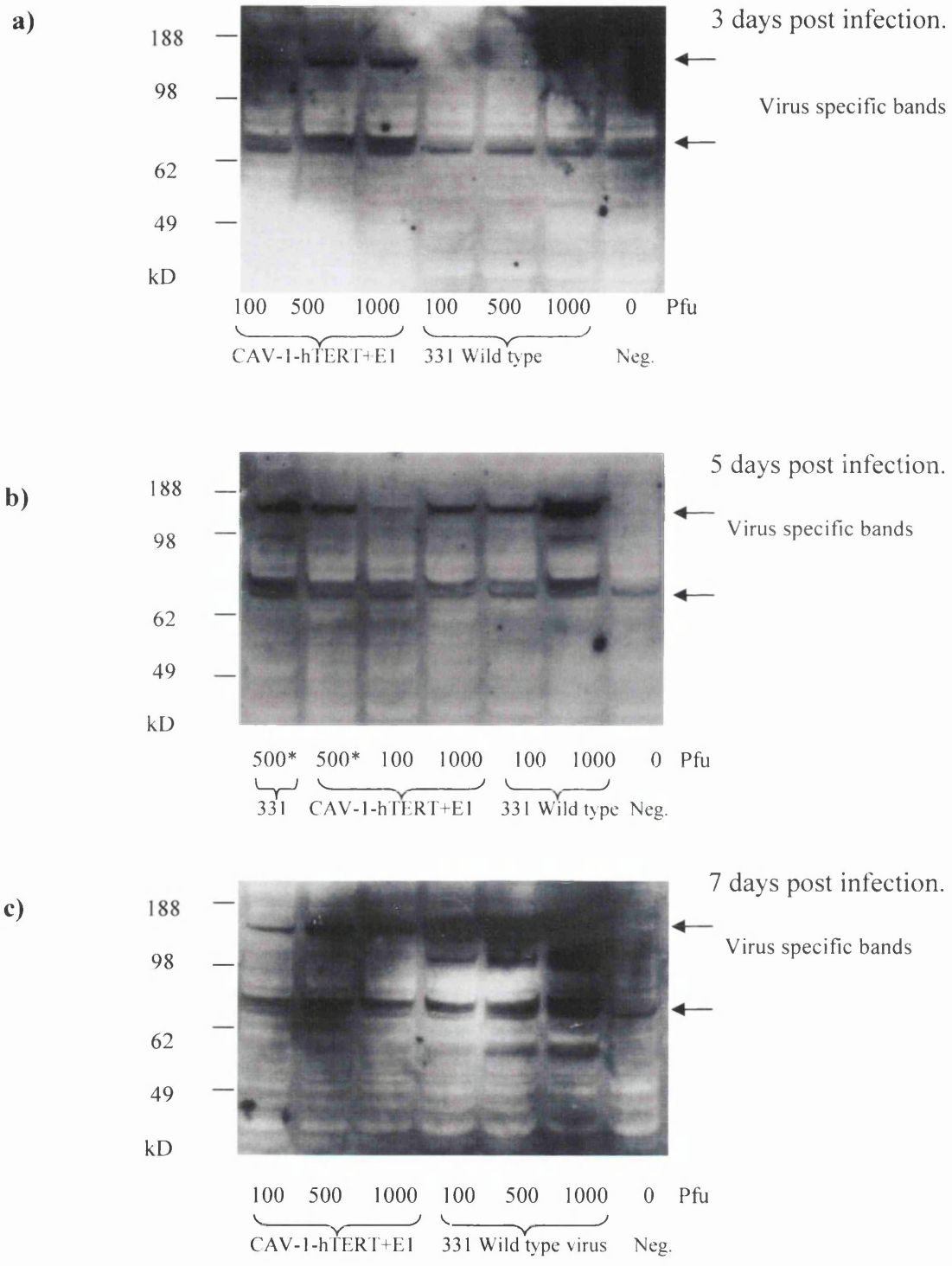

a)
188
98
62
49
kD
3 days post infection.
Virus specific bands
100 500 1000 100 500 1000 0 Pfu
CAV-1-hTERT+E1
331 Wild type
Neg.
b)
188
98
62
49
kD
5 days post infection.
Virus specific bands
500* 500* 100 1000 100 1000 0 Pfu
331
CAV-1-hTERT+E1
331 Wild type
Neg.
c)
188
98
62
49
kD
7 days post infection.
Virus specific bands
100 500 1000 100 500 1000 0 Pfu
CAV-1-hTERT+E1
331 Wild type virus
Neg.

**Figure 3.2.21: Western blot detecting CAV-1 using canine serum antibodies.**

Western blot showing the expression of CAV-1 proteins in infected MDCK cells using pooled canine serum as a primary antibody. Cell lysates were harvested 3, 5 and 7 days post infection and protocol carried out as for normal western blot as described in materials and methods. The location of the molecular weight marker (kDa) is shown to the left of the blot. Location of viral proteins is indicated by arrows to the right of the blot. a) 3 days post infection, b) 5 days post infection, note the discontinuity of the samples as noted by *, c) 7 days post infection.

# Chapter 4.

## Discussion

### 4.1 Development of the two step amplification system for targeting cancer cells.

Many gene therapy trials, even though proven to be safe, have shown no treatment efficacy (Nathwani et al. 2004, Trent & Alexander 2004, Selkirk 2004) demonstrating that improvements in current systems are necessary. In order to increase cell specific transgene expression, various systems have been developed. The TSTA system used in conjunction with the Gal4VP16 fusion protein has shown great potential to increase specific transgene expression (Fang et al. 1998, Gu & Fang 2003). In this project an alternative to Gal4VP16, VP16E2 was used in a bid to improve the TSTA approach.

#### 4.1.1 Comparison of the Gal4VP16 and the VP16E2

To improve transgene expression for gene therapy it was proposed that the VP16E2 transcriptional activator fusion protein, which has been shown to be a strong transcriptional activator in mammalian cells in previous work (Vance et al. 1999, Vance et al. 2001), could offer a potential improvement to the Gal4VP16 TSTA system due to the origin of the E2 DNA binding domain from a virus specialised in replicating in mammalian cells. This was tested by comparing the Gal4VP16 and the VP16E2 in a TSTA system under control of a CMV promoter followed by use of the hTR and hTERT telomerase promoters. Figure 3.1.2 shows that in conjunction with the CMV promoter, the VP16E2 transcriptional activator may elicit a higher level of reporter gene expression than Gal4VP16. Western blotting (figure 3.1.3) shows that VP16E2 is better expressed than Gal4VP16 and this may relate to differential transcriptional activity. Although both proteins are expressed from the CMV promoter, the VP16E2 protein may be more stable than Gal4VP16, explaining the difference observed.

The two step amplification system with incorporation of the hTR and hTERT promoters was tested in 3 different cell lines known to have telomerase activity (figure 3.1.5-3.1.7). As expected the reporter gene expression was not as strong in conjunction with the two step amplification mechanism initiated by the two telomerase promoters

compared to the CMV promoter due the relative low transcriptional activity of the telomerase promoters. These experiments demonstrated that the VP16E2 transcriptional activator induces a stronger level of reporter gene expression than the Gal4VP16 in all cell lines. The advantage of the VP16E2 over the Gal4VP16 transcriptional activator is particularly pronounced in the MCF-7 and the C33a cell lines. In these two cell lines the difference between the reporter gene expression between VP16E2 and Gal4VP16 is 10 fold. In comparison the maximal difference observed in the 293-T cells is only 3 fold. The C33a cell line is a keratinocyte cell line and it is therefore possible that the pronounced effect from the VP16E2 transcriptional activator in this cell line could be associated with the epitheliotrophic origin of the E2 DNA binding domain. This suggests that the VP16E2 transcriptional activator could be especially potent in treating cancers originating from epithelial cells.

The VP16 sequences used in both the Gal4VP16 and the VP16E2 fusion proteins were the same, suggesting that the difference seen in transcriptional activation between these two fusion proteins could be related to the DNA binding domains and their abilities to bind to specific DNA sequences, their stability and their interaction with the VP16 and cellular transcription factors. Both the Gal4 and the E2 bind to their specific DNA binding sites as a dimer (Walker et al. 1993, McBride & Myers 1996, Hedge 2002, Traven et al. 2006) and fused to the VP16 this binding recruits cellular transcription factors to the site. The full length Gal4 is a regulatory gene in yeast *Saccharomyces cerevisiae* and is important for inducing transcription of genes for galactose metabolism (Traven et al. 2006). It is however only the Gal4 DNA binding domain of the Gal4 regulatory domain that has been incorporated into the Gal4VP16 construct. The E2 used in the VP16E2 construct consists of the C-terminal 161 amino acids of the E2 gene from the epitheliotrophic bovine papillomavirus type 1. The N-terminal part of the E2 gene encodes a transcriptional activation domain (Hegde 2002), however this sequence is not included in the VP16E2 construct. One hypothesis is that the derivation of the E2 from a mammalian virus renders this protein more potent than the Gal4 protein which functions in yeast cells. Even though there are many similarities in transcription between yeast and mammalian cells there are also differences (Levsky et al. 2007) which could account for the lower activation by Gal4VP16. Both the E2 and the Gal4 DNA binding domains and their interaction with DNA binding sites have been studied and the crystal structure determined (Lohr et al. 1995, Hedge 2002). Though there are differences in the way that the two DNA binding domains fold and bind to

their recognition sequence, it has not been established if this accounts for the difference in transcriptional activation. The Western blots suggested that the VP16E2 was more stably expressed than the Gal4VP16 which is likely to be related to the nature of the E2 domain.

A condition that should be addressed is the spatial placement of the E2 binding sites versus the Gal4 binding sites in the reporter vector (figure 3.1.2 a)). It has been shown that spatial separation of the DNA binding sites from the promoter can reduce the transcriptional activity (Ray et al. 2004). This could explain why the Gal4VP16 shows a lover level of transcriptional activity than the VP16E2. This would however not explain the reduced protein expression seen in the Western blots (figure 3.1.3 and 3.1.8) and it is therefore believed that the spatial separation of the Gal4 DNA binding sites from the promoter in the reporter vector does not account for the difference in transcriptional activity induced by Gal4VP16 and VP16E2.

The potential increase in transcriptional activity by VP16E2 compared to Gal4VP16 in combination with the telomerase promoters provides an opportunity to improve current gene therapy protocols. The fact that transcriptional activity is observed with the VP16E2 at concentrations where no activity is seen with the Gal4VP16 allows for an increase in sensitivity of the TSTA system and potentially widening of the therapeutic window. As described in chapter 1.3, many disease and tissue specific promoters have been isolated and have potential to replace the hTERT promoter in the TSTA system. The survivin promoter, which is active in many cancer forms, would be suitable for an alternative approach targeting multiple cancer types (Zhu et al. 2004, Uchide et al. 2004). A more cancer type specific system could be developed by replacing the hTERT promoter with the prostate specific antigen promoter and enhancer and thereby restricting the use of the system to prostate cancer (Diamandis et al. 1998, Zhang et al. 2002).

### 4.1.2 Testing the specificity of the two step transcriptional amplification system in conjunction with hTR and hTERT.

A potential hazard of developing a very potent TSTA system is the risk of promiscuity of the system which could have detrimental effects to non target cells. Therefore the two step amplification system was tested in conjunction with the VP16E2 and the hTR and hTERT promoters in telomerase negative cells to determine if the

system would provide specificity for telomerase expressing cells (figure 3.1.10). The activity from the hTR and hTERT promoters from these experiments indicates that the hTR promoter is less specific for telomerase activity since it shows a high level of transcriptional activity in the GM-847 and MRC-5 cells in particular. This observation that hTR promoter activity is not reflective of telomerase activity is consistent with previous studies (Weinrich et al. 1997, Bodnar et al. 1998, Yin et al 2004). It has been shown that telomerase activity can be induced in telomerase negative cell lines by transfecting these cell lines with a hTERT expressing plasmid (Bodnar et al. 1998, MacKenzie et al. 2000), indicating that the other elements of the telomerase complex including the RNA template may be continuously expressed.

Activity of the hTERT promoter was observed in the MRC-5 cells. These cells are primary human fibroblasts that had been cultured for several passages at the time of use and a selection for telomerase expressing cells could have occurred. In the TRAP assay performed (figure 3.1.13), telomerase activity was detected in this cell line which could account for the transcriptional activity observed in conjunction with the hTERT promoter.

The results in the telomerase negative cells support the hypothesis that the hTERT promoter is suitable for specific targeting of telomerase expressing cells and also indicates the potential promiscuity of the hTR promoter. It is difficult to directly compare transcriptional activity seen between the different telomerase positive and negative cell lines, however if one examines the transcriptional activity relative to the pCG-VP16E2 transfected cells, there appears to be a therapeutic window at which low transcriptional activity is observed in the telomerase negative cells transfected with the hTERT-VP16E2 and a relatively high level of activity is seen in the telomerase positive cell lines.

### 4.1.3 Testing the system in canine cells.

To be able to use the canine population as a model for human gene therapy it is necessary that the promoters are transcriptionally active in canine cells and are still restricted to the same transcriptional specificity as in human cells. The two step amplification system was therefore tested in the canine telomerase positive MDCK cell line and in the canine primary chondrocyte cell line which was expected to be telomerase negative.

The two step transcriptional amplification system in conjunction with the hTR and hTERT promoters was able to elicit a similar level of reporter gene expression as the CMV promoter in the telomerase positive MDCK cell line (figure 3.1.11) demonstrating that the system can indeed be translated between the human and canine species without losing its potency. The activity of the hTERT, and in particular the hTR, promoter seen (figure 3.1.12) in the primary canine chondrocyte cell line was unexpected. Human primary chondrocytes cultured *in vitro* have however shown to exhibited telomerase activity reflected by expanded life span (Parsch et al. 2004). Indeed the primary canine chondrocytes used demonstrated telomerase activity in the TRAP assay (figure 3.1.13).

The activity of the TSTA system in conjunction with the human telomerase promoters in canine cells also confirms the work done by others showing the homology between the human and canine TERT promoter sequence and function (Long et al. 2005).

### 4.1.4 Testing the transfection efficiency

The TSTA results discussed so far depend upon co-transfection of two plasmids. It was therefore relevant to test that in cell lines which have a transfection efficiency of less than 100% that transfected cells would be transfected with both plasmids. Figures 3.1.15-3.1.17 indicated that the transfection with two plasmids observed by the use of florescent GFP and RFP reporter plasmids overlapped. This effect is important for both *in vivo* and *in vitro* use of the two step amplification system as a two vector system, since transfection with either vector alone would have no therapeutic effect.

### 4.1.5 Evaluation of the one vector system.

The incorporation of the two step amplification system into a one vector system has been shown by others to increase the efficacy of the system. This effect has been observed using adenovirus vectors (Zhang et al. 2002) and a plasmid vector system (Fukuzawa et al. 2004).

When comparing the forward and reverse construct of the 6xE2-3'BpTATA-hTERT-VP16E2 a slight difference in activity was seen between these in telomerase negative and positive cells. As illustrated (figure 3.1.19) the reverse construct showed a

tendency towards greater activity. The increased activity of the 6xE2-3'BpTATA-hTERT-VP16E2 REV in the telomerase negative cells in particular makes the reverse construct less favourable to use.

The general transcriptional activity of the one plasmid system in telomerase positive cells was reduced compared to the two plasmid system. This reduction in activity was most likely to be caused by two effects: Firstly the transcriptional activator is very potent and therefore the rate limiting effect for transcriptional activity is the concentration of the second promoter transcribing the reporter gene. When testing the TSTA system as a two plasmid system the reporter plasmid was added at a dose of 1000 ng regardless of the concentration of the hTERT-VP16E2 plasmid. In the one plasmid system the concentration of the reporter vector follows the hTERT-VP16E2 concentration, accounting for the lower transcriptional activation. To be able to directly compare the efficacy of the one vector versus two vector system one would need to titrate in dilutions of the reporter vector. Another reason for observing a reduced transcriptional activity with the one plasmid system is the potential interference that can occur between the primary and secondary promoters due to their close proximity in the plasmid which can have debilitating effects on the system. Others have managed this problem by using adenovirus vectors and inserting the primary and secondary promoters in distinct regions within the virus, allowing for good spatial separation (Sato et al. 2008). It should be noted that the promoter driving the transcription in the reporter vector used with the two vector TSTA system in figure 3.1.5 is different from the 6xE2-3'BpTATA which was used in the one plasmid system. It is however not expected that this has any influence on the reduced transcriptional activity since the 6xE2-3'BpTATA is a very responsive promoter as seen when tested in the canine cells figure 3.1.11 and as others have described (Vance et al. 2001).

When comparing the 6xE2-3'BpTATA-hTERT-VP16E2 with the Gal46xE2-3'BpTATA-hTERT-Gal4VP16 the advantage of the VP16E2 transcriptional activator compared to the Gal4VP16 is conserved in the one vector system. In previous studies with the two plasmid system (figure 3.1.8), the VP16E2 protein in Western blots was detected as a double band significantly stronger than the Gal4VP16. In the one plasmid system the VP16E2 protein appears as a single band of similar intensity as the Gal4VP16 indicating that the one plasmid system might have a compromising effect on VP16E2 expression (figure 3.1.23). The interaction between the papilloma virus E2 and the telomerase activity in papilloma virus infected cells has been investigated and a

negative correlation was found between E2 expression and telomerase activity (Lee et al. 2002, Seo et al. 2004, Nishimura et al. 2006). It has been suggested that this is an indirect effect in relation to infection with papilloma virus and E2's effect on the viral E6 and E7 expression (Seo et al. 2004, Nishimura et al. 2006). However, others have suggested that E2 binds directly to the hTERT promoter and down regulates transcription (Lee et al. 2002). This interaction of E2 with the hTERT promoter was studied using a full length E2 protein and it is therefore not known if the E2 region contained in the VP16E2 fusion protein has retained this ability. Since no repressive effect was seen by the E2 used in the two plasmid system and since there was a similar reduction in transcriptional activity in the one plasmid system with the Gal4VP16 (which is not known to interact with hTERT) then it is not likely that the VP16E2 has a repressive effect on the hTERT activity but more likely that the reduction in activity is due to limiting concentrations of the 6xE2-3'BpTATA promoter.

When looking at the transcriptional activity of the one plasmid system in telomerase positive and negative cells, it is seen that relative to the activity of the PCG-VP16E2 construct there is a potential therapeutic window in which a concentration would allow for transgene expression in telomerase positive cells whilst not in telomerase negative cells.

### 4.1.6 Killing of telomerase positive cells by incorporation of TRAIL into the TSTA system.

The TRAIL gene has been exploited for the use in cancer gene therapy due to its ability to induce apoptosis in cancer cells but not normal cells (Wiley et al. 1995, Lin et al. 2002). The canine TRAIL (cTRAIL) gene was isolated and cloned to determine if the canine gene shared functional homology with its human counterpart. The cTRAIL and hTRAIL share 87% sequence homology (figure 3.1.26) and the function of the TRAIL gene in inducing apoptosis is preserved in human and canine species as observed by detection of activation of apoptosis related proteins in Western blots (figure 3.1.27-3.1.29). Work has been published showing that canine cancer cells are sensitive to TRAIL induced apoptosis by treatment with soluble human TRAIL (Spee et al. 2006). The isolation of canine TRAIL and its ability to induce apoptosis in canine cells supports these findings.

The results from the TUNEL assays (figure 3.1.31) demonstrated that the full length TRAIL in conjunction with the TSTA system could be used in telomerase targeted cancer gene therapy as it induces apoptosis in telomerase expressing cells only. The characteristics of the TRAIL gene to induce apoptosis in cancer cells whilst not in most untransformed cells is an extra security barrier in the system since unspecific TRAIL expression in normal cells would not necessarily be toxic (Wiley et al. 1995, Lin et al. 2002). There is a concern when using such a system to target telomerase expressing cancer cells that ALT dependent cells will be selected (Yan et al. 2002, Reddel 2003, Harley 2008, Nittis et al. 2008). Therefore it is important that the transgene used for cell killing shows a pronounced bystander effect allowing for apoptosis induction in cells that are not expressing the transgene. Full length TRAIL has been shown to carry out a marked bystander effect (Kagawa et al. 2001). Since TRAIL is a trans-membrane protein it is most likely that this bystander effect is induced by cell-cell interactions rather than via diffusion or gap junctions (Kagawa et al. 2001).

Unlike the U2OS cell line which has been shown to be TRAIL sensitive (Garnett et al. 2007), the 293-T cell line used for the TUNEL is commonly used for generating TRAIL expressing virus vectors (Ma et al. 2005, Kim et al. 2006) and is therefore considered partially TRAIL resistant. The Western blot (figure 3.1.27) and the TUNEL stain, however, show that 293-T cells were susceptible to TRAIL induced apoptosis as others also have reported (Ozoren et al. 2000). The full length TRAIL has been used in combination with our TSTA system although it is possible to use versions of the TRAIL gene encoding recombinant soluble TRAIL forms to allow for secretion of the TRAIL gene (Kim et al. 2004a), Ma et al. 2005, Kim et al. 2006). This effect would significantly increase the bystander effect of the treatment, however, it has been shown that some cancer cells are resistant to soluble TRAIL but are still sensitive to killing by full length TRAIL (Voelkel-Johnson et al. 2002, Seol et al. 2003). Therefore full length TRAIL was chosen for use in our system.

Many other genes, and in particular suicide genes, have shown a great potential for cancer gene therapy as described in chapter 1.1 and the TRAIL gene in our TSTA system could be replaced with any of these genes to alter the treatment. In combination with other promoters our TSTA system could also be applied to other types of gene therapy in which a tight regulation of transgene expression would be required.

Our TSTA system in conjunction with TRAIL shows telomerase specific cell killing, demonstrating this system has potential for cancer therapy. To apply a system

like this to *in vivo* therapy one would have to consider how to deliver the DNA to cells. Most work using full length TRAIL for gene therapy has been done using adenoviruses as gene delivery vectors which has a synergistic effect since some adenoviral proteins sensitise cells to TRAIL induced apoptosis (Voelkel-Johnson et al. 2002). It is important however to remove the adenoviral E3 region since this region encodes proteins that down regulate TRAIL receptor expression and hence inhibit apoptosis (Howitz 2004). As described in chapter 1.2 there are now many ways of increasing cellular uptake of DNA and therefore the TSTA plasmid system could potentially be applied to *in vivo* using physical cellular delivery mechanisms to improve the uptake of the plasmid DNA.

### 4.1.7 Alterations to improve the TSTA system.

As described by others (Zhang et al. 2002) the TSTA system is a flexible system with many variables that can be altered to improve the efficacy. This includes altering the promoters and transcriptional activator elements but also spatial alterations that can alter the system (Zhang et al. 2002, Fukuzawa et al. 2004, Sato et al. 2008). As described in chapter 3.1.7, the spatial effect of incorporating the TSTA system into one vector was studied and did not show any obvious improvement to the system. In order to study the impact of other elements in the telomerase responsive TSTA system in conjunction with the VP16E2, alterations of the telomerase promoter, the VP16, the E2 and the E2 binding sites were studied.

It was investigated whether a xenogeneic versus an allogeneic TERT promoter would increase the transcriptional activity as others have reported when using the mouse TERT promoter in human cells (Horikawa et al. 2005). We observed (figure 3.1.36) a 2 fold increase in transcriptional activity with the 665 base pair dogTERT promoter compared to the human hTERT promoter when administered at a dose of 1000ng in human cells. This effect was not however replicated in the 2000ng dose and therefore it was concluded that no clear advantage was seen using a xenogeneic promoter in either canine nor human cells. The results from this study did however demonstrate that the hTERT and dogTERT promoters function to a similar extent in both species and that systems developed for treatment in either species can be translated into use in the other which could be useful when using canine patients as models for human cancer treatment.

The impact of incorporating an extra VP16 domain into the TSTA system was studied. Others have reported a 3 fold increase in transcriptional activity of the TSTA system when incorporating a tandem repeat of the VP16 sequence compared to a single construct (Zhang et al. 2002). This effect was observed with the VP16 sub-domain (amino acids 413-454) which have been shown to be sufficient for activating a promoter with multiple binding sites for the transcriptional activator fusion protein (Walker et al. 1993, Flint & Shenk 1997). Our results (figure 3.1.39) demonstrated an increase in transcriptional activity with the incorporation of the VP16 in tandem versus the single construct although the effect was not as pronounced as reported by others (Zhang et al. 2002). The VP16 sequence used to construct the VP16 in tandem consisted of amino acids 410-487 of the VP16 protein. Amino acids 455-487 are not necessary for activation of the 6xE2-3'BpTATA promoter since this promoter has several DNA binding sites for the transcriptional activator fusion protein and perhaps a more pronounced difference in effect with the tandem protein would be seen in conjunction with a shorter VP16 fragment. Work we have done comparing the effect of a shorter VP16 versus a longer VP16 fragment in conjunction with the Gal4VP16 transcriptional activator did not reveal any significant difference in transcriptional activity and therefore the VP16 fragment used in the VP16E2 fusion protein could be reduced to a shorter fragment by removing the terminal 454-487 amino acids (Walker et al. 1993, Flint & Shenk 1997).

Increasing the number of transcriptional activator binding sites has been shown by others to improve the efficacy of the TSTA system. We studied this effect by using the TSTA system in conjunction with the BPV-4 PV2 promoter with 4, 8 or 12 upstream E2 binding sites. No improvement in transcriptional activity was observed by increasing the number of E2 binding sites upstream of the PV2 promoter in 293-T cells (figure 3.1.41). In C33a cells (figure 3.1.42), a small increase in transcriptional activity was observed. Others reported a 60 fold increase in transcription when increasing the number of Gal4 binding sites from 2 to 5 in conjunction with the Gal4VP16 transcriptional activator fusion protein and the TSTA system (Zhang et al. 2002). We do not observed such a of dramatic effect although the lowest number of E2 binding sites used in this study was 4 and perhaps a saturation effect had already been reached, allowing for only small increases in effect. The PV2 promoter is derived from BPV 4 and is a longer 127 base pair version of the 3'BpTATA promoter described by others (Vance et al. 2001). This promoter construct has retained an epitheliotropic predilection

from the original BPV 4 promoter from which it is derived. This could explain the lack of any effect from increasing E2 sites when the system was tested in 293-T cells while an effect was observed in the C33a keratinocyte cell line. The increase in E2 binding sites could be studied in conjunction with the 3'BpTATA promoter to determine the direct effect on our TSTA system although our results with the PV2 promoter suggested that no effect will be seen and therefore the inclusion of 6 E2 binding sites appeared sufficient.

Modifying the E2 protein in order to increase stability was studied also. Others reported a 20 fold increase in viral BPV-1 replication when mutating the E2 serine residue 301 to an alanine, thereby eliminating a major phosphorylation site (Mcbride & Howley 1991). We carried out the same point mutation in our VP16E2 fusion protein and examined protein expression from this construct. This mutation increased expression of the VP16E2 fusion protein (figure 3.1.43), but the effect of the mutated VP16E2 has not been studied in transcription assays however this should be done before incorporating it into the final TSTA system.

### 4.2 Evaluating the CAV-1 based CRAd

The CAV-1-hTERT+E1 virus is not suitable for use in human oncolytic therapy due to the species specific replication of CRAds renders them unsuitable for cell killing in species other than their original host species (Hemminki et al. 2003). Such species specificity is a problem for studying the effect and possible toxicity of CRAds in animal models. The many similarities in pathogenesis and aetiology between human and canine cancers, as well as other diseases, makes the dog a suitable spontaneous animal model for studying cancer and cancer therapy (Hansen & Khanna 2004, Paoloni & Khanna 2007, Paoloni & Khanna 2008). The dog has been used for a long time by the pharmaceutical industry for studying drug metabolism since the physiological similarities between humans and canines results in drugs being utilised in a similar fashion between these species. This has led to a recent interest in studying cancer in dogs as a comparative model for humans (Mack 2006). The development of the CAV-2 based CRAd to specific target killing of osteosarcoma cells in canines is an attempt to develop a cancer treatment for dogs with the aim of developing a potential treatment for human cancer (Le et al. 2006). As in humans, dogs commonly have antibodies against canine adenoviruses making this a suitable comparative animal model system. The

CAV-1-hTERT+E1 was developed in order to study the effect of a CRAd in an animal model system. This system has in comparison to the osteosarcoma targeted canine CRAd a broader application since the telomerase promoter is active in a wide range of cancers, with up to 95% of canine cancers testing positive for telomerase activity (Yazawa et al. 1999). The incorporation of the TSTA system increases the sensitivity of the CRAd compared to conventional CRAds which do not contain an amplification system. The CAV-1 which was used to create the CAV-1-hTERT+E1 is a highly pathogenic virus which, unlike CAV-2, can cause severe disease and can be found in many tissues following an infection (Decaro et al. 2007). This pathogenesis can be seen as a threat to the safety of the virus for cancer treatment. However, since most dogs are vaccinated against CAV-1, the potential for this virus or for the mutant CAV-1-hTERT+E1 to cause disease is limited. The ability of CAV-1 to replicate in multiple tissues and its pathogenic potential supports the use of this virus to induce cell lysis in different cancer types originating from various cell types. The use of the CAV-1 fiber protein to generate hybrid human gene therapy vectors with an increased tropism illustrates the value of this virus in targeting many tissue types (Zheng et al. 2007). The presence of neutralising antibodies against CAV-1 is not seen as a direct disadvantage of the system but rather as a safety precaution assuring that the virus will not induce disease and perhaps lead to an increase in treatment efficacy by immunological support as seen with immunity against oncolytic herpes viruses in mice models (Zhu et al. 2007). There are several routes by which oncolytic virus treatment can be administered. The CAV-1-hTERT+E1 is developed with the intention of being used for local cancer therapy by intratumoural injection. This mode of delivery circumvents the problem regarding neutralisation by circulating antibodies but still maintains the benefit of immunological support to eradicate the tumour cells. In clinical trials in humans, antitumoural effects have been observed with all oncolytic viruses delivered intratumourally (Liu et al. 2007). On the other hand, systemic delivery has not shown to result in a similar clinical effect (DeWeese et al. 2001, Small et al. 2006, Alton et al. 2007). The reduced response observed following systemic delivery could be the result of too low viral concentrations at the tumour site. Ideally an oncolytic virus should be administered systemically for the virus to be able to target distant metastases, however this application remains a challenge. Anti-tumoural responses in metastases after intratumoural oncolytic injection of the primary tumour have been observed, suggesting

that the immune response plays a supporting role in the anti-tumour activity of the virus treatment (Liu et al. 2007).

### 4.2.1 CAV-1-hTERT+E1

The CAV-1-hTERT+E1 virus was able to induce cell lysis in telomerase positive cells while no cytopathic effect was observed in telomerase negative cells as reflected in the $TCID_{50}$ assays (chapter 3.2.3). In comparison, the wild type virus 331 killed both telomerase positive and negative cells, with higher levels of cell killing observed in telomerase negative cells. The observation that no cell toxicity at all was observed in the telomerase negative cells infected with the CAV-1-hTERT+E1 implies that no toxic effect occured in the absence of viral replication. The specific replication of the CAV-1-hTERT+E1 in telomerase positive cells and the ability to kill such cells suggests that this virus can be used to selectively kill telomerase expressing cancer cells without harming normal cells. The incorporation of the TSTA system into a replication competent virus is a novel approach which has not been reported previously and therefore our work has taken the TSTA system forward into a replication competent viral system with promising results.

### 4.2.2 Consideration about the CAV-1-hTERT+E1 design.

The viral titres of the CAV-1-hTERT+E1 and the wild type 331 virus secondary virus stock varied markedly, as did the tertiary virus stock. The reason for this great difference has not been determined but there are many possible explanations.

The CAV-1-hTERT+E1 virus depends on telomerase expression in cells in order to replicate. Low activity of the telomerase promoter could therefore limit virus production despite the amplification from the TSTA system in cells which are not sufficiently telomerase positive.

The TSTA is, as the name implies, a two step system and the viral replication with this system will potentially be delayed compared to wild type virus since the transcription and expression of the VP16E2 transcriptional activator is required to activate the transcription of the virus E1 gene and initiation of viral replication. This temporal delay could allow for cellular defence mechanisms to be activated and perhaps premature cell death, before viral particles had matured.

Another factor that could result in lower production of mutant virus is the removal of the E1 promoter region in the CAV-1-hTERT+E1. This region is located in the proximity of the viral packaging signals. Comparison of the CAV-1 sequence with other adenovirus sequences suggest that the viral packaging signal was not affected by the removal of the proposed E1 promoter region. Studies in the CAV-2 virus show that alterations in certain sequences between the inverted terminal repeat sequence and the transcription start site for the E1 can significantly alter the viral packaging efficiency (Soudais et al. 2001). In this context it was shown that deletion of the CAV-2 sequence base pairs 302-356, 248-259 and a double deletion from 201-218 and 248-259 reduced the viral packaging efficiency 50, 200 and 500 fold respectively as determined by replication competition assays (Soudais et al. 2001). The sequences of interest in this study were the 5'- TTTA- 3' and 5'-TTTG- 3' motifs located in the CAV-2 virus between base pairs 200-400 in the viral genome. The E1 promoter deletion in CAV-1-hTERT+E1 is from base pairs 282 to 415 in the CAV-1 genome. In this sequence there is both a 5'-TTTG -3' and a 5'-TTTA- 3' motif and it is therefore possible that the removal of the E1 promoter region in the CAV-1-hTERT+E1 has compromised the viral packaging efficiency. This problem could be addressed by studying viral replication and cytopathic effect of different viral mutants containing different E1 promoter deletions.

Wild type human adenoviruses should be able to carry up to 4 kbp foreign DNA without compromising the viral replication (Cao et al. 2004). In the CAV-1-hTERT+E1 1591 base pairs of foreign DNA in total was inserted in the ΔE1 promoter region and the ΔE3 region. The ΔE1 promoter region and the ΔE3 region that were excluded from the virus contained 979 base pairs, hence 621 base pairs of extra DNA were inserted into the virus. It is not known if these additional base pairs could affect the packaging or stability of the virus, however, when the relatively small size of this fragment is compared to the whole viral genome and the capacity of the human adenoviruses to carry additional DNA is considered, then this is most unlikely. It has been reported as well that the CAV-1 can package between 106 to 109% of wild type virus genome size (Morrison et al. 2002)

Another possible reason to account for the low viral production is the viral sequence which could have a cis acting influence on the inserted hTERT promoter. A polyAAA sequence was inserted upstream of the 6xE2-3'BpTATA promoter to

eliminate any up or down regulation of promoter activity by cis acting viral sequences on this promoter. It has been shown that polyAAA sequences are able to terminate any upstream transcriptional activity (Hoffmann et al. 2005, Nag et al. 2006). The hTERT promoter was inserted into the virus without an upstream polyAAA signal. Others have experienced that cis acting virus sequence can compromise the activity of exogenous promoters when inserted into a human adenoviral delivery system (Blackwood & Argyle 2004). It is therefore possible that the low viral replication is a result of a compromised hTERT promoter resulting in low initiation of the TSTA system and hence low viral replication. This could be addressed by inserting a polyAAA or another insulator sequence upstream of the hTERT promoter in the virus.

A further aspect to be considered with regard to the low virus titer is the exclusion of the viral E3 gene in the CAV-1-hTERT+E1 virus. It is known that the viral E3 gene is not essential for adenovirus replication *in vitro* however the region does have other functions which are important for infection (Linne 1992). In human adenoviruses the viral E3 gene region encodes proteins important for down regulation of MHC I expression, down regulation of tumour necrosis factor induced apoptosis and down regulation of FAS and TRAIL receptors. The proteins important for these functions are all expressed as early genes during viral replication (Horwitz 2004). In addition the E3 region encodes a pro-apototic protein named adenovirus death protein (ADP) which has been shown, unlike the other E3 proteins, to be expressed by a major late promoter (Horwitz 2004). The complete CAV-1 E3 region has been deleted in the CAV-1-hTERT+E1 virus. This deletion could have impacted upon the ability of the virus to induce cell lysis and could explain the low virus titer of the CAV-1-hTERT+E1 virus stocks. The CAV-1 E3 gene region is significantly different both from the human adenovirus E3 region and from the CAV-2 E3 region (Linne 1992, Morrison et al. 1997). A comparison between the CAV-1 and CAV-2 E3 regions has shown that they share conserved N and C terminal regions however that there is a significant difference in the majority of the E3 region and the open reading frames. The CAV-2 virus E3 region contains 5 open reading frames whilst the CAV-1 E3 contains only 2 (Linne 1992). It is not known exactly what the effect of this difference is, but it is hypothesised that the difference could account for the difference in pathogenesis between the CAV-1 and CAV-2 viruses (Linne 1992). The exact function of the CAV-1 E3 region is not known and it is therefore not known if it encodes a protein with a similar function as the ADP found in human adenoviruses. It is therefore uncertain if the low titer of the CAV-

1-hTERT+E1 could be caused by compromised viral cell lysis due to the lack of the E3 coding region. The great differences between the CAV-1 and other adenoviruses in the E3 region suggest that this region might be dispensable for cell lysis in CAV-1. The importance of the E3 region for viral induced lysis could be studied by infecting cells with CAV-1 virus with and without a deletion of the E3 coding region. Subsequently this, a simple comparison of the cytopathic effect on the cells infected with virus with and without E3 should demonstrate the effect of the E3 deletion.

### 4.2.3 Detection of virus DNA and proteins

PCR analysis (figure 3.2.14) confirmed that there was no contamination between the wild type and mutant viruses in the virus stocks and provided an indirect measurement of the amount of virus template present in the stocks. The presence of a PCR product from the 331 virus after only 15 PCR cycles in comparison to the CAV-1-hTERT+E1 which only produced a product after 20 cycles suggested that the 331 virus was more concentrated than the CAV-1-hTERT+E1. This result could reflect the difference observed in the virus titre between these two viruses. The result from the PCR could therefore indicate that either defective virus particles are produced with the CAV-1-hTERT+E1 infections or that viral particles are not released efficiently. This is supported by the fact that PCR was performed on viral stocks which were generated by lysing infected cells allowing for intracellular virus particles to be released.

In figure 3.2.19 a Western blot is shown from cells infected with various amounts of CAV-1-hTERT+E1 and 331 virus. Two interesting points emerged from this experiment. Firstly the antibody used to detect the CAV-1 virus in this experiment was a polyclonal antibody raised in rabbit against Ad-5. It is evident from the Western blot that cross reactivity exists between antibodies against Ad-5 and CAV-1. One of the arguments for developing canine adenoviruses as human gene delivery vectors has been that this provided a means to circumvent the problem with humans having pre-existing antibodies against the human adenoviruses (Kremer et al. 2000). If human adenovirus antibodies cross react with canine adenoviruses, then using a canine adenovirus as a gene therapy vector will not change the neutralising effect of circulating antibodies. The second thing that was interesting about the Western blot was that in agreement with the cytopathic effect that was observed on visual inspection of the infected cells, virus proteins from the CAV-1-hTERT+E1 virus were detected at 3 days post infection while

wild type virus proteins are only detected at 5 days after infection. This observation does suggest that the concentration of the secondary CAV-1-hTERT+E1 viral stock was higher than suggested from the $TCID_{50}$ assays.

In the Western blots stained with canine serum antibodies (figure 3.2.21) supported by pictures (figure3.2.20) the same effect was observed with virus proteins being observed at an earlier time point for the CAV-1-hTERT+E1 than the 331 virus. Another interesting thing about this western blot is the pattern of cell killing which was observed on the supporting pictures. For the CAV-1-hTERT+E1 sporadic cytopathic effect was seen at concentrations of 500 and 1000 PFU per plate after only 3 days and more pronounced at 5 days. In comparison the wild type virus showed cytopathic effect localised to plaques but only after 5 days of infection. This observation does suggest that theactual concentration of the CAV-1-hTERT+E1 stock is higher than determined by the $TCID_{50}$ assay but that the virus may not replicate as efficiently as the wild type virus ormay not be released. At day 7 after infection the cytopathic effect was more pronounced in the 331 infected plates, than the CAV-1-hTERT+E1 infected plates indicating that although the wild type virus was slow initially to generate a cytopathic effect itthen replicated faster than the CAV-1-hTERT+E1 virus.

The Western blots were not quantitative for viral replication but did provide evidence of viral replication as seen by the increase in viral protein between early and late harvested plates. This, supported by the cell killing observed in the pictures, provided evidence that viral replication and cytopathic effect are correlated.

Putting all these observations together the data suggest that the CAV-1-hTERT+E1 virus is compromised in replication, cytopathic effect or packaging compared to the wild type virus and possible modifications could be carried out to improve the viral replication and cytopathic effect. The good telomerase specificity observed with the CAV-1-hTERT+E1virus is however an important strength for the virus and alterations to be made should not compromise this.

### 4.2.4 Bystander effect of the virus.

As with the TRAIL telomerase targeted gene therapy, there is a risk that a positive selection pressure will be put on ALT dependent cells when using the telomerase controlled CRAd to kill cancer cells (Yan et al. 2002, Reddel 2003, Harley et al. 2008, Nittis et al. 2008). There is no direct bystander effect from the virus on surrounding cells although the immune response may play an important role since viral

replicaton can induce an immune response against cancer associated antigens and can therebyincrease the anti-tumoural efficacy of the virus (Rein et al. 2006, Liu et al. 2007). Armed oncolytic viruses carrying a cytotoxic or a suicide gene provides means of incoporating a bystander effect to the oncolytic viral system (Ye et al. 2005). In this system,expression of the transgene will enable targeting surrounding cells which might not support viral replication. In this context our CRAd could be reinforced with a TRAILgene. The adenovirus E3 region which is known to down regulate expression of TRAILreceptors (Horwitz 2004) is removed in our CRAd and therefore TRAIL could be verypotent in inducing cellular apoptosis in our system. Due to the strength of the VP16E2 transcriptional activator, an incorporated TRAIL gene could be under the transcriptional control of a second 6xE2-3'BpTATA promoter as illustrated in figure 4.1, since the VP16E2 would be able to activate two 6xE2-3'BpTATA promoters as demonstrated when testing low levels of VP16E2 in conjunction with high levels of reporter vector.

Oncolytic viruses are often used in combination with chemotherapy (Liu et al. 2006). Some of the stem cell cancer models suggest that stem cells are highly resistant to mostconventional therapies such as chemotherapy however that they possess strong telomerase activity and use of a combination of telomerase targeted therapy and chemotherapy may eradicate both cancer and cancer stem cells (Shay & Keith 2008).

## 4.3 Conclusion

The major obstacles in gene therapy are specific targeting of transgene expression, adequate levels of transgene expression and efficient *in vivo* transgene delivery. The achieving work in this thesis has been carried out in order to address each of these areas.

A novel TSTA system in conjunction with the VP16E2 transcriptional activator fusion protein, the 6xE2-3'BpTATA promoter and the hTERT promoter allows for a strong level of specific transgene expression in telomerase expressing cells, at a higher level than the commonly used Gal4VP16 system. The system in combination with the full length TRAIL as a transgene allows for specific apoptosis induction in telomerase expressing cells without exerting toxicity to non telomerase expressing cells.

The development of the CAV-1-hTERT+E1 under replicative control of the hTERT promoter and with incorporation of the TSTA system allows for specific killing of telomerase expressing cells and addresses the difficulty of *in vivo* delivery. This CAV-1-hTERT+E1 is a valuable tool for investigating telomerase targeted oncolytic cancer treatment in an immune competent spontaneous canine cancer model system. Further development of this virus will have application for gene therapy in canines and humans.

**Figure 4.1**

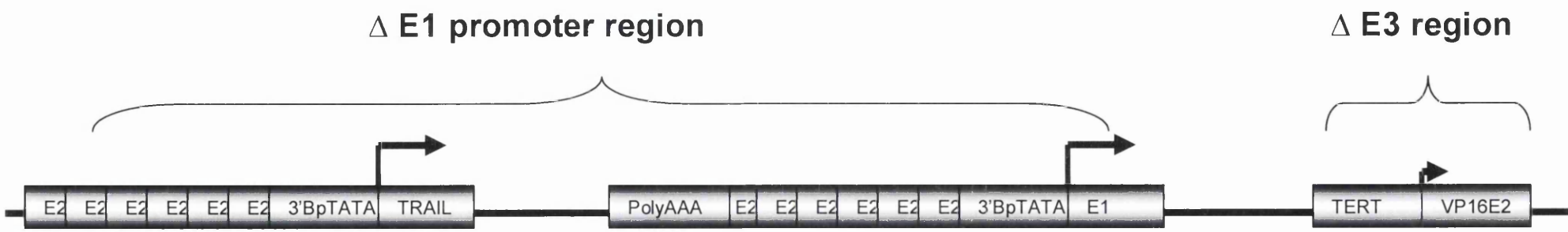



**Figure 4.1 illustration of incorporation of a lethal gene in the CRAd.**

Figure shows schematic presentation of how a lethal gene such as TRAIL could be incorporated into our CRAd. The TRAIL gene and the promoter could be placed in another location in the virus such as the E3 region upstream of the TERT promoter.

### 4.4 Future work

The plasticity of the TSTA system and the many possible applications in gene therapy leaves this project with a broad range of possible study aspects to be carried forward. As seen in figure 4.2 both the hTERT promoter and the transgene can be exchanged for other units to target the system towards other diseases. This opens the possibility for studying the use of the TSTA system for targeting other cell types and other diseases.

The transcriptional activator fusion protein can also be altered to optimise the system further such as by altering the length and the number of VP16 coding sequences. Other approaches to improve the TSTA system further could be incorporation of control elements such as tetracycline activation or repressor elements upstream of the hTERT promoter allowing for a possible *in vivo* control of transgene expression.

A positive feed back reinforcement of the TSTA system could easily be incorporated by placing E2 binding sites upstream of the hTERT promoter. In this form the transcription of the VP16E2 would act as a positive feedback on further VP16E2 transcription by activation of the hTERT promoter as seen in figure 4.3. It is important to ensure that modifications which offer increased efficacy of the TSTA system do not compromise the specificity of the system.

There are several aspects of the CAV-1 based CRAd that should be improved in future work. The viral stock of the CAV-1-hTERT+E1 generated to date is relatively dilute and therefore a more concentrated viral stock should be produced. This can be achieved by using more cells in larger volumes of media which when harvested are subject to ultracentrifugation in order to concentrate viral particles in a small volume. The current viral stocks originated from one cosmid clone after the second step of homologous recombination. Other clones have been isolated during the process and these could be studied to see if they would generate a more potent CRAd in case any compromising mutations may be present in the CAV-1-hTERT+E1genome. A negative control virus based on the CRAd but without the E1 insert should be generated since this would act as a good negative control to ensure that non replicating virus particles do not exert a deleterious effect on their own. This virus could be generated in an E1 complementary cell line. It would also be interesting to look at the effect of virus replication and cell lysis in viruses with and without deletion of the viral E3 region. A more simplistic CRAd with the hTERT driving the transcription of the E1 gene without

the TSTA system could also be interesting to study to see if the advantage of the TSTA system seen in the plasmid system is retained in the viral system. Examining different E1 deletion sequences could be useful to determine if an alteration could improve viral packaging.

Real time PCR with a probe and primer set directed against viral DNA sequences should be carried out to evaluate the replication of viral particles in culture. In this aspect it would be interesting to monitor the amount of viral DNA present in infected cells and media at different time points after infection. Comparing the intra and extra cellular viral DNA level could give valuable information regarding the replication and release of the virus as has been studied by others (Morrison et al. 2001). It would however not differentiate either defective viral particles or virus DNA which is not packaged from functioning infectious viral particles.

A future perspective of this work is to apply the CAV-1 based CRAd CAV-1-hTERT+E1 to a clinical setting. Injecting of the CRAd into viable resected tumour tissue and following fixation and staining of the tissue could give useful information on short term cellular uptake of viral particles and immediate distribution of the virus. In order to look at viral replication and actual spread of the virus the CRAd could be applied to terminally ill canine cancer patients prior to euthanasia. This would require generation of large quantities of purified virus which would be generated in a commercial lab. Virus could be applied to terminally ill canine cancer patients prior to euthanasia at a time point which would allow for viral replication to occur before euthanasia. It could also be applied in a similar fashion locally to tumour tissues prior to surgical removal. Post mortem or surgically resected tissues could then be evaluated to determine viral spread and replication. Blood samples could be evaluated alongside to determine whether local viral injection would give rise to acute systemic adverse effects. Despite the argument that the CAV-1 based CRAd circumvents the use of rodent animal models it is still essential for safety purposes that rodent studies are performed to examine *in vivo* toxicity and potential carcinogenicity of the virus. Once treatments have been considered safe and effective in these model systems, the goal would be to take this forward to phase I, II and III clinical trials for canine cancer patients, most likely as a combination therapy in conjunction with surgery and chemotherapy.

**Figure 4.2**

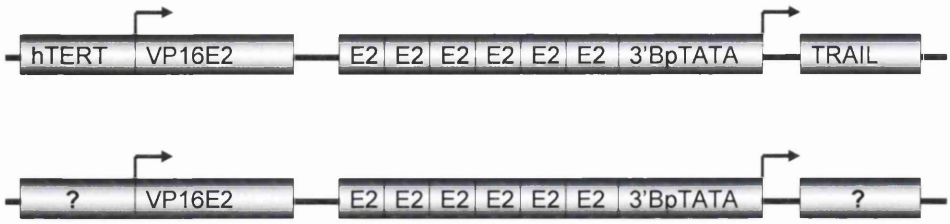



**Figure 4.2 Promoters and transgenes can be exchanged in the TSTA system.**

The plasticity of the TSTA system allows for promoters and transgenes to be exchanged to target specific diseases and treatments.

**Figure 4.3**

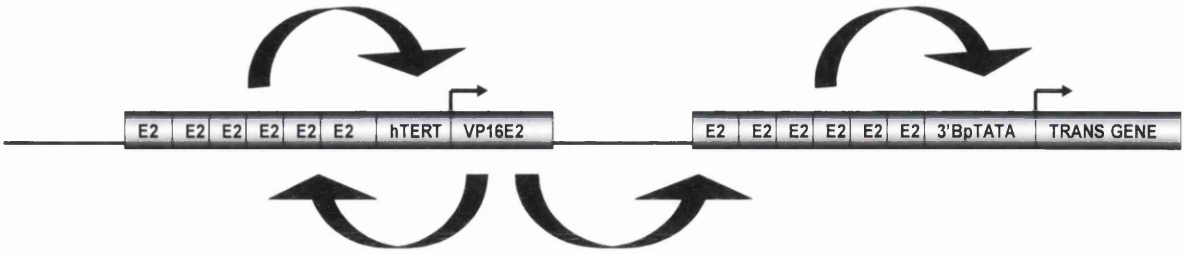



**Figure 4.3 Positive feedback activation of the hTERT promoter**

Illustration of insertion of E2 binding sites upstream of the hTERT promoter to act as a reinforcing positive feedback mechanism on promoter activity.

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