Telomere Dynamics and Telomerase Expression in Chronic Myeloid Leukaemia

by

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Related Publications

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Dedication

This work is dedicated to my wife Angela, and my children Finlay and Erica.

Author's Declaration

Unless otherwise stated I declare that all the work presented in this thesis is my own.

31/7/03

Definitions and Abbreviations

A	Adenine
7AAD	7-amino-actinomycin D
+α+β	Full-length, functional hTERT (containing A and B RT domains)
+α-β	Non-functional hTERT, with deletion of B RT domain.
-α-β	Non-functional hTERT with deletion of A and B RT domains
-α+β	Non-functional hTERT with deletion of A RT domain
ALL	Acute Lymphoblastic Leukaemia
AlloSCT	Allogeneic Stem Cell Transplant
ALT	Alternative Lengthening of Telomeres
AML	Acute Myeloid Leukaemia
AP	Accelerated Phase CML
ATP	Adenine Tri-Phosphate
BCR	Breakpoint-Cluster Region Gene
BCR-ABL	The BCR-ABL Protein Tyrosine Kinase
BCR-ABL	The BCR-ABL fusion gene
BM	Bone Marrow
BP	Blastic Phase CML
BSA	Bovine Serum Albumin
CCR	Complete Cytogenetic Remission
cDNA	Complementary DNA
CLL	Chronic Lymphocytic Leukaemia
CLP	Common Lymphoid Progenitor
CML	Chronic Myeloid Leukaemia
CMP	Common Myeloid progenitor
CP	Chronic Phase CML
CV	Co-efficient of Variation
C	Cytosine
ddH_20	Double-distilled water
DEPC	diethyl pyrocarbonate
DKC	Dyskeratosis Congenita
D-Loop	Displacement Loop
DMSO	Di-methyl Sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine Tetra-Acetic Acid
estCP	Established Chronic Phase
FACS	Fluorescence-Assisted Cell Sorting
FCS	Foetal Calf Serum
FISH	Fluorescence *in situ* Hybridisation
FITC	Fluoroscein Isothiocyanate
FNA	Fine Needle Aspirate
FRET	Fluorescence Resonance Energy Transfer
FSC	Forward Scatter
G	Guanine
G0	Gap-phase 0 (of cell cycle)
G1	Gap-phase 1 (of cell cycle)
G2	Gap-phase 2 (of cell cycle)
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
HDF	Human Diploid Fibroblast
HR	High-Risk Hasford Score
HSC	Haemopoietic Stem Cell

hTERT	The catalytic component of human telomerase
hTR	The RNA component of human telomerase
IFN	Interferon
IR	Intermediate-Risk Hasford Score
KO	Knock-Out
LBP	Lymphoid BP
LDL	Low-Density Lipoprotein
LR	Low-Risk Hasford Score
M-phase	Mitotic phase of cell cycle
M0	Mortality 0
M1	Mortality 1
M2	Mortality 2
MACS	Magnetic Cell Sorting and Separation
MBP	Myeloid BP
MESF	Molecular Equivalent of Soluble Fluorochrome
MNC	Mononuclear Cell
mRNA	Messenger RNA
NHEJ	Non-Homologous End-Joining
PBGD	Porphobilinogen Deaminase
PBL	Peripheral Blood Leucocytes
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
Ph	Philadelphia Chromosome
PHA	Phytohaemagglutinin
PI	Propidium Iodide
PNA	Peptide Nucleic Acid
POT1	Protection of Telomeres 1
Q-FISH	Quantitative FISH
Q-RT-PCR	Real Time RT-PCR
rHu-G-CSF	Recombinant Human Granulocyte Colony Stimulating Factor
RB	Retinoblastoma tumour suppressor protein
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROS	Reactive Oxygen Species
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAA	Severe Aplastic Anaemia
SD	Standard Deviation
SE	Standard Error
SNBTS	Scottish National Blood Transfusion Service
snoRNA	Small Nucleolar RNA
S-phase	Synthesis phase (DNA) of cell cycle
SSC	Side Scatter
T	Thymine
TBP	Telomere Binding Protein
T-Loop	Telomere Loop
TRAP	Telomere Repeat Amplification Protocol
TRF	Terminal Restriction Fragment
TRF1	Telomere Repeat Binding Factor 1
TRF2	Telomere Repeat Binding Factor 2
TSA	Trichostatin A
UCB	Umbilical Cord Blood
WBC	White Blood Cell
WHO	World Health Organisation

Abstract

INTRODUCTION: Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder of the haemopoietic stem cell (HSC), with a variable clinical course. Chronic phase (CP) disease, typically of 4-5 years duration, progresses to accelerated (AP) and then blastic phase (BP), with the latter behaving as a particularly aggressive acute leukaemia of either myeloid (MBP) or lymphoid (LBP) lineage. Treatment is most successful when delivered in CP, and accurate prognostic indices are required to individualise treatment. Increased telomere shortening has been described during progression of CML, and may be of prognostic relevance. Paradoxically, telomerase activity (TA, as determined by the TRAP assay) has been shown to be elevated in the CP stem/progenitor cell ($CD34^+$) compartment; however this may not accurately reflect telomere maintenance *in-vivo*. We sought to further define the prognostic significance of telomere shortening at diagnosis of CML, monitor the rate of telomere loss during the disease and characterise expression of the major telomerase components (hTR and hTERT) in $CD34^+$ selected cells at diagnosis and during disease progression.

METHODS: Peripheral blood leucocyte (PBL) telomere length measurement was performed by flow-FISH on cohorts of normal individuals, patients at diagnosis and all stages of CML. To define the degree of telomere shortening in individual patients at diagnosis *ex-vivo* expanded (*BCR-ABL*$^-$) T-cells were used as an internal control for 'normal' somatic cell telomere length. Expression of hTERT and hTR was quantified by Q-RT-PCR and hTERT mRNA splice variants detected by RT-PCR. $CD34^+$ selected cells from CML patients were confirmed as *BCR-ABL*$^+$ by FISH. TA was determined by TRAP assay.

RESULTS: Telomere shortening in CP and AP CML patients progressed at 10-20 times the rate of age-related shortening observed in the normal control group. Furthermore, high-risk prognostic score patients at diagnosis had significantly shorter telomeres than low-risk patients. High purity $CD34^+$ selected cells from CML, as compared to normal, demonstrated increased TRAP activity which correlated with the proportion of cycling cells. However, hTERT mRNA expression was not significantly elevated. Unexpectedly, Q-RT-PCR for hTR demonstrated a mean five-fold reduction in levels in the CML samples, raising the possibility that telomere homeostasis is disrupted in CML. In BP samples, hTERT expression was significantly lower in MBP than LBP and this was mirrored by a corresponding shift in hTERT splicing patterns. MBP hTERT expression correlated inversely with telomere length.

CONCLUSIONS: In summary, increased TRAP activity is not synonymous with telomere maintenance in CML, and dysregulated expression of hTR may contribute to the telomere loss observed in these patients. Indeed TRAP activity appeared largely dependent on the proportion of cycling cells. In the context of progressive (i.e. BP) disease, hTERT expression is lineage and telomere length dependent, thus explaining inconsistent reports of TA levels in BP samples. We have also demonstrated that subtle shifts in splicing of hTERT mRNA is likely to have a regulatory role in primary HSC. In prognostic terms telomere shortening in CML is greatest in high-risk score patients at diagnosis, and occurs rapidly during disease progression. These data further emphasise the potential clinical utility of telomere length measurement for prognostic modelling and monitoring of disease progression.

1. Introduction and Aims

1.1 Normal haemopoiesis

Haemopoiesis is the process by which pluripotent haemopoietic stem cells (HSC) generate the multiple cell lineages necessary to sustain life, a process which is estimated to generate in the region of 10^{11} cells per day. All mature circulating blood cells are derived from a relatively small number of HSCs via a successive series of intermediate progenitors displaying steadily increasing lineage commitment (**Figure 1-1**). Such a hierarchical model of haemopoiesis has provided a paradigm for the development of other tissues and, more recently, leukaemogenesis and tumourigenesis.

Candidate human HSCs can be isolated and characterised functionally (e.g. by the ability of single cells to reconstitute multilineage haemopoiesis in a mouse model or by the ability to establish long-term cell culture *in-vitro*) and by immunophenotyping. The latter is commonly performed in the clinical and laboratory setting using the CD34 antigen (a surface glycoprotein) as a surrogate HSC marker (Berenson, *et al* 1988). The properties of $CD34^+$ cells are well characterised from both a research and clinical perspective (in the context of bone marrow and peripheral blood stem cell transplants), although it is now apparent that even the $CD34^+$ progenitor cell population is in itself extremely heterogeneous.

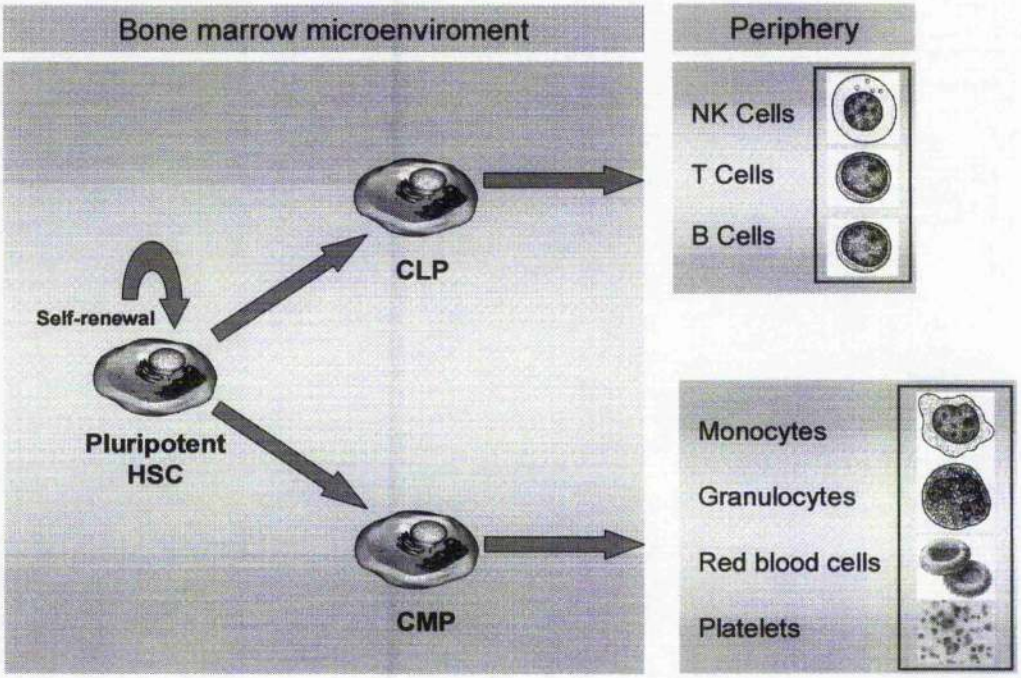


Figure 1-1 A summary of haemopoietic development

A simplified schematic of haemopoietic development from a pluripotent HSC. Such cells are characterised by ability to self-renew, pluripotency, extensive proliferative capacity and rarity (approximately 1 in 10^5 total nucleated bone marrow cells). Each HSC has an enormous capacity to generate large numbers of mature cells over many years, perhaps even for the duration of a human life; in the mouse a single HSC can reconstitute the entire haemopoietic system for the lifespan of the animal (Osawa, *et al* 1996). Lineage–committed primitive progenitors are derived from the HSC, and are functionally and immunophenotypically defined as common lymphoid and common myeloid progenitors (CLP and CMP respectively). Their mature, terminally-differentiated progeny are broadly divided into two groups of cells; lymphoid (small box, top right) and myeloid (medium box, lower right). The term 'myeloid' in its proper sense refers to all cells that are not lymphoid. In its most common usage however it generally refers to granulocytes (neutrophils) and monocytes. While in general terms neutrophils survive only for a few hours, lymphocytes may survive for decades. The implications of this hierarchical organisation, for both the clinical utility of HSC and leukaemic cell biology, are largely self-evident; only true HSCs can contribute to long-term multilineage regeneration post stem cell transplant (with committed progenitors providing only transient engraftment) (Jordan and Lemischka 1990) and leukaemias arising in the HSC compartment may give rise to multi-lineage clonally-derived progeny. Chronic myeloid leukaemia (CML) is the archetypal example of an HSC malignancy.

True HSCs (as defined above) most likely represent a tiny proportion of $CD34^+$ cells: $CD34^+$ cells that are negative (or low) for CD38, thy-1 and CD71 are enriched for HSC activity (Miller, *et al* 1999). Such primitive cells are

predominantly quiescent (i.e. in G_0) (Gothot, *et al* 1997), and it has been estimated by mathematical modelling that they divide, on average, only once every 1-2 years (Vickers, *et al* 2000). Unfortunately such cells are difficult to study *in-vitro*; in addition to their rarity, the mechanism of self-renewal *vs.* differentiation is not clear, and candidate HSC will expand and differentiate in culture conditions, with acquisition of lineage specific markers and concomitant loss of stem cell phenotype. The majority of cells displaying $CD34^+$ therefore represent progenitor populations of varying lineage commitment. That said however, the utility of the CD34 antigen remains of central importance with regards stem / progenitor cell isolation, manipulation and therapies.

Although HSC have an enormous capacity to self-renew and /or differentiate, this capacity is finite; murine HSC can only be serially transplanted 5-7 times in mice before haemopoiesis is exhausted (Harrison, *et al* 1978). Although it is possible that extrinsic stress may contribute to this observation (*i.e.* ex-vivo handling of cells, engraftment in a hostile bone marrow microenvironment *etc.*) the concept that an intrinsic mechanism is operating, perhaps by registering accumulated cell divisions, is an attractive one. The discovery therefore that telomeres (DNA-protein 'caps' which exist at all eukaryotic chromosomal termini) shorten with each cell division *in-vitro* (Harley, *et al* 1990), and with age *in-vivo* (Vaziri, *et al* 1994) subsequently implicated this as one such potential mechanism. In support of these data, the widely conserved enzyme telomerase (via its ability to reinstate lost telomeric DNA-repeat sequence, **Figure 1-2**) enables cells to bypass replicative senescence (the consequence of unchecked telomere shortening) and confer cellular immortality (Bodnar, *et al* 1998). In a haematological context, telomerase deficient murine HSC are considerably less able to undergo serial transplantation than wild-type (Allsopp, *et al* 2003a) and human HSC from umbilical cord blood (UCB, with long telomere sequence) have greater proliferative potential than BM

HSC from older donors (Holyoake, *et al* 1999b). Thus for successful *ex-vivo* expansion of HSC (e.g. for tissue engineering or gene therapy, all long-held ambitions of HSC biologists) the ability to manipulate telomerase is likely to be critical.

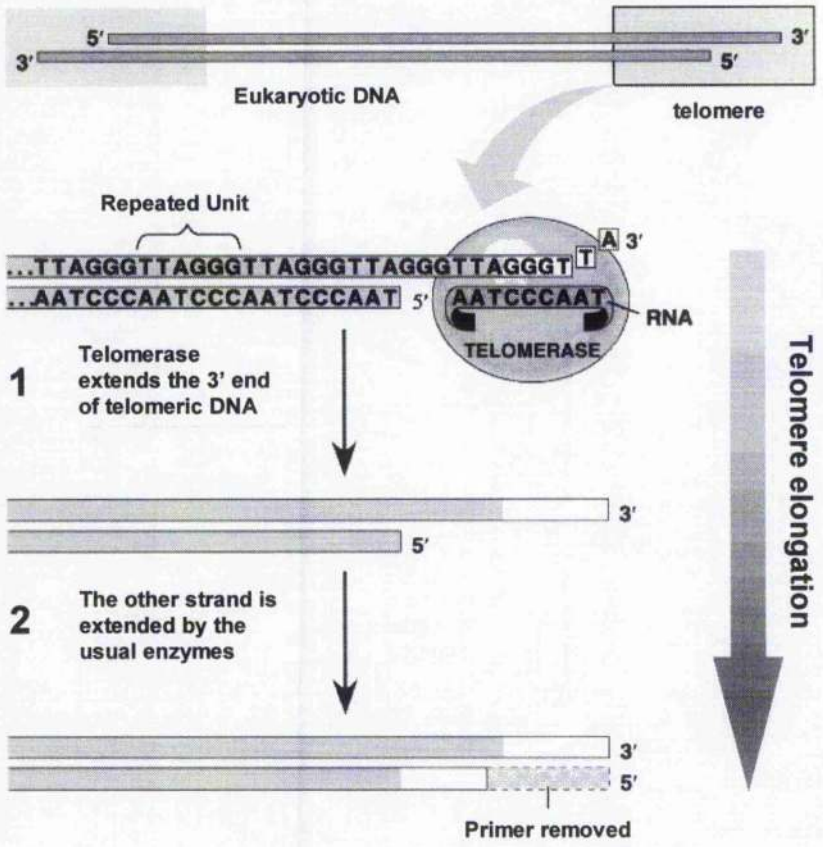


Figure 1-2 Telomere lengthening is mediated by telomerase

Eukaryotic chromosomal DNA ends in a 3' overhang of G-rich telomeric DNA. In conjunction with the RNA template component of telomerase (hTR), the reverse transcriptase hTERT catalyses the addition of telomeric repeats. The 5' end is extended by conventional DNA-synthesising machinery. By this process cells can maintain telomere length and abrogate the effects of replicative loss. In certain circumstances telomere elongation is also possible.

In parallel with an increased understanding of normal HSC biology, there has arisen a steadily increasing groundswell of opinion that considers many leukaemias (and indeed many cancers) to be disorders arising at the stem cell

level. Both acute and chronic myeloid leukaemia (AML and CML respectively) are now well characterised as stem cell malignancies (with varying degrees of downstream maturation or differentiation-arrest) (Lapidot, *et al* 1994). What seems increasingly likely is that the conventional approach to therapy for these disorders (e.g. combination chemotherapy) does not target the leukaemic stem cell population; this leads to relapse in a large proportion of cases. Conversely therapies that do eliminate these cells (e.g. allogeneic stem cell transplant, or alloSCT) are often curative. Novel approaches, tailored to target the malignant stem cell compartment, are therefore required. One such target is telomerase, given its ability to maintain the replicative ability of these putative target cancer stem cells. Determining how telomerase is expressed (and ultimately regulated) and defining the telomere dynamics within these disorders is paramount to developing future anti-telomerase treatment strategies.

1.2 Chronic myeloid leukaemia

It is fair to say that CML, despite its relative rarity (it has an incidence of approximately 1 in 100,000), is the most extensively characterised of the haematological malignancies. Interest in the pathogenesis of CML was the catalyst which led to the evolution of modern cancer cytogenetics and molecular biology, the advent of chemotherapy, the establishment of alloSCT as a routine (and curative) procedure, effective immunotherapy (in the form of interferon therapy, donor lymphocyte infusion and non-myeloablative allogeneic transplant) and, most recently, the proof-of-principle that molecularly targeted therapy works, in the form of imatinib mesylate. From a pathological viewpoint it is a fascinating disorder to study: in its early or chronic phase (CP) it is a single-gene disease (*i.e.* a consequence of the *BCR-ABL* fusion gene), and behaves in a relatively benign fashion characterised by myeloid expansion. Typically, excess neutrophils,

basophils, eosinophils (at all stages of maturation) and platelets accumulate in the haematopoietic compartment, and if unchecked may result in a grossly elevated white blood cell count (WBC, occasionally in excess of 400-500 x 10^9/l) with clinically apparent sequelae of hypervicosity (Sawyers 1999). Crucially these cells, although produced in vast excess and with immature forms visible in the peripheral blood, are functional. Patients may therefore survive for several years with relatively innocuous cell-count controlling chemotherapy such as hydroxyurea. Without disease modifying treatment however (such as interferon-alpha, alloSCT or very likely imatinib mesylate therapy), there is an almost 100% certainty of disease progression to blastic phase (BP, a particularly aggressive acute leukaemia characterised by maturation arrest in the lymphoid or myeloid lineage) and death, in conjunction with additional cytogenetic and molecular abnormalities.

CML was the first human malignancy to be linked to an acquired genetic abnormality, in which the 3' end of the c-ABL gene from chromosome 9 replaces the 3' end of the BCR gene on chromosome 22 (Groffen, *et al* 1984, Shtivelman, *et al* 1985). Detection of the resulting abnormally small chromosome 22 (the Philadelphia or Ph chromosome (Rowley 1973)) by bone marrow (BM) cytogenetics continues to be the mainstay of CML diagnosis and monitoring (typically with quantitative karyotypic analysis of 20-30 metaphases from a BM aspirate). More recently however, with the spectacular success of imatinib mesylate (a selective BCR-ABL kinase inhibitor (O'Brien, *et al* 2003)), sensitive peripheral blood *BCR-ABL* real-time (Q) RT-PCR has become important for monitoring of these patients (Lin F, Drummond MW et al, Blood *In Press*).

The molecular and phenotypic consequences of *BCR-ABL* acquisition in the HSC compartment have been extensively studied and are too numerous to discuss fully here. Briefly, the *BCR-ABL* product is most commonly a 210 kDa oncoprotein

($p210^{BCR-ABL}$) that has transforming activity in primary mouse BM and cell lines, and can induce many of the typical phenotypic features of CML cells upon transfection into primary human $CD34^+$ cells (Daley, *et al* 1990, Evans, *et al* 1993, Zhao, *et al* 2001). Crucial to the transforming ability of the *BCR-ABL* oncoprotein, in conjunction with its constitutively active tyrosine kinase activity, is its cytoplasmic location (in contrast to the nuclear location of $p145^{c-ABL}$) (Konopka and Witte 1985, Van Etten, *et al* 1989). Autophosphorylation of $p210^{BCR-ABL}$ (Pendergast, *et al* 1993) leads to recruitment of GRB2, a small adapter protein that can activate the RAS pathway (Ma, *et al* 1997). Nuclear signalling is mediated by phosphorylation of JAK2 and STAT1/STAT5 (Ilaria and Van Etten 1996), and together such signals contribute to the growth factor independence of *BCR-ABL* containing cell lines. Additionally, $p210^{BCR-ABL}$ mediated activation of the PI(3)K/Akt pathway (Skorski, *et al* 1995) and increased expression of BCL-2 (Sanchez-Garcia and Grutz 1995) increases resistance to apoptosis. Given the complexity of these downstream events, there is considerable potential for BCR-ABL tyrosine kinase activity to alter telomerase activity. As yet such effects remain uncharacterised, but worthy of further study.

In terms of CML HSC biology, a crucial consequence of *BCR-ABL* acquisition appears to be increased cell turnover, as demonstrated by 3H-thymidine suicide assays (Ponchio, *et al* 1995) and high-resolution cell cycle analysis of primitive leukaemic progenitors (Holyoake, *et al* 1999a). The demonstration of increased telomere shortening in the clonally derived peripheral blood leucocytes (PBL) of CML patients (Iwama, *et al* 1997) is supportive of these *in-vitro* observations, and has formed the basis for subsequent studies (Boultwood, *et al* 1999, Boultwood, *et al* 2000, Ohyashiki, *et al* 1997b)) including this current body of work. Utilising

telomere length measurement as a surrogate marker of *BCR-ABL*$^+$ HSC turnover (and hence disease progression) may potentially a useful prognostic tool.

The need for such tools is significant: despite all evidence to date pointing to *BCR-ABL* acquisition as a single genetic event in CP CML, patients display a particularly heterogeneous clinical course with respect to duration of CP. Although the median duration of CP is approximately 4 - 5 years, in clinical practice this varies widely, with some 10% of CML patients living beyond 8 years (Cervantes, *et al* 1994). At least some of this heterogeneity is due to the recently described deletions on the derivative-9 chromosome, which occur in 15% of patients (apparently at the time of the Philadelphia translocation) and result in poor prognosis disease (Huntly, *et al* 2001). It appears likely that this is due to the loss of an as yet unidentified tumour-suppressor gene. Thus, the classical description of CML as a 'single-gene' disease would appear to be misleading, from both a molecular and clinical standpoint. One of the few certainties of the disorder is that without effective disease modifying treatment all CP CML patients will eventually progress to BP, often via a less well defined accelerated phase (AP, **Figure 1-3**) (Spiers 1977), (Kantarjian, *et al* 1987). Predicting disease progression is therefore crucial for individual patient management decisions.

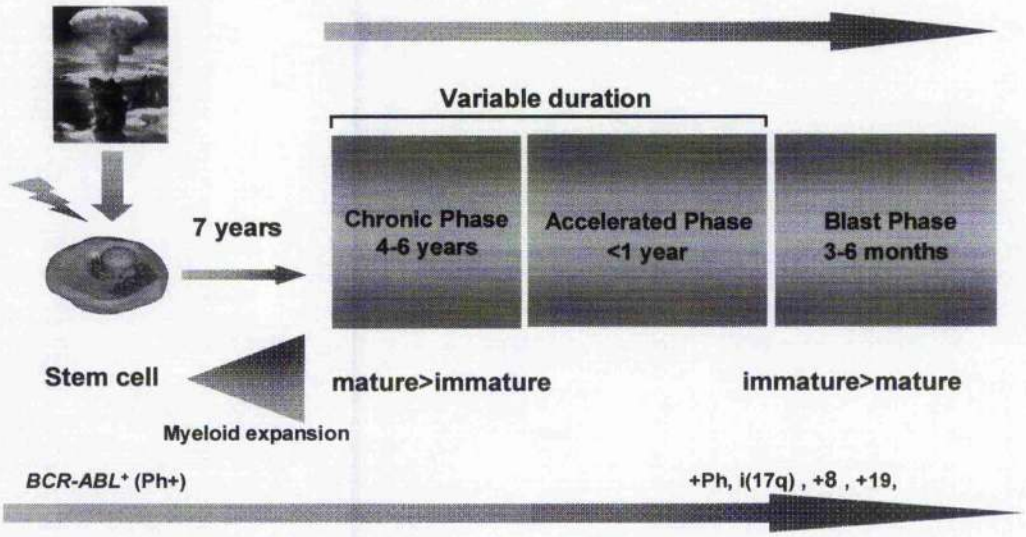


Figure 1-3 Timecourse of CML progression, and relationship to clinical, morphologic and cytogenetic features.

Ionising radiation remains the only clear aetiological factor in the causation of CML. Based upon look-back studies by the Hiroshima Foundation, the mean time to clinical development after the atomic explosion was seven years (Ichimaru, *et al* 1981). A typical triphasic disease pattern is illustrated, with median duration of each phase shown. Occasional patients may have a biphasic picture, and progress rapidly to BP without the ill-defined AP. Patients may even present in BP. A common feature of disease progression is the development of additional non-random cytogenetic abnormalities (in approximately 70-80% (Griesshammer, *et al* 1997) of patients), the most common of which are illustrated. In about 15% of patients progressive disease is associated with -7, -17, +17, +21 and –Y (Deutsch, *et al* 2001) . At the molecular level, loss of wild-type p53 function is a relatively common abnormality, being associated with 25% of myeloid BP (Ahuja, *et al* 1989). (The WHO clinical and laboratory criteria which define these different stages, and which are used to classify our patient material, are defined in **Table 2-1** and **Table 2-1** (Vardiman, *et al* 2001) in the Materials and Methods Section)

1.3 Defining the clinical problem

Because treatment becomes less effective with advanced disease, identification of patients at risk of rapid disease progression at diagnosis is vital for the development of risk-adapted treatment strategies. AlloSCT, the only established curative intervention for CML, achieves optimal survival benefit when carried out within the first year of diagnosis (Gratwohl, *et al* 1998). This procedure carries considerable transplant related morbidity and mortality however (up to 30%, even for well selected patients), and it is therefore likely to be of greatest benefit to (and most acceptable to) patients who are likely to progress quickly. A major (and ongoing) focus of CML research has therefore been the development of robust, accurate and routinely applicable prognostic scoring systems (Hasford, *et al* 1998, Sokal, *et al* 1984). The most contempary of these, the European or Hasford score (Hasford, *et al* 1998), utilises 6 routinely collected clinical data points (age, spleen size, platelet, blast, eosinophil and basophil counts) to generate a high-, intermediate- or low-risk score for any individual patient, with regards to response to interferon therapy. In general terms, for a patient in the low risk category receiving interferon a median survival of 98 months may be expected (or longer if the patient undergoes a complete cytogenetic response on BM cytogenetics). Such an individual may therefore elect to pursue the route of conventional (*i.e.* non-transplant) therapy. Conversely, a high-risk patient, with no likelihood of a significant benefit from interferon therapy and an expected median survival of 42 months, may assess the 'up-front' risk of alloSCT as acceptable.

While such systems are very useful in studying populations of patients (e.g. for comparing two or more arms of a clinical trial) they are somewhat less applicable to individual treatment decisions. Identification of biological markers of prognosis

is therefore of the utmost importance. It is within this context that telomere length measurement, both as an indicator of HSC turnover and a surrogate marker of genomic instability, may be a useful prognostic tool.

CML has served as a paradigm for the concept of haematopoietic stem cell (HSC) malignancy and tumour evolution, and the relative ease with which primary human material can be obtained, manipulated and studied has undoubtedly facilitated this. In terms of telomere dynamics and telomerase it is therefore a logical disease to study: telomere length can give insight into Ph^+ HSC turnover and (indirectly) genetic stability, the role of telomerase in early and late tumourigenesis can be determined and (dys)regulatory mechanisms identified, and finally (and ultimately most importantly) the clinical significance of these observations can be assessed. Critically, the myriad downstream effects of the BCR-ABL protein may well impact on telomerase function, although to date no conclusive studies have been performed. Incorporating knowledge of telomere biology into meaningful prognostic indices and novel and effective treatment strategies must be seen as the ultimate aim.

1.4 An introduction to telomere and telomerase biology

A functional definition of the telomere was initially provided by early genetic studies. It was observed that maize chromosomes with 'broken ends' were unstable, resulting in end-to-end fusions, ring or dicentric forms, thus demonstrating that the structural integrity of chromosome termini was a requirement for genomic stability (McClintock 1941, McClintock 1942). These early observations have been confirmed experimentally in yeast, where loss of a telomere results in eventual chromosomal loss (Sandell and Zakian 1993). Upon

disruption of telomeres the cell will attempt to repair the break, thereby generating fusions between the telomeres of different chromatids. Such fusions occur when the number of telomere repeats drops to a critical level and in the absence of an effective telomere maintenance mechanism (Blasco, *et al* 1997). Observations such as these therefore support the notion of telomere length as an indicator of telomere function, namely as a stabilising DNA 'cap'.

The non-coding DNA of telomere sequence is unique; an identical hexameric telomere repeat sequence has been described for trypanosomes (Blackburn and Challoner 1984), slime moulds (Forney, *et al* 1987), humans and other vertebrates (Moyzis, *et al* 1988): namely $(TTAGGG/CCCTAA)_n$ often repeated thousands of times . Although different sequences have been described in other organisms (for example $G_{1\text{-}3}T$ for the baker's yeast *Saccharomyces cerevisiae* (Shampay, *et al* 1984)) they share the common property of a G-and C-rich strand orientated in the 5' to 3' direction containing multiple sequence repeats (from a few hundred base pairs in length in ciliates and yeasts to thousands in vertebrates). In addition to these properties, an overhanging single-strand 3' G-rich terminus is also a highly conserved feature (Henderson and Blackburn 1989). Such conservation almost always implies biological importance.

How the telomere maintains genomic stability without being recognised as a double stranded DNA break has been the subject of intense research. In addition to its capping role, the telomere is also involved in the alignment of chromosomes during metaphase (Liu, *et al* 2002) and the spatial organisation of chromosomes within the nucleus (de Lange 1992). Although recent evidence for a telomere-position effect on transcription of sub-telomeric genes in human cells (Baur, *et al* 2001) has further broadened the list of potential telomeric functions, in comparison to the capping role they remain relatively less well characterised.

1.4.1 Telomere shortening

Semi-conservative replication of DNA presents a unique problem: the process only works in the 5' to 3' direction, and DNA polymerase requires binding of an RNA primer. Olovnikov and Watson predicted the consequences of this long before the telomere was characterised, and termed it the 'end-replication problem' (Olovnikov 1971, Watson 1972). This predicted the loss of a small 5' nucleotide segment as DNA synthesis took place, with progressive replication induced telomere shortening (**Figure 1-4**).

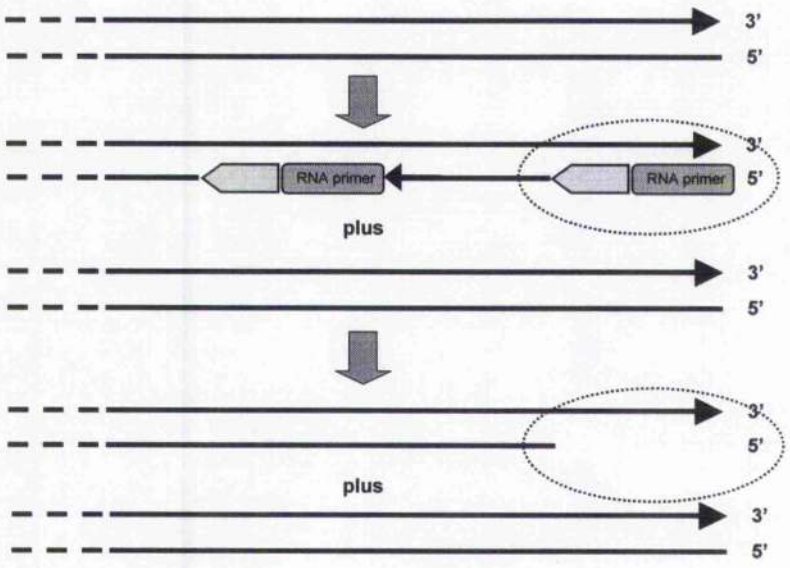


Figure 1-4 Replication of a linear DNA duplex molecule and the 'end-replication' problem.

The 3' end of the molecule is replicated continuously to the very end, having started at the opposite, and 5', terminus. However synthesis of the lagging strand (also in the 5' to 3' direction) is discontinuous because of the requirement for RNA primer binding and unidirectional growth of the new strand. Upon removal of the RNA primers, the gaps in the discontinuous lagging strand are filled in and ligated. However there is no provision for such a process at the immediate 5' end, thus leaving a gap. Since the DNA duplex is antiparallel each daughter molecule will be shortened on their 5' end after replication with successively shorter daughter chromosomes resulting from further cycles of cell division.

This mechanism has the attractive feature (both from an experimental and biological standpoint) of behaving as a 'mitotic clock' (Harley, *et al* 1990), suggesting a mechanism whereby cells could sense a 'maximum' number of divisions, and then stop dividing (senesce). Such cell behaviour has been observed *in-vitro* since the 1960s (Hayflick and Moorhead 1961). It was however clear that telomere shortening did not occur in all cells, as those that proliferate indefinitely (such as unicellular eukaryotes, immortalised cell lines and germline cells) were shown to maintain their telomeres at a constant length over time. Indeed sperm telomeres are longer than somatic telomeres and do not decrease in length during organismal aging (Allshire, *et al* 1989, Cooke and Smith 1986, Levy, *et al* 1992) as do somatic cells. In most eukaryotic cells this is achieved by the presence of an enzyme called telomerase, an RNA dependent DNA polymerase with the ability to add telomeric repeats, abrogate replicative loss and, as described below, bypass senescence.

Mammalian telomeres show a species-specific length setting (Kipling and Cooke 1990): in humans telomere length varies between individuals (approximately 5 - 10kb in peripheral blood cells) due to differences in the number of hexameric base pair sequences (de Lange, *et al* 1990, Lansdorp, *et al* 1996), with different strains of mice showing a greater degree of variability of between 12 and 80kb (Zijlmans, *et al* 1997). Even among individuals of the same age, considerable variation in telomere length of peripheral blood lymphocytes has been demonstrated (Rufer, *et al* 1999) and twin studies suggest that most of this variation is genetically determined (Slagboom, *et al* 1994). A novel locus on chromosome 2 has recently been proposed as having a role in the regulation of telomeric length in the mouse (Zhu, *et al* 1998), although no such locus has yet been described in humans. There is also variation in telomere length between individual chromosomes, with 17p having the smallest number of telomere repeats in humans (Martens, *et al*

1998). The significance of these interchromosomal variations remains unclear however, and the majority of published studies use average telomere length measurements for experimental purposes.

1.4.2 The biology of cell senescence

Leonard Hayflick, in a seminal series of experiments performed some 40 years ago, was first to suggest the existence of a finite limit to diploid cell division *in vitro* (Hayflick and Moorhead 1961). He utilised human diploid fibroblasts (HDFs) to demonstrate irreversible cessation of cell division after 60-80 population doublings (the 'Hayflick limit' or Mortality 1 (M1)), with concomitant development of a phenotype that he described as "senescent" ('old or withered', as opposed to *quiescence* which is generally assumed to be reversible) (Hayflick 1965). Consequently it was assumed that all normal cells had an intrinsically limited replicative lifespan. The term 'replicative senescence' is now used to describe these observations.

Despite a state of cell-cycle arrest, senescent cells remain metabolically active, and may remain viable for years with appropriate attention to supporting culture conditions. It is now known that this state is achieved by activation of the p53 and retinoblastoma (RB) tumour suppressor pathways (Shay, *et al* 1991). Consequently, the introduction of oncogenes that inhibit these control mechanisms – such as SV40 large T antigen (Ikram, *et al* 1994) – allows cells to undergo further cell division (Counter, *et al* 1992). However their replicative capacity again remains finite, and after a further 20-30 population doublings, the cells enter a period of 'crisis', characterised by genomic instability and apoptosis (M2). Occasional immortalised clones may emerge from this chaotic state (Counter, *et al*

1992), albeit rarely (at a frequency of less than 1 in 10^7). These *in-vitro* observations have largely been explained in terms of telomere dynamics.

1.4.3 Replicative senescence

The most attractive 'counting' mechanism to mediate replicative cell senescence is that of telomere shortening. Harley *et al* showed that HDF telomeres progressively shorten in culture (Harley, *et al* 1990), and it was demonstrated that their replicative capacity correlated with absolute telomere length rather than with the age of the fibroblast donor (Allsopp, *et al* 1992). In keeping with these studies is the observed accumulation of short telomeres prior to senescence in human fibroblast cultures (Martens, *et al* 2000). In this study, HDFs lost 50 – 150 bps of telomere sequence with each division. Telomeres of 1-2Kb or shorter were observed immediately prior to senescence and, interestingly, the onset of senescence was significantly correlated with the mean telomere length (rather than with that of the shortest chromosomes). Telomere shortening is thought to induce senescence once some telomeres have become sufficiently short to activate a DNA damage signal (Harley 1991). In summary these early studies pointed to a tight relationship between telomere length (and hence genomic stability), DNA-damage surveillance and repair mechanisms and cell-cycle arrest / tumour suppressor pathways. Disruption of such regulatory elements could clearly be of importance in the development and evolution of tumours.

A causal link between telomere shortening and replicative senescence was ultimately achieved in two ways. Firstly, Bodnar *et al* demonstrated that human retinal pigment epithelial cells and foreskin fibroblasts transfected with hTERT, the rate limiting component of telomerase, underwent at least 20 additional population doublings (Bodnar, *et al* 1998). Furthermore transfected cells demonstrated

telomere stabilisation or even lengthening. Secondly, it was shown that when telomerase was inhibited in telomerase-positive immortal cells, telomere shortening followed leading to chromosome-to-chromosome fusions and cell death (Hahn, *et al* 1999b, Zhang, *et al* 1999). These important observations have been confirmed and extended by other groups (Vaziri and Benchimol 1998, Zhu, *et al* 1999). It has been assumed that replicative senescence also occurs *in-vivo*, although no definitive proof has yet been published. Telomere shortening is certainly demonstrable in many somatic cell types, including PBL (Rufer, *et al* 1999), which demonstrate a rapid loss in the early years of life, with subsequent slowing of loss in later years. However there is as yet no conclusive proof that this contributes to organismal ageing. Indeed a truly senescent phenotype has not been definitively described *in-vivo* (Severino, *et al* 2000) and somatic cells from aged donors can be easily made to grow *in-vitro* (Cristofalo, *et al* 1998). Ageing is clearly therefore, a more complicated process than *in-vitro* replicative senescence.

Using HDFs *in-vitro*, von-Zglinicki has demonstrated that the major mechanism of telomere loss in this cell-type under specified culture conditions is via induction of single strand breaks (von Zglinicki, *et al* 2000) and oxidative damage (von Zglinicki, *et al* 1995). Telomeric DNA is less well repaired than the rest of the genome (and indeed appears 5 to 10-fold more susceptible to oxidative damage in the first place (Oikawa and Kawanishi 1999)), thus telomere shortening may simply be representative of genomic damage sustained as a whole. Alternatively, telomere shortening by whatever means (oxidative damage or replicative loss) may be a crucial final common pathway. As DNA damage signals mediate the final phenotype of these cells, it is likely therefore that many different stimuli can give rise to a senescent-like state, resulting in a stochastic loss of cell viability. It seems likely that upregulation of $p16^{INK4A}$ is of particular importance with regards telomere-independent senescence (Munro, *et al* 2001), a phenomenon which has

been termed M0. The term 'culture shock' has also been used to define replication-independent mechanisms of senescence *in-vitro*, emphasising the need for meticulously controlled cell culture conditions when studying replicative senescence.

The straightforward telomere shortening / Hayflick limit relationship has also been questioned recently by Blackburn (Blackburn 2000), who has proposed a more complex model of replicative senescence (see Section **1.5.3** below). The basis for this hypothesis is largely twofold; firstly that the telomere is a complex DNA-protein structure, and that telomere sequence length is unlikely to be the primary determinant of 'capping' ability; and secondly, that telomere length is not always a good indicator of cell ageing and viability (Yang, *et al* 1999). In the latter study hTERT transfected human endothelial cells underwent >100 population doublings despite exhibiting shorter telomeres than that of a senescent control population. Thus it appears that the presence of telomerase is crucial for determining replicative capacity, not necessarily because of its telomere-lengthening ability but perhaps via a stabilising or capping role.

1.4.4 Replicative senescence as a tumour suppressor mechanism

Extension of initial observations on telomere-limited growth *in-vitro* to the *in-vivo* situation has led to the hypothesis that telomere shortening and replicative senescence may have evolved as a tumour suppressor mechanism, by preventing excessive cell division. The multistep nature of carcinogenesis dictates the need for multiple mutations (Vogelstein and Kinzler 1993) with extensive population doublings after each mutation for a reasonable chance of further events occurring. A replicative barrier of approximately 80 doublings has been estimated to limit the

expansion of the pre-malignant clone, and serve to generate senescent cells which contribute to the ageing phenotype. Given the high rates of apoptosis in most tumours however, it is difficult to model the number of divisions required to generate a clinically significant tumour. However, as 90% of human tumours express telomerase, it would appear that telomere stabilisation is of crucial importance for their continued growth (Kim, *et al* 1994). There is a growing consensus that telomere shortening represents a barrier to unlimited replicative ability, and akin to the checkpoint function of p53, provides a 'telomere checkpoint' function (Verfaillie, *et al* 2002). Any such mechanism will likely not act in isolation, but in concert with other tumour suppressive 'checkpoints' such as immune surveillance, cell-cycle arrest and DNA repair. Identifying the relevant importance of these mechanisms is likely to prove very difficult, as studies of cultured cells have shown.

1.5 Telomere structure and dynamics

1.5.1 The T-loop and other structures within telomeric DNA

A recent twist in the tale (literally) of telomeric structure came with the discovery that mammalian telomeres (including those of human cell lines and normal peripheral blood leucocytes) end in a large duplex loop formed by intercalation of the 3' overhang back into the duplex telomeric strands in a TRF2 (a double-stranded telomere binding protein) mediated process (Griffith, *et al* 1999). Consistent with this finding is the ability of TRF2 to negatively regulate telomere length, presumably by facilitating loop formation and preventing telomerase access (Smogorzewska, *et al* 2000). This would appear to be a neat structural solution to prevent the ends of chromosomes from being recognised as double

stranded DNA breaks, with subsequent activation of DNA repair mechanisms or apoptosis. These loop structures are termed t-loops (telomere loops), and result in the formation of a small displacement loop (D-loop) where the G-strand overhang rejoins the duplex (**Figure 1-5**). An additional consequence of these loop structures is to limit the access of telomerase to the telomeric end, which may be further hindered by the presence of 'G-quartet' structures. These are planar tetrameric arrangements of guanine nucleotides within the G-rich strand, which can adopt a number of different topologies as visualised by nuclear imaging techniques (Aboul-Ela, *et al* 1992). Given the requirement of the hTR template region of telomerase for a linear non-folded DNA primer, it is entirely plausible that these structures provide a further control on access of telomerase to the telomere (Zahler, *et al* 1991). It seems likely that reduction of telomere length below a critical threshold could disrupt these stabilising structures.

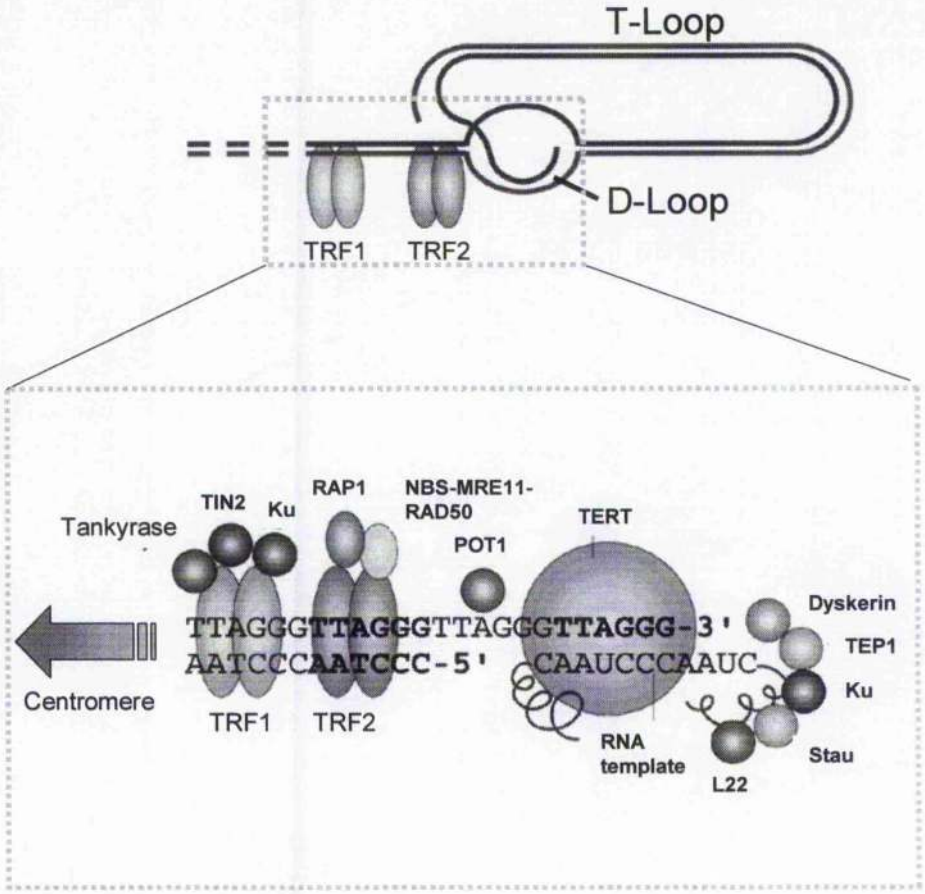


Figure 1-5 Telomere structure and telomere associated proteins

A schematic illustrating the higher order DNA structure (namely T- and D-loops) of the telomere, as well as a putative model of the binding proteins. For additional details see text. NBS-MRE11-RAD50 is a DNA-repair complex that binds to telomeres, an association mediated at least partly by TRF1 and TRF2 (Wu, *et al* 2000). Ku is a component of the DNA dependent protein kinase complex (DNA-PK) involved in double strand DNA repair (Bailey, *et al* 1999). The telomerase associated proteins dyskerin, TEP1, Stau and L22 are shown to convey the potential complexity of the entire telomere-telomerase complex, and are described in **Section 1.6.9.1**. Adapted from Hodes *et al.* (Hodes, *et al* 2002).

1.5.2 Telomere binding proteins

Several telomere-binding proteins (TBPs) have now been characterised as important for telomere stability and length regulation. The first to be characterised, two related TBPs (TRF1 and TRF2), bind the duplex array of T_2AG_3 repeat sequences via direct interaction with double-stranded DNA (Bianchi, *et al* 1997)

(Broccoli, *et al* 1997). Van Steensel has shown that TRF1 negatively regulates the action of telomerase, possibly at the level of individual telomeres, by preventing telomerase access. In those studies over expression of TRF1 in human cell lines resulted in telomere shortening (van Steensel and de Lange 1997). As regards TRF2, a small ubiquitously expressed protein, inhibition of its function in many cell types (including primary human lymphocytes) results in loss of the G-rich overhang, cell cycle arrest and apoptosis via the ATM / p53-dependent DNA damage checkpoint pathway (Karlseder, *et al* 1999). Indeed TRF2 would appear to play such a central role in telomere homeostasis that de Lange has proposed that the role of T_2AG_3 repeats is to provide TRF2 binding sites (de Lange 2002). It is likely that some (if not all) of these characteristics of TRF2 are dependent on its ability to stabilise the T-loop structure described above, via its binding to the base of the loop (Griffith, *et al* 1999). From these data TRF2 would appear to be a major protective factor operating at chromosome ends.

Although much less well characterised than TRF 1 and 2, other TBPs also exist, although a full description of these is beyond the scope of this section (**Figure 1-5**). Briefly, Pot 1 (protection of telomeres 1), has recently been described in humans as a single strand DNA binding protein necessary for protection of telomeric ends (Baumann and Cech 2001). Others, such as TIN2, TANK1 and hRAP1 have profound effects on telomere length when over expressed (Kim, *et al* 1999, Li, *et al* 2000a). Recently described associations between DNA repair proteins and the telomere are less well understood however. For example the DNA repair component DNA-PKc (the catalytic component of the DNA-dependent protein kinase complex, a non homologous end-joining, NHEJ, and repair enzyme) has been shown to have a role in telomere capping and maintenance, with dual DNA-PKc and telomerase knock-out (KO) mice showing greater rates of telomere shortening than telomerase KO mice alone (d'Adda di Fagagna, *et al* 2001,

Espejel, *et al* 2002). Intriguingly DNA-PKc is down regulated by BCR-ABL, highlighting the potential importance of this association in terms of the telomere loss observed in CML (Deutsch, *et al* 2001).

Although telomerase is discussed fully in the next section, it is important to emphasise that as well as having the ability to 'reset' the mitotic clock via telomere elongation / stabilisation, it also plays a 'capping' role via telomere stabilisation in cells with very short telomeres ((Yang, *et al* 1999), (Zhu, *et al* 1999) This would appear to be independent of its role in telomere elongation and may simply involve a telomerase-telomere physical interaction to form a protective cap. In conclusion it seems that there are many ways to stabilise a telomere; by analogy there must also be many ways to destabilise it.

1.5.3 Telomere structure, function and dynamics: a synthesis

What is now becoming clear is that telomere length *per se*, although important, need not be the sole determinant of its functional state. Three crucial factors participate in stabilising the telomere: a minimum number of telomeric repeats, an intact G-strand overhang and a complex set of associated telomere binding proteins. Elizabeth Blackburn has proposed a model of telomere dynamics in which telomeres exist in capped and uncapped states (Blackburn 2000). In this model the presence of DNA repair proteins at the telomere is postulated to be crucial for the action of telomerase, which involves transient uncapping. In long telomeres, with extensive protein binding sites, the chances of the telomere being uncapped is low. After successive divisions this possibility becomes more likely and, in a stochastic fashion, the telomere may become uncapped. At this stage one of three things may happen: telomerase could act transiently at the uncapped

site, the telomere could undergo homologous replication (and thereby maintain or even increase its length), or NHEJ will occur and fuse telomeres (resulting in genomic instability at the next cell division). If the cap is not restored via any of these mechanisms, the cell can exit the cell-cycle and may undergo apoptosis. Crucial to this model is the stochastic nature of the process: indeed such a model neatly explains the stochastic nature of cell division *in vitro*. As Blackburn states: "whether a telomere will become uncapped is expressed as a probabilistic function influenced by several factors, only one of which is length".

In experimental terms, the complete determination of the functional state of a given telomere may therefore require quantification of multiple proteins, telomeric length measurement and visualisation of secondary DNA structure etc. From a practical aspect this is difficult (and laborious, particularly where large numbers of samples require to be analysed), and the mainstay of assessing telomeric status continues to involve measurement of telomere length.

1.6 Telomerase

1.6.1 Background

It is now accepted that the apparent tumour-suppressive role of telomere shortening can be circumvented by the presence of telomerase, a ribonucleoprotein reverse transcriptase first described by Elizabeth Blackburn and Carol Greider in *Tetrahymena* (Greider and Blackburn 1985). The two crucial constituents of telomerase (which together can reconstitute telomerase activity *in-vitro* in a rabbit reticulocyte-lysate model (Weinrich, *et al* 1997)) consist of a catalytic protein subunit known as hTERT (Greenberg, *et al* 1998, Harrington, *et al*

1997) and an RNA template molecule of complimentary sequence to the telomeric DNA (hTR) (Blasco, *et al* 1995, Feng, *et al* 1995, Greider and Blackburn 1989).

While hTR can be detected in most tissues, hTERT is much more restricted in its expression (Harrington, *et al* 1997, Meyerson, *et al* 1997, Nakamura, *et al* 1997). Introduction of the latter is sufficient to reconstitute telomerase activity and this has led to the concept that hTERT is the primary rate-limiting component of telomerase. This is however a simplistic view as hTR can also be limiting for telomere maintenance in some circumstances (described in detail below). For this reason most studies of telomerase regulation have focused on transcriptional control of TERT, with comparatively few on regulation of hTR or post-translational mechanisms. These are now being seen as increasingly important.

Present in 90% of tumours (Kim, *et al* 1994), telomerase is thought to confer immortality via addition of telomere repeats, although other non-telomere dependent functions of this tightly regulated enzyme are a possibility. A second, less well-characterised, telomerase-independent mechanism of telomere maintenance known as alternative lengthening of telomeres, or ALT, has also been described (Bryan, *et al* 1995). This mechanism would appear to occur infrequently however, being detected most commonly in tumours of mesenchymal origin (Bryan, *et al* 1997). Human ALT cells are rare (thus far seen in a few tumours and cell lines only) and are characterised by lack of telomerase activity with great heterogeneity in telomere length. The latter is due to recombination events involving telomere repeats, the mechanism of telomere maintenance operating in these cells. In general terms the overall contribution of ALT to malignancy appears small. This is perhaps partially explained by a recent study which found ALT tumours from mTERT$^{-/-}$ Ink4a/ARf$^{-/-}$ mice unable to generate metastases, a property which was restored upon reconstitution of mTERT (Chang,

et al 2003). Considerable interest in ALT remains however, not least because of it being a potential mechanism of resistance to anti-telomerase therapies.

1.6.2 Measurement of telomerase activity

The majority of the studies described in the following sections have utilised the telomeric repeat amplification protocol (TRAP assay) to detect enzymatic activity in the extracts of the cells or tissue in question. As originally described by Kim (Kim, *et al* 1994) (with subsequent modifications (Kim and Wu 1997)), the TRAP assay is based on the ability of telomerase (within the sample extract) to extend a DNA primer, with the products subsequently amplified via a PCR step and visualised on a polyacrylamide gel. A number of control samples are incorporated: a heat – or RNAse- treated negative control sample (to inactivate the catalytic or RNA components respectively), a positive control (an immortal telomerase-positive cell line sample) and an internal DNA standard (with flanking sequences for the TRAP assay primers, e.g. ITAS) to control for inhibitors of Taq polymerase (which can be present in the sample). Although highly sensitive, the technique is at best semi-quantitative, as it depends on a PCR step. Additionally it is sensitive to protease interference, as it relies on the presence of the intact telomerase holoenzyme. Furthermore, many TRAP studies are undoubtedly confounded by contaminating telomerase positive cells (*eg* lymphocytes) in the samples, and pre-assay homogenisation of the sample precludes localisation of the enzymatic activity. As originally described the assay was moderately laborious (and therefore expensive) and probably unsuitable for high-throughput screening of clinical samples in a diagnostic setting, as had been originally hoped. Further advances as regards simplicity have resulted in an ELISA based TRAP, which may yet allow it to enter a clinical setting.

Whether telomerase activity *in-vitro*, as measured by TRAP, actually reflects telomere maintenance *in-vivo* however is open to some considerable doubt; for example KO mice deficient in a crucial telomerase component do not display any alteration in TRAP activity yet have profound defects in telomere maintenance (Hathcock, *et al* 2002). This will be discussed in more detail in Chapter 6.

A complementary approach to TRAP is the direct measurement of the hTR or hTERT components. hTR (cloned and sequenced prior to hTERT (Feng, *et al* 1995)) was initially detected using Northern blotting, although an early report failed to show any correlation between hTR levels (expressed both in normal and tumour samples) and telomerase activity in immortalised cells and tumours (Avilion, *et al* 1996). Such studies were partly responsible for the (still) often repeated and misleading term 'ubiquitous expression of hTR', thereby implying that it is not regulated in any particularly relevant manner. As detailed below, interest in this molecule as a regulatory element of telomerase activity, and as a mediator of human disease, has been rekindled of late. Other means of detecting hTR include in-situ hybridisation (Soder, *et al* 1997a) and RT- and Q-RT-PCR (Takahashi, *et al* 2000). hTERT, the apparent rate-limiting component of the telomerase holoenzyme, is generally accepted to be a surrogate marker of telomerase activity: levels of hTERT are generally correlated with TRAP activity (Kilian, *et al* 1997, Meyerson, *et al* 1997, Nakamura, *et al* 1997) and hTERT transfection confers telomerase activity to telomerase negative cells (which express hTR, see below). hTERT measurement by sensitive and accurate Q-RT-PCR is therefore particularly useful in addition to the semi-quantitative TRAP assay.

1.6.3 Cellular immortalisation and telomerase

1.6.3.1 Transfection of hTERT into human cell lines

Subsequent to the observation that transfection of hTERT into retinal pigment epithelial and foreskin fibroblast human cell lines conferred increased growth in culture (Bodnar, *et al* 1998), extensive phenotypic studies on hTERT immortalised fibroblasts have shown that they lack the characteristics of neoplastic cells (namely anchorage independent growth, reduced serum requirements and tumour formation in nude mice), have a normal karyotype and have intact DNA damage checkpoint controls (Ouellette, *et al* 2000). Thus up-regulation of telomerase alone does *not* confer oncogenic ability.

Hahn and Weinberg have determined the genetic 'requirements' for the creation of a human tumour cell *in-vitro* (Hahn, *et al* 1999a). In this study, which also emphasised the need for tumour cells to overcome M1 and M2 checkpoints, only cells expressing hTERT, SV40 large-T antigen and H-*ras*V12 were truly tumourigenic on injection into nude mice. This '4-hit' model therefore incorporates loss of p53 and Rb checkpoint functions (via SV40) and growth signals (via an activated *ras* gene) in addition to a telomere maintenance mechanism (hTERT). Such a model fits very neatly within the classical hallmarks attributed to cancer *in-vivo*; namely self-sufficiency in growth signals; loss of suppressor / checkpoint functions; avoidance of apoptosis; limitless replication potential; angiogenesis; and invasion and metastasis (Hanahan and Weinberg 2000). In terms of leukaemia it is likely that the latter two characteristics assume much less importance in disease evolution, due to bone marrow origin of the disease and likely circulation of the target malignant cell. Telomere maintenance is almost certainly a requirement however.

1.6.3.2 Modulation of telomerase activity in human cell-lines

Using an alternative approach to define the role of telomerase, transfection of immortalised human glioblastoma cell lines with a vector expressing antisense telomerase RNA was found to induce either apoptosis or differentiation (Kondo, *et al* 1998). More recently, Herbert *et al.* demonstrated that oligonucleotide inhibition of telomerase immortalised human breast epithelial cells led to progressive telomere shortening and apoptosis (Herbert, *et al* 1999). In addition to demonstrating the importance of telomerase in the maintenance of most immortalised cell lines, these studies also provide support for telomerase as a valid target for anti-cancer strategies.

1.6.3.3 Telomere independent functions of telomerase

As suggested above, there is some evidence that telomerase may have proliferative effects on the cell that are independent of its action at the telomere. In mice that overexpress mTERT, (in basal keratinocytes within the skin via the bovine keratin 5 promotor, while retaining normal telomere length and skin histology), there is a twofold increase in the rate of skin wound healing and skin tumour development from chemical carcinogenesis (Gonzalez-Suarez, *et al* 2001). These data imply that telomerase may have direct growth-promoting effects in cells with sufficiently long telomeres. These effects can be aggravated when mice overexpressing mTERT also lack p53, and in these double-transgenic mice there was a significant reduction in lifespan due to a high incidence of neoplastic and pre-neoplastic lesions in the affected tissues (Gonzalez-Suarez, *et al* 2002). In another model, forced cardiac-myocyte expression of mTERT resulted in hyperplasia and hypertrophy of the cells (Oh, *et al* 2001).

In-vitro studies of human cells have gone some way to explaining these observations. In one study hTERT expressing human mammary epithelial cells become resistant to the anti-proliferative effects of TGF-β (Stampfer, *et al* 2001), thus gaining a proliferative advantage. More recent work on hTERT transfected human cells has implicated hTERT mediated upregulation of epiregulin (a potent growth factor) and epidermal growth factor receptor in HDFs and human mammary epithelial cells respectively (Lindvall, *et al* 2003, Smith, *et al* 2003). Furthermore, in rodent neural cells, mTERT expression was seen to behave as a cell-protective mechanism by conferring resistance to apoptosis (Fu, *et al* 2000). This has recently been confirmed for hTERT human T-lymphocytes *in-vitro*, which displayed resistance to oxidative stress-induced apoptosis (Luiten, *et al* 2003). Further work to determine the significance of these findings for normal tissue homeostasis and cancer biology is required.

Although the signalling and effector mechanisms for these potential additional actions of telomerase (and ultimately their *in-vivo* significance) remain unclear; they further support the strategy of targeting telomerase within anti-tumour strategies.

1.6.4 The telomerase-negative mouse

The telomerase knock-out (KO) mouse was developed by Blasco *et al.*, with telomerase activity confirmed to be completely absent in cells of animals homozygous for the null mTR allele (Blasco, *et al* 1997). Q-FISH techniques demonstrated steady telomere shortening, with disappearance of the T_2AG_3 signal on some chromosomes (consistent with shortening of telomeres to a few hundred bps long). An increase in end-to-end chromosomal fusions was observed in later generations of mice, all of which remained viable for at least the six generations studied.

However, in a subsequent report involving later generation animals, mice displayed defective spermatogenesis and infertility, uterine and intestinal villous atrophy, impaired mitogenic responses of lymphocytes, ulcerative dermatitis, an increased rate of apoptosis and compromised proliferative capacity of cells within the haematopoietic system (Lee, *et al* 1998, Rudolph, *et al* 1999). The impaired germinal centre reaction with B-cell telomere shortening observed in these animals after immunisation (wild-type animals demonstrated germinal centre B-cell telomere lengthening) also provided support for a telomere-dependent model of immune senescence (Herrera, *et al* 2000). However of particular interest was the fact that these telomerase-absent mouse cells *could* be spontaneously immortalised in culture, transformed by oncogenes and (despite their severely shortened telomeres) formed tumours in nude mice. Recent studies have also shown that the ALT mechanism can maintain, and indeed lengthen, telomeres in telomerase KO murine cell lines in some circumstances (Hande, *et al* 1999) and it may be this process which allows immortalisation to take place.

If telomerase is not then required for unlimited cell growth in cell lines derived from these mice, it raises questions as to the role of mouse telomerase *in vivo* where activity has been repeatedly demonstrated in mouse tumours (Blasco, *et al* 1996, Broccoli, *et al* 1996). Indeed there is the possibility that mouse telomerase activation is simply a surrogate marker of clonal evolution and proliferation in tumour cells, inferring that caution should be exercised when extrapolating these data to human biology. Arguing against this however, $mTR^{-/-}$ mice developed 33% fewer skin tumours in response to chemical carcinogens than wild type telomerase competent mice, indicating that telomerase does indeed have a role in mouse tumourigenesis (Gonzalez-Suarez, *et al* 2000).

It is now apparent that different tissues would appear to respond to lack of telomerase in different ways. A thorough study of the phenotypic features of late generation telomerase KO mice showed an increased incidence of cytogenetic abnormalities and a 4-6 fold increase in spontaneous cancers, at a younger age than the wild type mouse (Rudolph, *et al* 1999). That most of these tumours arose in highly proliferative cell types where telomere shortening (and hence chromosomal abnormalities) should be most marked (lymphomas and teratocarcinomas), is compelling evidence for telomere instability having a direct role in tumour formation. That there should be an increased risk of cancer at all, in the face of a general reduction in cell viability in these mice, is somewhat contradictory. One explanation is that telomerase independent mechanisms facilitate long-term tumour growth after the initial period of genetic instability and carcinogenesis. Data from *S. cerevisiae* has confirmed that obtained from mice: the mutation rate in the target gene (*CAN1*) increased 10-100 fold in strains with absent telomerase and telomere dysfunction (Hackett, *et al* 2001).

These data suggest a biphasic role for telomerase in tumorigenesis: absence in the early stages promotes genetic instability and tumour evolution, with subsequent upregulation (and/or other mechanism of telomere maintenance) allowing clonal expansion and tumour growth. The human disorder dyskeratosis congenita (DKC, described below), a hereditary disorder of telomerase function, directly supports such a mechanism in humans.

Ultimately the genetic background of a cell with critically short telomeres, particularly in relation to its p53 or Rb status, is likely to determine its fate. p53 is an extremely efficient tumour suppressor, and DNA damage (for example secondary to ionising radiation) results in rapid phosphorylation of its N terminus with stabilisation of the molecule (Shieh, *et al* 1997), (Siliciano, *et al* 1997). The

consequence of this critical regulatory step is either cell cycle arrest (with DNA repair) via expression of p21, or apoptosis. Studies using an $mTR^{-/-}$ $p53^{-/-}$ mouse model have demonstrated a p53-mediated response to critically shortened telomeres, with increased frequency of chromosomal fusions (3-fold) and transformation in cells lacking both mTR and the p53 checkpoint function (Chin, *et al* 1999). Presumably the latter cells are able to accumulate more genetic damage without undergoing cell cycle arrest or apoptosis, increasing the likelihood of a 'second hit ' .

1.6.5 Patterns of telomerase activity in humans

The initial report of telomerase activity in human tissues (Kim, *et al* 1994) (*i.e.* a specific association with tumour cells, immortal cells and germ cells, with no activity in somatic tissues) has not been borne out by subsequent studies. For example, high levels of telomerase activity have been demonstrated in activated lymphoid cells (Weng, *et al* 1996) and proliferating endometrium (Brien, *et al* 1997), with low levels present in many other somatic tissues. It appears to be the case that such low-level expression results from rare telomerase expressing stem or progenitor cells rather than low-level expression in all cells in the specimen (Bonatz, *et al* 1998, Kolquist, *et al* 1998, Ramirez, *et al* 1997), although there may be more widespread expression in proliferating endometrium (Kyo and Inoue 2002).

Thus many somatic tissues, previously accepted as telomerase negative, have been shown to harbour subsets of telomerase competent, presumed progenitor cells. This hierarchical tissue model, with regards to telomerase expression and regulation, has been best studied in the haemopoietic system, and will be discussed in detail in subsequent chapters. In all tissue types however, telomerase activity appears to be tightly associated with proliferative status, although how this

occurs at a regulatory level remains unclear. One practical consequence is that many observations of telomerase modulators *in-vitro* (or of telomerase activity between different primary tissues) may be confounded by altered or inherently different cell cycle activation status rather than by direct modulation of telomerase activity *per se*. Some of the regulatory elements of telomerase activity, as discussed below, also regulate cell proliferation. This view is complicated even further if one accepts that telomerase itself may confer proliferative status on a cell directly, as discussed above.

Studies of telomerase regulation during cell cycle progression have produced conflicting results. Zhu *et al.* demonstrated upregulation of telomerase activity in human tumour cells in S phase, using a series of phase-selective cell cycle inhibitors (Zhu, *et al* 1996). However a subsequent study, using selected cycling cell populations based on DNA content (hence avoiding potentially cytotoxic cell cycle modulators), demonstrated no change in telomerase activity during cycle progression (Holt, *et al* 1997). When cells entered G_0 however, telomerase was repressed. In this study telomerase activity generally correlated with growth rate. By analogy, cells that are post-mitotic should therefore not express telomerase; indeed rapid down-regulation of activity has been described in several cell lines during terminal differentiation (Reichman, *et al* 1997, Sharma, *et al* 1995). This downregulation is apparently mediated by a rapid reduction in hTERT mRNA levels, and occurs independently of simple cell cycle arrest as a specific component of the differentiation programme (requiring de-novo protein synthesis) (Xu, *et al* 1999).

The implications of these observations are considerable, and are frequently overlooked. They would imply that a primary determinant of TRAP activity in a tumour or tissue sample is likely to be the proportion of cycling cells (i.e. stem or

progenitor whether they are normal or malignant) rather than true differences on a cell-to-cell basis. Studies on haematological malignancy support this notion; for example in early chronic lymphocytic leukaemia (a disorder characterised by slow accumulation of mature B-lymphocytes displaying resistance to apoptosis rather than increased turnover) TRAP activity is not raised until the disease transforms or accelerates (when cell turnover is increased) (Bechter, *et al* 1998). To determine whether there are genuine cell-to-cell or sample-to-sample differences in telomerase activity there is at least a requirement for cell-cycle profiling (with resolution of G_0 populations) of the samples under investigation and ideally, comparison of sorted cell-cycle phase-specific (eg S-phase) purified populations. Such studies can be performed relatively easily upon normal and malignant HSC and progenitor cells isolated from PB or BM.

1.6.6 Telomerase deficiency as a cause of human disease

Almost without exception, early studies of telomerase in humans concentrated on the patterns, associations, mechanisms and consequences of over expression, with the major emphasis on tumourigenesis. It was, therefore, of profound interest that an established human congenital disorder, dyskeratosis congenita (DKC), has been linked to mutation and / or aberrant expression of the hTR component of telomerase.

DKC is an inherited multisystem syndrome characterised by skin abnormalities (skin pigmentation, nail dystrophy and leucoplakia), pulmonary complications (a reduced diffusion capacity and / or restrictive defect), BM failure (85.5% of patients have a cytopaenia of one or more lineages) and a propensity to develop solid tumours, myelodysplasia or AML (Dokal 2000). Indeed the probability of BM failure

is 94% at 40 years of age, although expression of the other phenotypic characteristics is very variable. The majority of patients (67%) die from the direct consequences of BM failure, with the remainder succumbing to malignancy (Dokal 2000). Laboratory studies of BM from these patients have described a prematurely aged BM: namely a reduction in the proliferative potential of progenitor cells plus a reduced subplating cloning efficiency (Dokal 2000, Marley, *et al* 1999). DKC therefore appears to affect proliferative tissues, and could quite feasibly fit the bill as a disorder of telomere homeostasis, with its not dissimilar phenotype to late generation KO mice.

The X-linked form of the disease is caused by missense mutations in *DKC1*, the gene coding for dyskerin (Heiss, *et al* 1998), a highly conserved nucleolar protein with pseudouridine synthase activity. As well as binding to the H/ACA small nucleolar RNAs (snoRNA, responsible for ribosomal RNA processing) (Mitchell, *et al* 1999b), dyskerin also associates with hTR via its H/ACA RNA motif (Mitchell, *et al* 1999a, Mitchell, *et al* 1999b). It is assumed that mutations affect this interaction. In affected males there is a fivefold reduction in levels of hTR RNA, with reduced telomerase activity and markedly shorter telomeres in lymphoid cells from these individuals (Mitchell, *et al* 1999b). How dyskerin mediates these effects on hTR and telomerase is as yet unknown, however it may be required for telomerase RNP assembly or accumulation.

That telomerase dysfunction is the 'final common pathway' in this heterogeneous disorder has received further support from studies on the autosomal dominant form of the disease. Vulliamy *et al.* have shown that in some affected individuals studied there are destabilising mutations in one of the hTR alleles (Vulliamy, *et al* 2001). However in these patients hTR expression is not reduced (on Northern blot) and a 'dominant negative' effect of the mutated allele was proposed as the

mechanism for the significant telomere shortening observed in these patients. Unfortunately no TRAP analysis was performed in an attempt to resolve this issue. What is of additional interest is whether hTR abnormalities could play a role in other disorders with similar bone marrow failure phenotypes, for example severe aplastic anaemia (SAA) (Vulliamy, *et al* 2002). Of 17 SAA patients studied, 2 patients had hTR mutations not observed in 214 normal controls, and all patients demonstrated significant telomere shortening. Ultimately some form of gene therapy may be applicable to these individuals.

1.6.7 Telomerase (and telomeres) in tumourigenesis: a synthesis

A complex 'biphasic' relationship of telomerase and telomere function in the context of tumour evolution and growth has been suggested (Hackett and Greider 2002), as briefly outlined above. Progressive telomere shortening, observed in most tumours studied to date and assumed to be largely replicative (de Lange, *et al* 1990, Hastie, *et al* 1990), results in telomere loss and genomic instability. Such telomere loss may be facilitated by lack of telomerase, as observed in KO mice and DKC patients. Conversely, telomerase expression, often implicated as a late event during tumour evolution (Chadeneau, *et al* 1995, Tang, *et al* 1998) can stabilise telomere length (by 'healing' short dysfunctional telomeres (Rudolph, *et al* 2001)) and promote immortality.

This pattern of short telomere length and high telomerase activity is not uncommon, particularly in advanced haematological malignancies (Ohyashiki, *et al* 2002), and it is tempting to speculate that this is due to such sequential telomere shortening followed by telomerase upregulation. Several models incorporating a biphasic role for telomerase have been proposed in the context of human

malignancy, including CML (Brummendorf, *et al* 2000). Such models also imply prognostic significance for telomere length and / or telomerase activity, as according to this mechanism of tumourigenesis short telomeres and elevated telomerase would indicate advanced disease. This has been tested (and shown to be the case) for CML (Brummendorf, *et al* 2000), multiple myeloma (Wu, *et al* 2003) and myelodysplasia (Ohyashiki, *et al* 1994). As well as having prognostic import, such a model may alter the approach to anti-telomerase therapy. For example telomerase inhibitors may be most effective when administered in the context of short telomeres and telomerase dependence (i.e. in advanced disease). It may also suggest that their use should be approached with caution in early or pre-malignant disease, when telomere shortening and genomic instability may be promoted. It is probable that such questions will only be answered by appropriate animal studies or even at the Phase I/II stage of clinical trials.

1.6.8 Telomerase in cancer: diagnostic, prognostic and therapeutic potential

In a large study, Kim determined telomerase activity (as measured by TRAP) to have a specificity of 91%, sensitivity of 85%, positive predictive value of 93% and negative predictive value of 81% in the diagnosis of malignancy in biopsies from a range of tissues (Kim 1997). However these malignancies can, in general, be diagnosed accurately by conventional histological techniques, and one could argue that telomerase adds little in this context.

Detection of telomerase activity may therefore prove more useful in the screening of bodily fluids, *e.g.* urine or sputum, or fine-needle aspirates (FNA) where conventional cytological techniques are more demanding, less sensitive and to a degree, subjective. Hiyama *et al.* undertook a prospective study of telomerase activity in FNA samples from suspected breast cancer: the diagnostic accuracy of

TRAP analysis was significantly higher than conventional cytology (86 vs. 70%, $p<0.001$) (Hiyama, *et al* 2000). TRAP activity of sputum in suspected lung cancer had a sensitivity, specificity and diagnostic accuracy of 81.6, 100 and 86.5% respectively (Sen, *et al* 2001). These studies clearly illustrate the genuine clinical utility of telomerase detection; however, to our knowledge, these techniques have not entered routine clinical practice (at least in the UK).

In the era of intensive chemo- and radiotherapy protocols, improved prognostic indices are a prerequisite for administering risk-adapted treatment regimens. Numerous reports have dealt with telomerase as a prognostic determinant. In colonic adenocarcinoma, levels of telomerase activity are higher with increased tumour stage (Chadeneau, *et al* 1995) and are an independent predictor of outcome (Tatsumoto, *et al* 2000). Similar data has been obtained in non-small cell lung cancer (Marchetti, *et al* 2002) and breast cancer (Clark, *et al* 1997). In one of the most intriguing studies, telomerase negative stage IVS neuroblastomas exhibited short telomeres and frequent spontaneous regression, while those with high TRAP activity had a poor outcome (Hiyama, *et al* 1995). Thus telomerase may indeed, at least in this context, be a true indicator of biological behaviour and clinical outcome. Many reports are inconsistent however, and there are almost certainly issues concerning representative tissue sampling and contaminating telomerase-positive cells (e.g.lymphocytes).

In-vitro studies of telomerase inhibition in immortalised cell-lines (as described above) have provided encouragement for the development of anti-telomerase therapies *in-vivo*. The biology of telomere shortening predicts that after inhibition of telomerase there will be a significant lag-time before a critical length is reached, and this has been born out by studies demonstrating that timing of cell death is related to initial telomere length (Hahn, *et al* 1999b, Zhang, *et al* 1999). These

studies, which used dominant-negative constructs of hTERT, have two important implications: in tumours with long telomeres the patient may be dead before crisis is reached, and that tumours with short telomeres will be more amenable to telomerase inhibition. Importantly, these studies also demonstrated that wild-type p53 was not a prerequisite for apoptosis, which given how common loss-of-function p53 mutations are in human cancer, is reassuring. Additionally, surveys of telomere lengths in cell lines have revealed them to be universally short (5kb or so) (Chu, *et al* 2000). It will therefore be crucial to determine the appropriate clinical setting in which to test anti-telomerase strategies.

The ideal therapeutic approach to telomerase inhibition would involve the use of tailored small-molecule inhibitors. The broad range of expression of telomerase in human cancer makes it an attractive target for the development of such agents (Shay and Baccheti 1997). Crucial to their success will be selectivity or (ideally) specificity for tumour cells. This may be achieved because normal stem and progenitor cells generally have long telomeres, with low-level telomerase activity. Reverse transcriptase inhibitors (such as zidovudine) and nucleoside analogues (such as dideoxyguanosine triphosphate) have anti-telomerase properties in immortalised cell lines *in-vitro* (Strahl and Blackburn 1996). An alternative strategy targets the substrate (the G-quadruplex telomere structure) of telomerase, with several molecules recently described with potent activity in this respect (Harrison, *et al* 1999, Perry, *et al* 1998).

As with all other anti-cancer agents, concerns already exist over development of drug resistance, most likely via selection for ALT tumour cells. That such a mechanism may co-exist with telomerase-dependent telomere maintenance in human cells has recently been demonstrated, giving credence to concerns about resistance via this mechanism (Cerone, *et al* 2001). Alternative approaches to

targeting telomerase that may avoid this potential problem include immunotherapy. In a seminal study Vonderheide *at al.* demonstrated tumour cell lysis by $CD8^+$ cytotoxic T-cells specific for an hTERT peptide (Vonderheide, *et al* 1999). Phase I trials targeting hTERT by this strategy in advanced cancer are currently underway in the USA (Vonderheide 2002). In clinical terms therefore, telomerase has not yet even begun to fulfill its potential; however there are high hopes that the next 5-10 years will change this.

1.6.9 Structure of telomerase and regulation of its activity

The importance of telomerase, both in physiological and pathological systems, is apparent. In contrast, relatively little is known about the mechanisms regulating the expression of telomerase, particularly in primary human cells and during tumourigenesis. Given that most somatic cells repress telomerase activity and most cancer cells express it, it is logical to assume that it is tightly regulated. An understanding of the mechanisms involved is a prerequisite for attempting to manipulate telomerase activity successfully. One important, and as yet unresolved, issue is whether the emergence of telomerase positive tumour cells reflects the phenotype of the originating (*i.e.* stem or precursor) cell, or if there is a sequential breakdown of regulatory events within tumour cells resulting in telomerase expression. This section will deal with the current understanding of how telomerase activity is regulated, and what may go wrong in disease states.

1.6.9.1 The telomerase ribonucleoprotein complex

Exactly how telomerase acts at the telomere is not fully understood. It is assumed that telomerase binds to telomere primer and, using the RNA template of hTR, catalyses the addition of single DNA nucleotides to the telomere (Shippen-Lentz and Blackburn 1990). Once a complete TTAGGG sequence has been completed,

telomerase can translocate to the next binding site, and so on. The *catalytic activity* (the ability to elongate sequential telomeres) and *processivity* (the ability to add single nucleotides) would appear to be differentially affected by physicochemical conditions (specifically temperature and concentration of K^+ and substrate) (Sun, *et al* 1999). However it remains unclear even whether or not these reactions require ATP, and detailed information on the kinetics of the process are lacking. How (and indeed where) the telomerase ribonucleoprotein (RNP) complex is assembled, and what its key components are, is also an area that needs further study.

The estimated molecular mass of the telomerase RNP is approximately 1000kDa in unfractionated nuclear extracts (Schnapp, *et al* 1998), indicating that *in-vivo* it consists of more than simply hTERT plus hTR in combination (Figure 1-5) although this is the minimum requirement to reconstitute telomerase activity *in-vitro* (Beattie, *et al* 1998). *In-vitro* TRAP activity does not necessarily equate with active telomerase and telomere maintenance *in-vivo* however; the other constituents of the telomerase RNP are likely to be crucial to its action.

The molecular chaperones p23 and heat-shock protein (hsp) 90 have recently been shown to stably associate with hTERT *in-vitro* and in mammalian cells (Forsythe, *et al* 2001, Holt, *et al* 1999). Subsequent to binding hTERT, p23 and hsp90 direct assembly with hTR. Consistent with these data is the observation that hsp90 inhibitors blocked telomerase activity (presumably by disrupting its assembly process). It is also notable that p23 and hsp90 were present in the rabbit reticulocyte lysate model that was used to establish the functional significance of hTERT and hTR (Forsythe, *et al* 2001), suggesting that they could be an absolute functional requirement. TEP1 (telomerase-associated protein 1) on the other hand appears to be of doubtful relevance to telomerase activity (Liu, *et al* 2000a).

The human telomerase holoenzyme exists as a functionally co-operative dimer *in-vivo*, containing at least two *trans*-complementary hTERT molecules (Beattie, *et al* 2001) and two interdependent hTR molecules (Wenz, *et al* 2001). hTERT stably associates with the RNA template via a small region at its amino terminus (Lai, *et al* 2001). The majority of evidence to date suggests that transcriptional, post-transcriptional and post-translational mechanisms of the major telomerase components (hTR, and particularly hTERT) all contribute to regulation of telomerase activity.

1.6.9.2 hTR: expression, genetics and molecular regulation

In comparison to hTERT (see below) very little is known about regulation of this intriguing molecule. Part of the reason for this may be the oft-quoted statement describing 'ubiquitous expression' of hTR, implying (wrongly) a lack of regulation and almost a 'housekeeping' presence. Initial reports using RT-PCR detected hTR at 'steady-state' in many telomerase-negative tissues (Feng, *et al* 1995), and hTERT has been demonstrated as the 'rate-limiting' component of telomerase activity in most circumstances (Weinrich, *et al* 1997) leading to it becoming the focus of attention. However, studies on lymphocytes demonstrated hTR levels that paralleled telomerase activity, with a 5-fold increase upon activation (Weng, *et al* 1997) indicative of dyanamic regulatory mechanisms in primary cells. Furthermore a detailed study of hTR expression during embryogenesis detected hTR at high levels in undifferentiated tissues that progressively became undetectable during differentiation. In adults, expression was predominantly limited to dividing cells, with particularly high levels in the primary spermatocytes and Sertoli cells of the testes (Yashima, *et al* 1998). hTR levels are often increased in immortal human cells and tumours, with descriptions of amplification and increased copy number in the literature (Soder, *et al* 1997a). The latter study, using *in-situ* hybridisation,

elegantly demonstrated hTR expression within tumours but not in surrounding normal tissue, a finding subsequently confirmed by other groups (Heine, *et al* 1998). These data, in addition to its role in the pathogenesis of DKC, point to hTR as a crucial telomerase component.

The gene for hTR has been mapped to chromosome 3 (specifically 3q26) (Soder, *et al* 1997b) and is transcribed by RNA polymerase II, which, after processing at its 3' end, results in a mature transcript of 451 nucleotides (Feng, *et al* 1995). A telomerase RNA subunit has now been cloned from more than 25 species, from ciliates to human. Despite their striking difference in size (in ciliates it may only be 147 nucleotides in length (Greider and Blackburn 1989)) and sequence (hTR and mTR share only 65% sequence homology (Blasco, *et al* 1995, Feng, *et al* 1995)) their common feature is a template sequence complimentary to approximately 1.5 telomeric repeats. The introduction of mutations to this crucial site results in predictable alterations to the telomere sequence with cell senescence as a result of telomere dysfunction (Yu, *et al* 1990). In telomerase negative cells, the half-life of hTR is approximately 5 days, and this can be increased some 1.6-fold by the presence of hTERT, via both an increase in transcription and (possibly) half-life duration (Yi, *et al* 1999).

How transcription of hTR is controlled is poorly understood. The elements required for hTR promoter activity are contained in a 231bp region between –272 bp and –42 bp upstream of the transcription initiation site (Zhao, *et al* 1998). The hTR gene is sited within CpG islands, and methylation of the promoter region has been demonstrated in some ALT cell lines, which failed to express hTR (Hoare, *et al* 2001). Interestingly, this region contains consensus sites for the binding of haemopoietic transcription factors, such as GATA-1, PU.1 and C/EBP (Hohaus, *et al* 1995, Scott, *et al* 1994, Zon, *et al* 1991), which is highly relevant to the

regulation of telomerase activity in normal haemopoiesis and leukaemia. Binding of the transcription factor complex NF-Y (also known as CBF) to the CCAAT region of the promoter is essential for hTR promoter activity (Zhao, *et al* 2000), and Sp1 and pRb also activate it. In contrast Sp3 is a potent repressor, raising the possibility that altered Sp1/Sp3 ratios regulate overall gene expression. Indeed Sp1 also activates hTERT expression (Kyo, *et al* 2000), and the possibility of a common mechanism of transcriptional control cannot be discounted. NF-Y can interact with histone acetyltransferase (HAC) enzymes (which antagonise chromatin induced repression), and may serve as a means of keeping the chromatin 'open' to allow transcription to occur (Jin and Scotto 1998). Considerable work is still required to understand regulation of this intriguing molecule.

1.6.9.3 hTERT: genetics and molecular regulation

The hTERT gene is located on chromosome band 5p15.33 (Bryce, *et al* 2000) and extends over 35kb (with 16 exons and 15 introns (Cong, *et al* 1999)). As most somatic cells do not express hTERT to any great degree it is largely assumed that the primary means of regulating hTERT is at the level of transcription.

1.6.9.3.1 Epigenetic regulation

The subtelomeric location of the *hTERT* gene has raised the possibility that its expression is influenced by telomere position effect (TPE), whereby subtelomeric genes undergo reversible silencing. Some evidence for this mechanism exists in human cells (Baur, *et al* 2001) and it would provide a neat 'feedback' mechanism whereby progressive telomere shortening negated TPE with derepression of *hTERT*. The recently published complete *hTERT* sequence pinpoints the gene as greater than 2Mb away from the telomere however (Leem, *et al* 2002), and Cong

has pointed out that this may be too distant for the TPE to function (Cong, *et al* 2002).

CpG islands are target sites for methylation, a process involved in normal development (e.g. X-chromosome inactivation, imprinting and suppression of extraneous DNA sequences (Li, *et al* 1993, Panning and Jaenisch 1998)). The methylation status of cancer cells is markedly different from that of their normal counterparts: a general hypomethylated state (Goelz, *et al* 1985) is interspersed with areas of CpG hypermethylation which generally results in preferential transcriptional silencing of tumour suppressor genes (Merlo, *et al* 1995). However both transcriptional activators and repressors may be methylation sensitive as far as binding to promoter sequences (which may or may not be methylated). Thus promoter methylation is not a simple matter of an 'on-off' switch. The observed hypomethylation of the *hTERT* promoter in somatic cells with hypermethylation in cancer cells has been explained as involving a methylation-sensitive transcriptional repressor binding site (Devereux, *et al* 1999). Conversely one heavily methylated *hTERT*-negative fibroblast cell line, SUSM-1, expressed *hTERT* subsequent to treatment with the demethylating agent 5-aza-2'-deoxycytidine (Devereux, *et al* 1999). *In-vivo*, *hTERT* promoter methylation has been correlated to low telomerase activity in a subset of B-CLL tumour samples, however numbers were small in this study (Bechter, *et al* 2002). The true contribution of methylation status to *hTERT* regulation and expression *in-vivo* thus far remains doubtful.

Likely to be of more relevance is the role of chromatin status: addition of trichostatin A (TSA), an inhibitor of histone deacetylase (HDAC), induced *hTERT* expression in telomerase-negative human cell lines (Cong and Bacchetti 2000), resting T-cells, normal fibroblasts and in cancer cell lines (Hou, *et al* 2002). It

should be noted that TSA does not lead to global changes in gene expression, with acetylation associated with specific target genes (Struhl 1998). Sp1 binding sites, located at the proximal end of the *hTERT* promoter, were found to be required for the action of TSA (Hou, *et al* 2002). *hTERT* promoter histone hypoacetylation has been observed to occur during the differentiation of HL60 cells, concomitant with a reduction in *hTERT* expression (Xu, *et al* 2001). Importantly, this reduction was prevented by addition of TSA. Supporting the existence of this mechanism *in-vivo*, upregulation of *hTERT* expression subsequent to T-lymphocyte activation has been shown to involve histone hyperacetylation (Hou, *et al* 2002). How chromatin remodeling enzymes are recruited and activated at specific promoter sequences and how they interact with known transcriptional factors are currently areas of intense research.

1.6.9.3.2 Transcriptional regulation

Transcriptional regulation is thought to be the most important regulatory element controlling telomerase activity in cells (Ducrest, *et al* 2001), although the evidence for this is not absolute. The core promoter region is contained between 330bp upstream of the transcription initiation site and the second exon (Cong, *et al* 1999), and luciferase-promotor constructs demonstrate inactivity within normal somatic cells with increased activity in immortalised cells, consistent with their observed telomerase activity (Cong, *et al* 1999). The core promoter region also contains numerous transcription factor-binding sites (Cong, *et al* 1999), including E-boxes and Sp1 binding sites.

Transfection of mammary epithelial cells with *C-MYC* expressing viral vectors resulted in a 50-fold increase in *hTERT* mRNA levels (Wang, *et al* 1998). c-Myc expression is a prerequisite for the G_1-S transition, and is deregulated in a large number of human tumours. It is, therefore, not unreasonable to postulate that c-

Myc overexpression is the cause of increased hTERT levels in normal proliferating cells and in tumours (Fujimoto and Takahashi 1997). The c-Myc protein binds to Max and, as a heterodimer, binds to the E-box sequence in the core promoter region of *hTERT*, activating transcription (Blackwood and Eisenman 1991, Kyo, *et al* 2000). This step is independent of de-novo protein synthesis, and is therefore a direct effect (Greenberg, *et al* 1999). An additional level of control is provided by the Mad protein which complexes with Max, competes for E-boxes, and represses *hTERT* transcription (Xu, *et al* 2001). Kyo *et al* have shown that c-Myc cooperates with the transcription factor Sp1 (which binds GC boxes) to activate *hTERT* transcription (Kyo, *et al* 2000). Mutation of these GC-sites absolutely abolished *hTERT* transcriptional activity (Cong and Bacchetti 2000). Oestrogen, via two potential oestrogen response elements in the promoter region, can also directly upregulate hTERT in hormone responsive tissues (Kyo, *et al* 1999, Misiti, *et al* 2000). This mechanism neatly explains endometrial telomerase activity as described above. In the mouse, the highly-expressed and inducible transcription factor NF-κB can bind to the promoter region and activate mTERT transcription (Yin, *et al* 2000), and hTERT has recently been described as a downstream target of NF-κB in a human myeloma cell line (Akiyama, *et al* 2002). With regards to CML, BCR-ABL has direct effects on both c-Myc (Sawyers, *et al* 1992) and NF-κB (Reuther, *et al* 1998) leading to speculation that hTERT expression may be directly affected in *BCR-ABL*+ cells.

Repression of *hTERT* transcription may be an important mechanism in somatic cells: fusion of normal cells to immortalised (telomerase positive) cells results in telomerase repression (Wright, *et al* 1996). The transfer of chromosome 3, but not others, into human tumour cell lines repressed telomerase activity in a c-Myc independent fashion (Cuthbert, *et al* 1999, Ducrest, *et al* 2001). This activity has been mapped to two small regions that may contain telomerase repressors

(Tanaka, *et al* 1998), and which often display loss of heterozygosity or homozygous deletion in human cancers (Cuthbert, *et al* 1999).

The other major mechanism for suppression of *hTERT* transcription is via the 'classical' tumour suppressor genes p53, Rb and Wilm's tumour (WT) 1. By an as yet undefined Sp1 dependent (and p21 independent) mechanism, p53 appears to directly repress *hTERT* expression (Xu, *et al* 2000). The repressive effects of Rb are less well understood, and may be secondary to cell-cycle inhibition (Xu, *et al* 1997). WT1 on the other hand appears to repress *hTERT* via direct promoter interaction, and may be involved in tissue-specific *hTERT* repression during differentiation (Oh, *et al* 1999). Again, these observations are attractive in the context of tumourigenesis, as they link loss of tumour suppressor function with loss of inhibition of *hTERT* expression, arguably the two most common abnormalities in human cancer.

1.6.9.3.3 Alternative splicing

Estimates of the number of human genes with alternately spliced forms range from 35 to 59% (Consortium 2001, Modrek and Lee 2002) of the 35,000 or so genes in the human genome, and it is apparent that this process contributes significantly to both the size of the proteome and the resulting phenotypic complexity. Although the mechanisms of splicing itself are well documented, its regulatory elements are not. Indeed alternative splicing would appear to be subject to tight regulation, both in a tissue specific (incorporating activation- and developmental-status specific elements) and inducible manner (Lynch and Weiss 2000, Min, *et al* 1997), thus providing a critical regulatory step for post-transcriptional gene expression.

The human *hTERT* gene has been shown to contain at least 6 alternate splice sites; to date 4 insertion and two deletion sites have been identified (Kilian, *et al*

1997, Wick, *et al* 1999). In Kilian *et al's* original report the most striking variation in patterns of splicing to be observed involved the deletions of one or both 36 and 182bp motifs from the common reverse transcriptase domains A and B (*hTERT*-α and –β, resulting in inactivity or premature translation termination respectively) (Kilian, *et al* 1997, Lingner, *et al* 1997) (**Figure 5-6**). The proximity of these sites allowed their simultaneous detection using one set of primers for RT-PCR. In no normal or tumour tissue so far studied has telomerase activity been detected in the absence of the full-length transcript (Ulaner, *et al* 1998, Ulaner, *et al* 2000). Additionally, the demonstration that the -α variant is a dominant negative inhibitor of telomerase activity has given further support for a regulatory role of alternatively spliced products (Colgin, *et al* 2000, Yi, *et al* 2000). Various normal tissues (colonic crypt and testes), cell lines and primary tumour tissues demonstrate marked differences in splicing patterns at these sites (Kilian, *et al* 1997, Ulaner, *et al* 1998).

It is possible that that splicing may regulate telomerase activity independently of transcription: in foetal heart tissue, loss of telomerase activity with gestation correlates completely with loss of expression of all *hTERT* transcripts (implying primarily transcriptional control) whereas in kidney it correlates with loss of full-length *hTERT* only, with ongoing expression of β-deleted *hTERT* (Ulaner, *et al* 1998). Conversely in solid tumour cell lines it has been demonstrated that there is a near uniform expression of splice variants, with only 5% of total transcripts being full-length (Yi, *et al* 2001). It is however, likely that the highly selected nature of cell lines confounds studies of telomerase expression. Indeed in studies of primary human tumours and normal tissues a wide variety of splice variants are expressed. Thus heterogeneous patterns (albeit generally exhibiting *hTERT*–β expression) are observed in both in melanoma (Villa, *et al* 2001) and neuroblastoma (Krams, *et al* 2001), while normal resting B and T lymphocytes fail

to show any splicing (Liu, *et al* 1999) with only full-length, functional transcripts observed. These patterns strongly support the regulated expression of hTERT splice-variant mRNA, leading to the description of splicing as the 'dimmer switch' for telomerase (Aisner, *et al* 2002).

1.6.9.3.4 Post-translational regulation

The location and activation status of the telomerase RNP would appear to be tightly regulated. The fact that overexpressed hTERT and hTR localise to the nucleus would support the hypothesis that assembly occurs there (Harrington, *et al* 1997, Hiyama, *et al* 2001). Preferential nuclear localisation would appear to be dependent on the 14-3-3 protein family, which bind hTERT and appear to prevent its nuclear export (Seimiya, *et al* 2000). Furthermore Yang *et al* demonstrated active nucleolar-nucleoplasmic shuttling of hTERT (Yang, *et al* 2002), with the nucleolar localisation signal residing in the extreme N-terminus of the hTERT protein. The release of hTERT from the nucleoli occurs in a synchronised manner, with nucleoplasmic hTERT levels enhanced at the expected time of telomere replication, namely S phase (Wong, *et al* 2002). Interestingly in tumour and transformed cells, hTERT remains almost completely dissociated from the nucleolus and is predominantly nucleoplasmic, suggesting that disruption of compartmentalisation is a mechanism operating during tumourigenesis.

Studies on primary human cells have produced similar findings: upon activation of T-lymphocytes, hTERT is phosphorylated and shifts into the nucleus, and the resulting increased TRAP activity occurs without a concomitant rise in hTERT protein levels (Liu, *et al* 2001). Such regulation of the active telomerase complex's proximity to the telomeres via its compartmentalisation is eminently logical. It is also apparent that conventional *in-vitro* whole-cell TRAP analysis will be 'blind' to

this process, as dramatic shifts in telomerase location may occur with no overall change in telomerase activity.

Phosphorylation of hTERT protein by c-ABL in response to ionising radiation, can negatively regulate telomerase activity (Kharbanda, *et al* 2000). c-ABL mediates growth arrest and apoptotic responses to DNA damage in normal cells: one may speculate that by preventing 'capping off' of broken chromosomes (via the action of telomerase), the propogation of cells containing damaged DNA may be avoided. It also raises intriguing questions about potential interactions between hTERT and BCR-ABL. The latter is largely cytoplasmic, and c-ABL is primarily nuclear: constitutive BCR-ABL tyrosine kinase activity may dysregulate cytoplasmic hTERT which could have significant effects upon telomere maintenance. Other signalling pathways, namely protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) via Akt kinase, have also been shown to control the phosphorylation and activation of hTERT (Kang, *et al* 1999, Li, *et al* 1998). These pathways are intimately involved in cell proliferation, apoptosis and carcinogenesis and promotion of telomerase activity may be crucial to their activity.

In summary, expression of telomerase activity is controlled at multiple levels; however by far the majority of these studies have utilised cell lines. It follows that the relevance of these observations to primary human cells, particularly the critical populations of interest (e.g. stem cells) remain unproven. Furthermore, given the apparent complexity of normal regulatory mechanisms, there is enormous scope for their disruption in cancer. Acquisition of a single oncogene such as *BCR-ABL* may have complex downstream effects upon telomerase activity, and these potential interactions are illustrated in **Figure 1-6**. As a first step in delineating these it is necessary to characterise the expression of the major telomerase components and telomerase activity in *BCR-ABL*$^+$ primary human HSC.

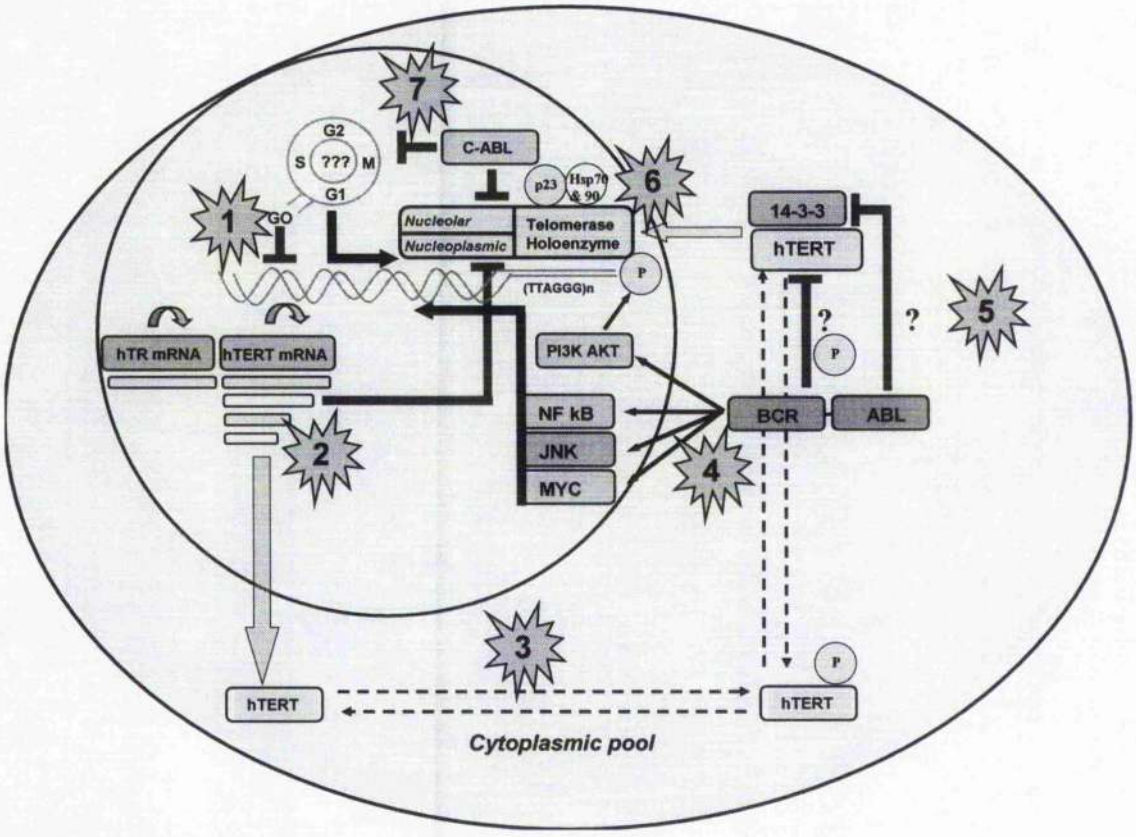


Figure 1-6 Potential interactions of BCR-ABL kinase activity with telomerase

The major regulatory pathways governing telomerase activity are illustrated, as are potential interactions (numbered 1-7) with BCR-ABL. These are as follows: 1) Via alteration of HSC cell cycle regulation (i.e. increased cycling), there may be indirect effects upon transcriptional or post-transcriptional regulation; 2) Alteration of hTERT mRNA splicing; 3) Altered homeostasis of cytoplasmic pool of hTERT, possibly via phosphorylation by the cytoplasmic BCR-ABL kinase; 4) Alteration of telomerase transcription via interaction with transcription factors known to act downstream of BCR-ABL; 5) Direct interaction with hTERT or 14-3-3 proteins altering nuclear-cytoplasmic shuttling; 6) Altered assembly of telomerase ribonucleoprotein complex and nuclear-nucleolar sub-localisation; 7) Loss of one normal c-ABL allele (as a result of the *BCR-ABL* transclocation), perhaps with loss of cell-cycle inhibition or altered phosphorylation status of telomerase. These interactions are entirely speculative, with perturbation of both inhibitory and stimulatory pathways.

1.7 Telomeres and telomerase in normal and malignant haemopoiesis: background and aims

Normal HSC express telomerase; however this is not reflected by stable telomere length (reviewed in **Chapter 3, Results 1**), loss of which is detected in total nucleated cells and $CD34^{+}CD38^{lo}$ cells from adult BM as compared to foetal liver or cord blood (Vaziri, *et al* 1994, Yui, *et al* 1998). Telomerase activity is upregulated in $CD34^{+}CD38^{+}$ (typically cycling) cells, and repressed in the (largely) quiescent $CD34^{+}CD38^{lo}$ group and their differentiated progeny (Yui, *et al* 1998).

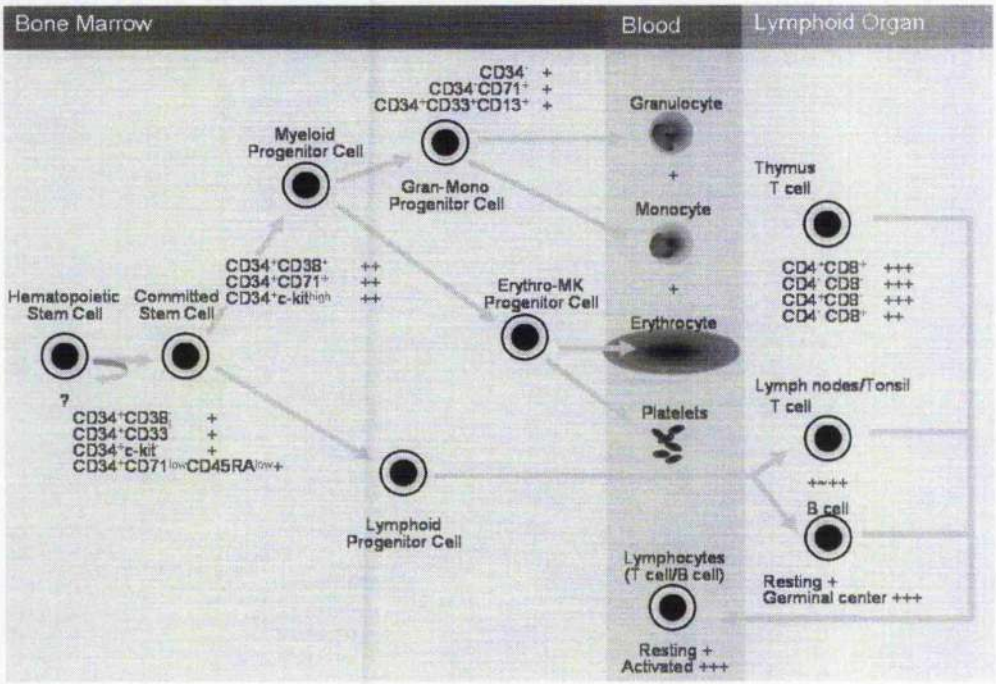


Figure 1-7 Patterns of telomerase expression during haemopoiesis

Telomerase activity (illustrated as +++, ++, +, or high, medium and low respectively) is associated with cycling progenitor cells (i.e. $CD34^{+}$ and lineage $marker^{+}$) and lymphocytes. Activity is particularly high in thymic T-cells and germinal centre B cells, both areas of extensive lymphocyte activation. Adapted from Ohyashiki *et al* (Ohyashiki, *et al* 2002).

This apparently tightly regulated and proliferation-associated pattern of expression may be mirrored to a degree in haematological malignancies of different lineage

and maturational status. For example in chronic lymphocytic leukaemia, a disorder typified by slow accumulation of relatively mature B-cells, TRAP activity is low (Bechter, *et al* 1998), most likely as a result of hTERT promoter methylation (Bechter, *et al* 2002). Progression of the disease to a more aggressive stage was however accompanied by a small (but significant) 2-4 fold increase in TRAP activity. In contrast, acute leukaemias are more often associated with high TRAP activity (from the outset) and short telomeres, and are characterised by the rapid accumulation of blast cells with varying degrees of maturation and lineage commitment (Ohyashiki, *et al* 1997a). TRAP activity in these groups falls with remission induction therapy (unsurprisingly, as the blast cells are killed) and tends to be higher at relapse (Tatematsu, *et al* 1996). The pattern of these results would suggest that telomerase expression in haematological malignancy partly mirrors that of the cell or lineage of origin (i.e. mature non-dividing B-cells express low activity, whereas the stem / progenitor cell population from which acute leukaemias arise have relatively higher activity). How this relationship develops during the course of malignant progression is less clear however: for example selection pressure exerted by short telomeres may mean that in telomerase-low malignancies, up-regulation of telomerase is selected for.

When HSC are put under conditions of replicative stress in-vivo (such as post SCT, see **Chapter 3, Results 1**), telomere sequence is lost more rapidly. In acquired aplastic anaemia (thought to result from immune-mediated damage to the HSC and progenitor-cell compartment) significant telomere loss detectable in PB granulocytes has been observed (Brummendorf, *et al* 2001b). Furthermore, the BM failure of dyskeratosis congenita has been convincingly linked to telomere shortening secondary to telomerase dysfunction (Dokal 2000). These observations have been synthesised with regards haematological neoplasia in a model that describes early replication induced telomere shortening, with telomerase activity

primarily determined by the proportion of cells in the proliferative pool as well as the lineage and maturational status of the tumour cells. Disease progression thereafter is characterised by the pattern of short telomeres and frequently, telomerase upregulation. This model has been partially validated for CML (discussed fully in **Chapter 4, Results 2**), however many questions remain unanswered. Such a model should ideally be tested on primary human material, with several specific questions in mind; firstly, does the degree of telomere shortening at diagnosis have prognostic implications, and how rapidly does it progress thereafter; secondly, whether there is any evidence of telomerase dysregulation during CP disease that may contribute to the rapidity of telomere loss; and thirdly what is the timecourse for telomerase upregulation during disease progression?

1.8 Aims

With regards telomere dynamics in CML,

1. To establish and validate the technique of flow-FISH for telomere length measurement

2. To establish the 'normal' age-related range of telomere length in our local population

3. To determine whether the degree of PBL telomere shortening at diagnosis of CML is of prognostic significance

4. To determine the rate at which telomere shortening occurs during progression of the disease

With regards telomerase expression in CML;

1. To establish and validate the technique of quantitative RT-PCR for detection and measurement of hTERT and hTR expression

2. To determine the expression patterns in CML PBL during disease progression (including hTERT splice variants) and determine any relationship to telomere shortening and BP lineage

3. To determine the expression patterns in CML $CD34^+$ cells *vs.* normal

These questions will be dealt with in turn, within the relevant Results Chapters. Each Chapter begins with a short, focused introduction to the data and culminates in a brief discussion.

2. Materials and Methods

2.1 Cell handling and culture

2.1.1 Red cell lysis

Red blood cells in whole peripheral blood samples or leucapheresis aliquots were lysed with ammonium chloride solution (0.083%, Sigma, Poole, Dorset, UK) at 37^0C for 10 minutes (at a 15:1 NH_4Cl to PB ratio), which was repeated at least twice to ensure complete red cell lysis for flow-FISH. Nucleated cells were recovered by centrifugation (250g for 10 minutes) and resuspended in PBS (Sigma)/0.1% BSA (Calbiochem, Nottingham, UK) for flow-FISH or PBS/2% FCS (Calbiochem).

2.1.2 Cell counting

All cell counts were performed on a Coulter MD18 Analyzer (Beckmann Coulter, Fullerton, CA), which utilised 12µl of sample.

2.1.3 Viability assessment

In most instances viability was assessed using Trypan Blue (Sigma) exclusion. Briefly 40µl of 1:10 Trypan Blue was added to 10µl of cell suspension and 8µl transferred to a haemocytometer. 200 cells were counted, with blue-staining cells counted as non-viable. For pure populations *i.e. ex-vivo* expanded T-cells and $CD34^+$ selected cells, viability assessment using propidium iodide (PI, Sigma) staining and FACS analysis was performed (see below). PI was added (after the appropriate fluorescent surface antibody had been washed off) as the final stage of cell staining at a concentration of 1µg/ml in PBS. After spinning at 250g for 10

minutes cells were resuspended in PBS prior to FACS analysis. PI stained cells were analysed in FL3, and the positively stained fraction (*i.e.* non-viable) gated out of subsequent analysis.

2.1.4 Freezing cells

$CD34^+$ selected cells, control T-cell acute lymphoblastic leukaemia (T-ALL) cells for flow-FISH and aliquots of expanded cell-lines were frozen down for subsequent use where appropriate. Briefly an appropriate cell number was aliquoted into a Nunc cryotube (Fisher Scientific, Loughborough, UK) and resuspended in 10% DMSO (Quest Biomedical, Solihull, UK)/ALBA (4.5% albumin solution, SNBTS, Edinburgh, UK) solution. Cryotubes were cooled steadily in isopropyl alcohol at –80°C in a cryofreezer container (Fisher Scientific) before being transferred to liquid nitrogen for storage. For TRAP analysis a dry cell pellet was immediately frozen at –80°C.

2.1.5 Recovering cells from frozen

Cells were cryopreserved in liquid nitrogen. Extreme care was taken to ensure successful thawing, particularly when a high proportion of granulocytes was anticipated. Cells were removed from liquid nitrogen and immediately thawed at 37°C in a water bath until all ice crystals had disappeared. One 15ml sterile tube (Greiner Bio-One, Longwood, Florida, USA) was used for each sample, and thawing solution added dropwise (i.e. *very* slowly) initially (2.5mM $MgCl_2$ (Sigma), 10U/ml pulmozyme (Roche Products Ltd, Welwyn Garden City, UK) and 1% human serum albumin (SNBTS) in Calcium and Magnesium free D-PBS (Sigma)). Importantly, this was performed at room temperature (RT) to facilitate the action of the enzyme pulmozyme. Once 15mls of this solution had been added, cells were

spun at 250g for 10 minutes. Cells were then washed in PBS/2% FCS, counted and checked for viability.

2.1.6 T-cell culture

T-cells were expanded *ex-vivo* from CML patients as a source of Ph^- cells on which to perform telomere length measurements by flow-FISH. It has previously been shown that this population of cells is indeed Ph^- (Brummendorf, *et al* 2000). $2.5x10^6$ unmanipulated fresh PBL (from CML PB specimens) were cultured in 2mls RPMI 1640 supplemented with 10% FBS, 2% L-glutamine (Sigma), 2% penicillin-streptomycin (Sigma), 50 U/ml recombinant human IL-2 (Pepro Tech, Rocky Hill, NJ, USA) and 1.0 µg/ml phytohaemagglutinin (PHA, Sigma). Adequate T-cell numbers ($>1x10^6$) were achieved after 8-10 days of incubation, at a purity of >85% of viable cells.

2.1.7 Cell – lines

Cell lines were obtained from previously cryopreserved stored aliquots at our institution or as a gift from Dr Junia Melo (Hammersmith Hospital, UK). They were maintained in suspension culture in RPMI 1640 medium (Sigma) supplemented with 10% FCS. Cells were maintained in $25cm^2$ tissue culture flasks (Corning Life Sciences, Koolhovenlaan, Netherlands). On reaching an approximate cell density of $1x10^6$/ml, they were passaged and re-cultured at $1x10^4$ / ml.

2.1.8 Primary human PBL

All material was collected with the approval of the Local Research and Ethics Committee and with written informed patient consent where appropriate. Fresh PBL from 55 normal healthy subjects over a wide age range (Median age 39,

range 0-99 years) were obtained from discarded routine or voluntary blood samples (n=43) and discarded umbilical cord blood (UCB) samples (n=12). All CML PBL samples were obtained from patients with 100% Ph^+ metaphases on their diagnostic or most recent marrow assessment, apart from in 10 patients who had achieved a complete cytogenetic response (CCR, *i.e.*100% Ph^-). Stage of disease was determined according to the WHO Classification of Tumours (Vardiman, *et al* 2001), **Table 2-1** and

Table 2-2). Diagnostic samples were obtained prospectively from a total of 32 consecutive patients (median age 54 years, range 33-75, 19 male and 13 female) prior to commencement of therapy in all cases apart from 2, who had each received hydroxyurea for 2 and 5 days respectively. Hasford prognostic score was calculated online at www.pharmacoepi.de/cmlscore.html. All patients at diagnosis were in CP apart from 2 who displayed cytogenetic evolution (+8 and +Ph respectively, both high-risk score) and were AP as per WHO criteria. Additionally, 24 patients in early CP (<2 years since diagnosis), 12 in established CP (estCP, >2 years since diagnosis), 15 in AP and 17 in BP were sampled. An additional 4 BP samples archived in TRIzol® (Life Technologies, Paisley UK) were made available for analysis of hTERT expression. All cells were analysed fresh whenever possible: however the use of paired CP and BP specimens from the same patient required analysis of cryopreserved material in 5/7 instances.

The Diagnosis of CML-AP may be made when one or more of the following are present:
-Blasts 10-19% of WBCs in peripheral blood and/or nucleated bone marrow cells
-Peripheral blood basophils ≥ 20%
-Persistent thrombocytopaenia (<100 x 10^9/L) unrelated to therapy, or persistent thrombocytosis (>1000 x 10^9/L) unresponsive to therapy
-Increasing spleen size and increasing WBC unresponsive to therapy-
-Cytogenetic evidence of clonal evolution

Table 2-1 Definition of AP CML as per WHO criteria

The Diagnosis of CML-BP may be made when one or more of the following are present:
-Blasts ≥ 20% of WBCs in peripheral blood and/or nucleated bone marrow cells
-Extramedullary blast proliferation
-Large foci or clusters of blasts in the bone marrow

Table 2-2 Definition of BP CML as per WHO criteria.

2.1.9 Selection of $CD34^+$ cells

All cell samples were obtained either from CML patients at diagnosis (n=16), on progression to BP (n=3), from normal allogeneic donors (mobilised with recombinant human granulocyte colony stimulating factor, or rHu-G-CSF, n=2) or from patients with 'non-stem' cell haematological malignancies (non-Hodgkin's lymphoma or multiple myeloma, n=14) mobilised with rHu-G-CSF subsequent to chemotherapy. As part of their initial clinical management, CML patients were leucapheresed using a Cobe Spectra continuous flow blood separator (Cobe

Laboratories, Quedgeley, UK) to reduce the peripheral WBC count and to obtain $CD34^+$ cells for potential later use in an autologous grafting procedure. From each of these leucapheresis products (and after appropriate consent was obtained) a sample was taken for subsequent enrichment of $CD34^+$ cells. All samples were processed within 24 hours of collection. Populations, enriched for $CD34^+$ cells, were obtained using either the Isolex® Immunomagnetic Cell Selection System (Baxter, Deerfield, IL, USA) or the StemSep™ Negative Selection System (Stemcell Technologies, Vancouver, Canada, both techniques performed by Dr Michael Alcorn or Mrs Linda Richmond according to manufacturers' instructions). With the Isolex® System, $CD34^+$ cells are positively selected. With the StemSep™ System, lineage-marker positive cells are removed, thus providing a population of cells enriched for $CD34^+$ cells. To obtain samples with >90% purity of $CD34^+$ cells, these pre-enriched samples were thawed and further selected using the MACS system (Miltenyi Biotec, Auburn, CA, USA) where necessary, according to the manufacturers instructions. The LS column size was used, allowing selection of up to 10^8 cell positively labelled with MACS microbeads, from up to $2x10^9$ total cells. The cells were washed once in PBS/2% FCS and resuspended in 2mM EDTA buffer (300µl per 10^8 cells, Sigma). Prior to magnetic labelling, 100µl of FcR Blocking Reagent per 10^8 total cells was added. 100µl of CD34 MicroBeads per 10^8 total cells were added and incubated at 6°C for 30 minutes. Cells were washed in 2mM EDTA and resuspended at $2x10^8$ cells/ml (maximum) in buffer before being filtered through pre-wetted (2mM EDTA) 30µm pre-separation filters directly onto a pre-prepared LS column in a midi-MACS magnet (all Miltenyi Biotech). After washing cells through (3x3ml of EDTA buffer), cells were eluted, and if resultant cell purity demanded it (based on target of >90% $CD34^+$ cells on FACS), selected for again. An aliquot of approx $5x10^4$ cells was used for FACS analysis to determine purity.

2.1.10 $CD34^+$ cell culture

Upon recovery from liquid nitrogen, $CD34^+$ cells were washed once in PBS/2% FCS (Sigma and Calbiochem respectively) and cultured in 35mm suspension dishes (Corning Life Sciences, Bucks, UK) at a concentration of $2x10^6$/ml for 3 days (at 37°C and in 5% CO_2) in strictly defined medium. This consisted of Iscove's modified Dulbecco medium (Sigma), supplemented with a serum substitute (BIT, Stem Cell Technologies), 1% penicillin/streptomycin (Sigma), 1% L-glutamine (Sigma), and 10^{-4}M 2-mercapto-ethanol (Gibco, Paisley, UK) and 40µg/ml LDL (Sigma). This was supplemented with rHu-IL-3 (50µg/ml, Novartis, Basel, Switzerland), rHu-IL-6 (50µg/ml, Cangene, Mississauga, ON, Canada), rHu-SCF (50µg/ml, Terry Fox Laboratory, Vancouver, BC, Canada), rHu-Flt-3 ligand (50µg/ml, Immunex Corporation, Seattle, WA) and rHu-G-CSF (10µg/ml, Chugai Pharma, London, UK). Cells were harvested on day 3 and washed in PBS/2% FCS prior to analysis.

2.2 Flow – cytometry techniques

2.2.1 Flow – FISH

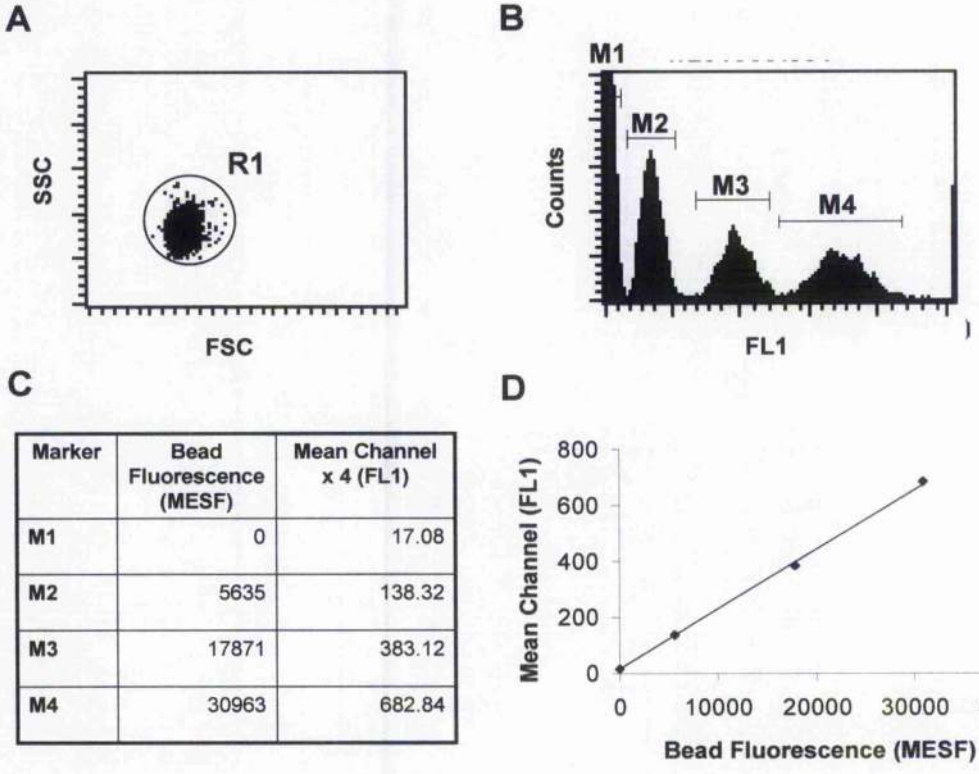
For measurement of telomere lengths in PBL, flow-FISH was established in our laboratory, as previously described (Brummendorf, *et al* 2000), (Rufer, *et al* 1999). This technique allows measurement of fluorescence in whole cells hybridised to a specific telomeric fluoroscein isothiocyanate (FITC)-labelled peptide nucleic acid (PNA) probe. The PNA probe preferentially hybridises to target DNA in conditions of low-ionic strength and is extremely stable. In addition, the reasonably preserved

cell morphology at the end of the procedure allows subpopulations of cells to be discriminated on forward scatter (FSC) *vs.* side scatter (SSC) on a flow cytometer. In almost every case this was performed on fresh PBL, within 48 hours of sampling and with appropriate storage (i.e. 4^0C) during this period. The 48 hour limit was strictly observed: early experience indicated a rapid loss of cell viability after this point. Five valuable paired CP and BP samples (*i.e.* from the same patient both at diagnosis and on disease progression) were analysed from frozen. Red cell lysis of PB samples was repeated at least twice until complete (to avoid potential quenching of the relatively weak FITC signal of the telomere probe by haemoglobin) prior to resuspension in PBS/0.1% BSA. Three x 10^5 viable cells were added to each of 4 Eppendorf tubes (Eppendorf, Cambridge, UK) containing 500µl PBS/0.1% BSA and spun briefly at 13000 rpm in a microfuge (Eppendorf). Stock PNA probe (of sequence FITC-OO-CCC-TAA-CCC-TAA, Applied Biosystems, Framingham, MA) was dissolved in ultra pure formamide (Gibco BRL): double distilled water (ddH_20, 1:1) to give a final concentration of 1µg/µl and then aliquoted and stored at –20°C in the dark. When required each aliquot was resuspended in 640µl of 1:100 TE buffer (Sigma), to a final concentration of 30µg/ml. Cells were resuspended in 300µl hybridisation buffer in duplicate either with telomere probe (20mM Tris pH 7.1(Sigma), 1% BSA, 70% ultra pure de-ionised formamide, 0.3µg/ml PNA FITC telomere probe in ddH_20) or without (20mM Tris pH 7.1, 1% BSA, 70% ultra pure de-ionised formamide). The ultra pure formamide was deionised using AG 501-X8 mixed-bed resin (Bio-Rad Laboratories, Hemel-Hempstead, UK). After careful resuspension of cells in the hybridisation mix (using a yellow tip with vigorous pipetting to ensure a single cell suspension) the tubes were transferred immediately to the thermomixer (CLF-Schutron, Emersacker, Germany, preset at 80°C) for a 10-minute denaturation step. The samples were removed and allowed to re-anneal for 2 hours in the dark

at RT. 1ml of wash buffer #1 was added to each tube (20mM Tris pH 7.1, 0.1% BSA, 70% formamide, 0.1% Tween 20 (Sigma) in ddH_20) which were vortexed and spun at 850g for 7 minutes. This was repeated x 1. Two washes with buffer #2 were then performed (0.1% BSA, 0.1% Tween in PBS). The cell pellet was resuspended in 300µl PI/RNAse (Roche, Welwyn Garden City, UK) solution (0.06µg/ml PI, 1,000 U/ml RNAse T1, 0.1% BSA in PBS). Cells were stored in RT (in dark) for 4 hours (or overnight at 4^0C) before FACS analysis.

Flow- cytometric analysis was performed on a Becton Dickinson FACScan with linear-scale measurement of green (FL1) fluorescence and calculation of telomere fluorescence (stained-unstained) in molecular equivalent of soluble fluorochrome units (MESF). The MESF unit is defined as "the stoichiometric unit for the number of fluorochrome molecules in solution required to produce the same fluorescence intensity as that measured on the labelled particle" (Henderson, *et al* 1998). MESF units are consistent for any given fluorochrome, and may be compared across standards and samples. Where possible, separate lymphocyte and granulocyte gates were set (normal volunteers, UCB and established CP samples) on the 2N (based on PI staining) population. This was not always possible with diagnostic and advanced disease samples; therefore total leucocytes were gated on. BP samples almost always had a discernible blast population (based on low SSC), and gates were set appropriately. Compensation for day-to-day shifts in linearity and laser intensity of the flow cytometer was achieved using a FITC-labelled fluorescent bead standard (Quantum™ 24 Premixed, Bangs Laboratories, Fishers, IN, USA). At the beginning of each experiment, the fluorescence signals from calibration beads suspended in PBS/0.1% BSA were acquired. The beads consist of 4 standard fluorescent (FITC) populations ranging from 3,0000 to 50,000 MESF (molecules of equivalent soluble fluorochrome) as well as a population of non-fluorescent beads. In FL1 (linear), voltage and amplification

were set in such a way so as to give a 'spread' of signal from the control beads up to channel 800 approximately (**Figure 2-1**). Identical FL1 settings were used for analysis of telomere length to allow calculation of the telomere fluorescence signal (mean FL1 channel stained – mean FL1 channel unstained) in kMESF. The equation used was Tel_F (MESF) = $(T_S-T_U)/G_{cal}$, where Tel_F is telomere fluorescence, T_S and T_U are mean channel in FL1 for stained and unstained telomere fluorescence channel number respectively, and G_{cal} is the gradient of the calibration curve.



Marker	Bead Fluorescence (MESF)	Mean Channel x 4 (FL1)
M1	0	17.08
M2	5635	138.32
M3	17871	383.12
M4	30963	682.84

Figure 2-1 Flow-cytometry fluorescence calibration using a fluorescent bead standard.

A bead suspension was gated on (R1), and analysed in FL1 (linear). The unlabelled beads (marker 1, M1) and 3 of 4 fluorescent standards (M2-M4) are shown in the FL1 channel. Mean channel readings were recorded and tabulated to obtain the calibration curve (MESF vs FLI mean channel). This was used to calculate telomere fluorescence in MESF units (see text), and to allow comparisons between different experiments.

In general, all values for telomere length are reported in kMESF units. To estimate telomere length in bps we used the equation as published by Rufer *et al*: base pairs (bp) = MESF units x 0.02604 x 0.019 x 10^3, which roughly equates to 1 kMESF = 0.5 kb. This was derived from a comparative study of flow-FISH and Southern blotting for telomere length in normal lymphocytes (Rufer, *et al* 1998), and subsequently confirmed as valid for granulocytes (Rufer, *et al* 1999).

In all experiments identical aliquots of T-ALL cells were included to analyse inter-experimental variation in flow-FISH results and allow comparison of samples from the same individual at different time-points. In all experiments at least 10^4 events were collected.

2.2.2 High resolution cell-cycle analysis

Telomerase activity appears inextricably linked to the cell-cycle status of the population of interest, although this is not fully understood. For analysis and interpretation of telomerase activity or expression in $CD34^+$ selected populations (both CML and non-CML) it was therefore necessary to resolve the cycle status of the cells. This was done using the method of Jordan *et al* (Jordan, *et al* 1996), which combines 7-amino-actinomycin D (7AAD, Sigma) staining of DNA (in FL3) with that of the nuclear activation antigen Ki67 (FL1, BD Biosciences, Oxford, UK). Importantly, for interpretation of telomerase activity, this technique allows discrimination between G_0 and G_1 phases of the cell cycle.

1x10^6 Cells were washed in PBS/FCS (2%) before being resuspended and fixed in PBS/0.4% formaldehyde (Sigma). After 30 minutes on ice an equal volume of PBS/0.2% Triton X-100 (Sigma) was added, and the cells incubated at 4^0C overnight. Cells were washed once and resuspended in 1ml PBS/2% FCS. This aliquot was split and either 20µl of Ki67 FITC labelled antibody, or appropriate

isotype control, was added to each sample. Cells were washed as before and resuspended (by vigorous pipetting through a yellow tip to ensure a single cell suspension) in PBS/2% FCS/7-AAD (1µg/ml) before being incubated at 4^0C overnight. FACS analysis was performed on a Becton Dickinson FACScan, with log-linear analysis on FL1 (Ki67) and linear analysis on FL3 (7-AAD). To distinguish cells from aggregates and debris the cell population was gated on using FSC vs. FL3. This gate was analysed in FL1 vs. FL3 and the relative percentages in each stage of the cell cycle calculated. In all experiments at least 10^4 events were collected.

2.2.3 Staining and analysis of specific leucocyte sub-populations

Enumeration of *ex-vivo* expanded T-cells, $CD34^+$ cells and CD45RO and CD45RA lymphocyte subset was performed as required using appropriate antibodies (CD3-PE or FITC, $CD34^+$- PE or FITC, CD45RO-PE and CD45RA–FITC respectively, all from BD Biosciences) according to the manufacturer's recommendations. In general live populations were gated on using PI staining (1µg/ml) to discriminate dead cells. Flow-cytometer compensation was set as necessary and isotype controls used as appropriate.

2.2.4 FACS sorting of specific leucocyte sub-populations

Mr Charlie Pearson or Mrs Susan Graham performed FACS sorting of $CD34^+$, $CD3^+$, $CD19^+$ and $CD15^+$ subpopulations using a Becton Dickinson FACSvantage instrument. Briefly, viable cells were sorted (using a PI negative gate), before setting gates appropriately on the positive stained cells of interest with reference to an isotype-control stained population.

2.3 Molecular techniques

2.3.1 RNA and DNA preparation

RNA was prepared from samples aliquoted in TRIzol®. Between 1 and $10x10^6$ cells of interest were stored in 1ml of TRIzol® at -80^0C until required. Fewer than $1x10^6$ cells were stored in 0.5ml TRIzol®. For RNA extraction 200µl of chloroform (Sigma) per 1ml TRIzol® was added and shaken vigorously for 10-15 seconds. Samples were microfuged at 11,000rpm (5 minutes) after standing at RT for 5 minutes. The supernatant was transferred carefully to a fresh Eppendorf tube and kept on ice. An equal volume of iso-propyl alcohol (Sigma) was added, inverted to mix and left at RT for 10 minutes. Centrifugation at 11,000 rpm (5 minutes) revealed a pellet of RNA. The supernatant was poured off and 70% alcohol added (to a volume equivalent to the original volume of TRIzol®), vortexed and spun at 7,500 rpm for 5 minutes. The supernatant was removed and the samples allowed to dry (30 –60 minutes at RT). Depending on anticipated yield of RNA either 10 or 20µl of diethyl pyrocarbonate (DEPC)-treated H_20 (Gibco BRL) was added at this stage before being heated at 55^0C for 10 minutes (to help the RNA go into solution). Standard precautions were taken to minimise RNAse mediated sample degradation *i.e.* use of gloves, solutions made up with DEPC-H_20 and sterile RNAse free plastics.

2.3.2 Quantitation of nucleic acids

This was done spectrophotometrically using a GeneQuant *pro* RNA/DNA Calculator (Biochrom, Cambridge, UK) according to the manufacturer's instructions. Absorbance at 260nm quantifies nucleic acids, and together with absorbance at 230 and 280 can determine purity (via 260/230 and 260/280 ratios)

that is compromised largely by protein contamination. RNA and DNA concentrations were differentially (and automatically) calculated using a correction factor for differing base composition. Pure DNA and RNA preparations have expected ratios of ≥ 1.8 and 2.0 respectively, and absorbencies should be ≥ 0.05 for accurate measurement. Briefly, 1µl of sample was diluted in 500µl of DEPC H_20 and transferred to a quartz cuvette for analysis. The instrument displayed appropriate absorbencies, calculated ratios and quantitated the nucleic acid in question. For quantitative RT-PCR on the LightCycler, RNA was further diluted to a working concentration of 50-100ng/µl. RNA and DNA were stored at –80^{0}C until required.

2.3.3 DNAse treatment of RNA samples

The Ambion DNA-*free*™ Kit (Ambion UK, Huntingdon, UK) was used to remove contaminating DNA prior to Q-RT-PCR for hTR (see below) according to the manufacturer's instructions. Briefly, RNA samples were diluted to 50-100 ng/µl, and DNAse added with incubation at 37°C for 1 hour. Using the inactivation 'slurry' provided in the kit, the DNAse was inactivated, spun down (to pellet the slurry) and the supernatant aliquoted into a fresh 1.5ml tube for later analysis. Typically this process resulted in < 0.1% contaminating DNA.

2.3.4 Synthesis of cDNA and GAPDH RT-PCR

cDNA was synthesized from 1µg of total RNA using the Abgene Reverse-iT™ 1st Strand Synthesis Kit (Abgene, Epsom, UK) according to the manufacturer's instructions. The integrity of generated cDNA was assessed by performing PCR for GAPDH as a housekeeping gene with the Taq PCR Core Kit (Qiagen, Crawley, UK) and using the primers 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' (forward, Clontech, Palo Alto, CA, USA) and 5'-CATGTGGGCCATGAGGTCCACCAC-3'

(reverse) to generate a 983bp product. Settings used were: 94^{0}C for 45 sec, 60^{0}C for 45 sec, 72^{0}C for 2 minutes (all repeated for 30 cycles) then 72^{0}C for 10 minutes

2.3.5 RT-PCR for hTERT altered splicing mRNA products

To analyse the relative expression of the alternatively spliced mRNA products, a single set of primers was used within a single PCR reaction, to allow calculation of relative amounts of each product. Primers, as previously described (Kilian, *et al* 1997), were used to generate a 457 bp product containing the A and B reverse transcriptase motifs, and therefore able to detect the α and β deletions when present (**Figure 5-6**). Splice variant products were amplified from 1µl cDNA using the hT2164F (5'-GCCTGAGCTGTACTTTGTCAA-3') and hT2620R (5'-CGCAAACAGCTTGTTCTCCATGTC-3') primers (Clontech). The Taq PCR Core kit was used with Hot-StarTaq reverse transcriptase (Qiagen). We used the following cycles: 94^{0}C for 15 mins, followed by 40 cycles of 95^{0}C for 30s, 64^{0}C for 45s, 72^{0}C for 45s, and finally 72^{0}C for 5 mins. PCR products were separated and quantitated on an Agilent 2100 Bioanalyser using a DNA 1000 LabChip™ (Agilent Technologies, Palo Alto, CA, see below).

2.3.6 Visualisation and quantification of PCR products

In place of conventional slab gel electrophoresis we analysed PCR products using the Agilent 2100 Bioanalyzer and the DNA 7500 LabChip™ kit. This system allows rapid chip-based separation of nucleic acids within micro-fabricated channels and automates detection, sizing and quantitation to allow digital data evaluation and analysis. Data can be displayed either as a gel-like image or an electropherogram plot. Fragments are detected by fluorescence within the 670 to 700nm wavelength range. To prepare a chip for sample loading, a gel-dye mix (pre filtered through a spin-filter) was injected under pressure into the loading well on a chip. Each

sample well (up to 12 can be analysed in each chip) was loaded with a marker (upper and lower) in buffer solution, followed by the sample of interest (*i.e.* RT-PCR products). A standard DNA sizing ladder in an additional well allowed accurate sizing of detected fragments. Chip-analysis on the instrument is automated, and was generally complete within 30 minutes. PCR product concentrations were calculated in ng/µl, and sized in bps.

2.3.7 Quantitative RT-PCR for hTR and hTERT

Measurement of telomerase activity by the TRAP assay is at best semi-quantitative. It is also limited by the requirement for enzymatically active telomerase in the sample with the associated problem of enzymatic instability during storage. The assay itself may generate non-specific amplification products or be inhibited by PCR inhibitors within the sample itself (Kim and Wu 1997). These limitations are effectively overcome by quantifying the expression of the two major components of the telomerase complex (hTR and hTERT) using the LightCycler instrument (Roche Diagnostics Ltd). Both these reactions were carried out using the relevant LightCycler kits (the LightCycler TeloTAGGG hTR and hTERT Quantification Kits respectively, Roche Diagnostics Ltd) and rely on the same principles of detection, namely a one-step RT-PCR in glass capillaries. The target sequence was reverse transcribed using commercially supplied primers, and the amplicon detected using a specific pair of hybridisation probes. These consist of a pair of oligonucleotides that hybridise to an internal sequence of the amplified fragment during the annealing phase of the amplification cycle. One probe is labelled at the 5'-end with LightCycler-Red 640 and the other at the 3' end with fluorescein. After hybridisation to the target DNA the two probes come into close proximity to each other. This results in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, fluorescein, the

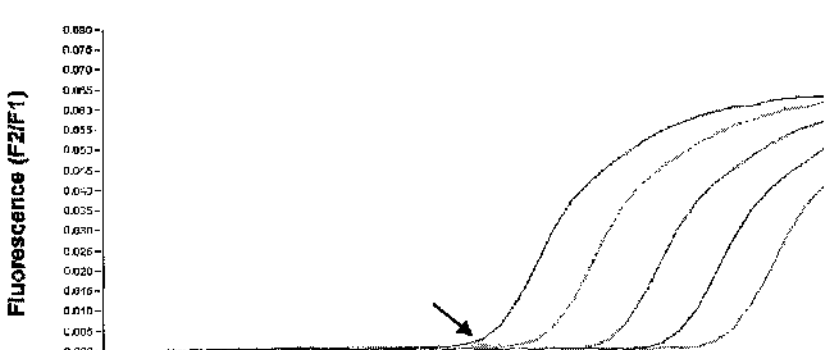
donor fluorophore, is excited by the LightCycler instrument light source and part of the excitation energy is transferred to the red (acceptor) fluorophore. The instrument then measures the emitted fluorescence of LightCycler-Red 640. Quantification is achieved by measuring the point at which the detected fluorescence crosses the detection threshold (the 'crossing point'), this being inversely proportional to the amount of target present. An absolute number of transcripts can be assigned to this point by incorporating a series of standard dilutions of RNA template (**Figure 2-2**). Correction for loading of RNA and PCR efficiency is achieved by a separate (although within the same PCR run) one step quantitative RT-PCR reaction for porphobilinogen deaminase (PBGD), a housekeeping gene. This acts as a reference for relative quantification, and is used in both kits; only when $>10^3$ PBGD transcripts were detected was the sample deemed suitable for inclusion in the final data set. All PCR reactions were run with a positive and negative control (for both hTERT or hTR and PBGD).

The assay was performed according to the manufacturer's instructions, using either RNA or cDNA. Briefly the appropriate quantities of master mix (MM, n+1) were made; 2µl of hTERT reaction mix, 0.1µl of reverse transcriptase, 2µl of hTERT or PBGD detection mix and 13.9µl of PCR-grade H_20 were added per sample (total 18µl). These were stored briefly on ice and protected from light until required. The RNA of interest was diluted (with PCR-grade H_20) to a final concentration of 100ng/µl. After addition of 18 µl of the appropriate master mix to each capillary, 2 µl of the sample RNA (*i.e.* 200ng) was added to each. A typical run for hTERT included 5 standards (hTERT template of a known copy number), 2 positive controls (hTERT and PBGD respectively, provided in the kit), 2 negative controls (PCR-grade H_20), and up to 11 samples of interest (with separate hTERT and PBGD reactions for each). The instrument settings used were: 60°C for 10 minutes then 90°C for 30 sec (once only), followed by 95°C for 1 sec, 60°C for 10

sec and 72°C for 10 sec (repeated for 40 cycles) then 40°C for 60 sec. Amounts of hTERT and hTR transcripts were expressed as a percentage relative to that of PBGD ([hTERT or hTR transcript copy number / PBGD transcript copy number] x 100). Use of the same housekeeping gene and identical PCR conditions in both the hTR and hTERT assays permitted direct comparison of transcript levels of each gene in the same sample.

The hTERT kit utilises a primer / probe set that detects only β-sequence containing transcripts (see **Figure 5-6**). (It should be noted that sequences of the primers and probes were not made available by Roche and repeated attempts to sequence the final product were unsuccessful). The hTR gene is intron-less and so requires incorporation of minus-RT controls to exclude amplification of contaminating DNA. Only samples that demonstrated <0.1% levels of DNA contamination (as calculated by [–RT control hTR transcript number / +RT hTR transcript number] x 100) were included for analysis.

A



RNA Standard	Number of transcripts	Crossing point (cycle number)
Standard 1	8.8×10^5	21.12
Standard 2	1.0×10^5	24.22
Standard 3	8.4×10^3	27.93
Standard 4	9.1×10^2	31.27
Standard 5	7.0×10^1	35.35

B

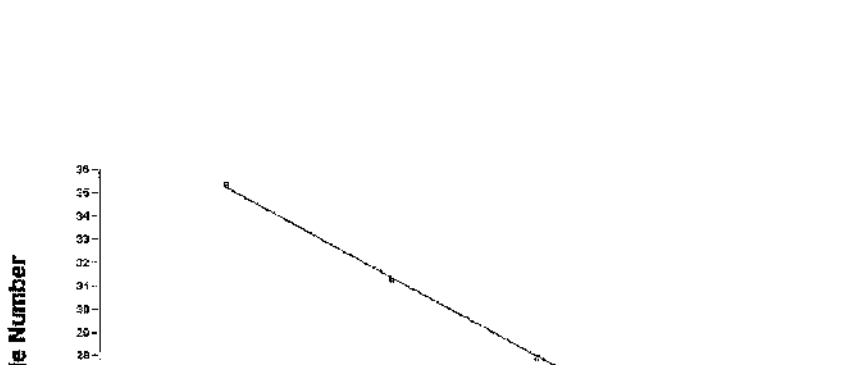


Figure 2-2 Generation of LightCycler standard curve for hTERT Q-RT-PCR

5 known standard concentrations of hTERT RNA template are included with each PCR run. The increase in sample fluorescence during the PCR reaction is visualised as a curve (A) that generates a 'crossing point', at which the fluorescence becomes detectable (arrow on Standard 1 curve). This is proportional to the amount of starting template and can be plotted to obtain a standard curve (B). Crossing points for 'unknowns' can therefore be converted into absolute numbers of transcripts.

2.3.8 FISH for the BCR-ABL translocation

To confirm that the selected populations of $CD34^+$ cells from CML patients were clonal in origin (i.e. *BCR-ABL* positive) we performed dual dolour FISH on an appropriately fixed aliquot. Cells were fixed according to a standard protocol. After washing in PBS, 1 x 10^6 cells were spun at 450g at RT and resuspended in prewarmed (37^0C) hypotonic solution (0.075M KCl, Sigma), mixed gently and allowed to stand for 10 minutes at RT. Cells were spun at 450g, resuspended in 2ml of freshly prepared fixative (methanol: acetic acid, 3:1, Sigma) and incubated at RT for 5 minutes. After a further spin cells were suspended in 10ml fixative. This step was repeated twice. Cells were transferred to an Eppendorf and resuspended in 1ml fixative (to a final density of approximately 1 x 10^6 cells / ml) and stored at –20^0C until required. Mrs Elaine Allan (SNBTS) performed FISH staining and scoring of cells. FISH staining was performed using the Vysis LSI *BCR-ABL* Dual Colour Translocation Probe (Vysis, Richmond, UK) according to the manufacturer's instructions and imaged an epi-fluorescence microscope with a 100-watt mercury lamp with appropriate single band pass filters (including DAPI (Sigma) for the counterstain).

2.3.9 Telomere repeat amplification protocol (TRAP)

Telomerase activity of PBL and $CD34^+$ selected samples was peformed (with assistance from Aileen Monaghan, Beatson Institute) using a modification of the telomeric repeat amplification protocol described by Kim *et al* (Kim, *et al* 1994). Dry cell pellets (10^5-10^6 cells) were stored at –80^0C until required, whereupon they were lysed with NP-40/NaDOC lysis buffer (Calbiochem). Protein concentrations were measured, and 10µL of appropriately diluted extracts (containing 1 and 0.1µg protein extract of each sample) was added to 40µL reaction buffer (50µM dNTPs, 1µg telomerase substrate (TS) primer [5'-AATCCGTCGAGCAGAGTT-3']

(Clontech), 20mM Tris/HCl pH 8.3, 1.5mM $MgCl_2$, 63mM KCl, 1mM EGTA, 0.1mg/ml bovine serum albumin, 2U Taq DNA polymerase (Invitrogen) and 1ug alternative complementary (ACX) primer [5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3']). Samples were incubated at room temperature for 30min and then heat inactivated for 10min at 85°C. Telomerase elongated product in samples was amplified: 90sec at 90°C then 31 cycles of 94°C for 30sec, 50°C for 30sec, 72°C for 90sec. An aliquot of each sample was heat-treated (10min, 85°C) before assaying to serve as a negative control. An internal telomerase assay standard (ITAS, 15 attogram), was included in the PCR buffer. For qualitative analyses, 5µL PCR product was analysed on a 12% non-denaturing polyacrylamide gel. Gels were stained for 20min with 1X SYBR Green (Invitrogen) and visualised on a UV transilluminator. Samples were scored as positive when compared to the GLC4 telomerase positive cell extract included as standard in each assay. Limit of detection was 100 GLC4 cell equivalents. All samples were assayed blind and experiments were performed in duplicate. For semi-quantitative analyses, 1µL of PCR product was analysed on the Agilent Bioanalyser, using a DNA 500 Chip (**Figure 6-2**). The concentrations (ng/µl) of the first six 6-bp incremental bands were added together (as previously described (Hamad, *et al* 2002)) and expressed as a percentage of 10,000 GLC4 cell equivalents. The first peak detected was 50bp in length, and consisted of the primers plus one telomere repeat (**Figure 2-3**). This was therefore excluded from analysis and the next 6 peaks analysed. All samples were assayed blind.

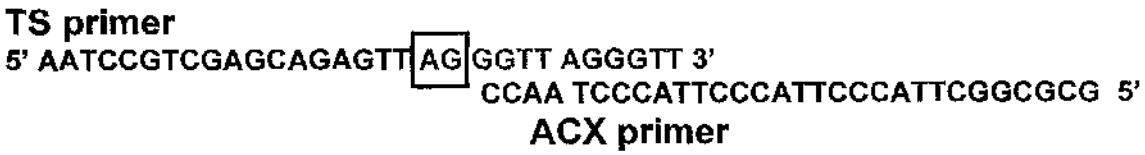


Figure 2-3 TRAP primers constitute the first detectable peak (50bp) on analysis

The TS primer is 18bp in length, and the reverse (ACX) primer is 30bp. Together with a single telomere repeat plus a degree of primer overlap, the overall length of the product is 50 bp. Consequently this peak was excluded from analysis, with the subsequent 6 6bp-incremental peaks quantitated.

2.3.10 Statistics

All statistical tests were two-sided. Comparisons between groups of continuous variables were performed using the Mann-Whitney U or Kruskall Wallace tests. Paired sample analysis was performed using the Wilcoxon signed rank test. ANOVA was used to determine the significance of regression analyses. GB-Stat Statistical Software for Windows (Dynamic Microsystems, Silver Spring, MD) was used throughout, and significance was assumed for p values < 0.05.

3. Results 1

3.1 Establishment and validation of flow-FISH

3.1.1 Introduction

Conventional telomere length measurement by Southern blot analysis utilises restriction enzymes which cleave internal (but not telomeric) sequences, plus a telomere-sequence specific probe [^{32}P-(CCCTAA)$_3$], and generates a smear of DNA fragments called terminal restriction fragments (TRF). Thereafter densitometry scanning of each lane allows determination of the amount of telomeric DNA and the mean TRF length (Harley, *et al* 1990). The actual length of the telomere sequences is some 2-5kb less than the TRFs as a result of the upstream location of the restriction enzyme sites (Harley 1991, Hultdin, *et al* 1998), and there is also data to suggest that the position of these sites vary considerably between individuals (Brown, *et al* 1990). Incorporation of sub-telomeric sequences in Southern TRF analysis will reduce the ability of this technique to resolve small, yet potentially significant, differences in telomere length (Rufer, *et al* 1998).

Fluorescent *in-situ* hybridisation techniques (FISH) allow measurement of the telomere sequences *per se*, via binding of a fluorescent PNA probe. Such probes contain an uncharged glycine backbone (rather than the charged backbones of oligonucleotide probes) (Egholm, *et al* 1993). They hybridise to the target DNA sequences under low-ionic strength conditions that do not favour the reannealing of the target strands. Therefore, under the hybridisation conditions used in the flow-FISH protocol, PNA-DNA interactions (as opposed to DNA-DNA or DNA-RNA) are selected for. The heat treatment of the cells (at 80°C in the presence of

formamide and PNA probe) allows for simultaneous denaturation of DNA, and fixing of the cells.

Flow-cytometric methods of detection measure average telomere length in test cell populations (flow-FISH), while digital-imaging techniques can measure individual telomeres in metaphase preparations, so-called quantitative-FISH (Q-FISH) (Poon, *et al* 1999, Rufer, *et al* 1998). However, the Q-FISH technique is labour intensive and time consuming, and as it measures cells in metaphase, may select for those with longer telomeres (*i.e.* those still able to divide) in populations nearing senescence or crisis. Flow-FISH only requires 3 x 10^5 cells per sample, as opposed to the 10^6 - 10^7 required for Southern blotting. Although the latter two techniques offer a mean telomere length for any given cell population, the distribution patterns of the hybridisation signal can allow inferences to be made on the distribution of telomere length in the sample. For these reasons we elected to use flow-FISH as a high throughput technique which could give a telomere length measurement within 30 hours.

The initial aim of our study was to establish flow-FISH in the UK, and validate the technique with a collaborative laboratory in Tubingen (Germany). These data are discussed in this chapter. Additionally, the measurement of age-related telomere decline in a healthy local population served as a baseline for later analysis of CML patient material, while further validating our dataset with published normal ranges of telomere length. Of further interest was whether we could model the increased rate of telomere loss observed for lymphocytes, as previously published, as being consistent with a shift from naïve to memory cells.

3.1.2 Measurement of telomere length in a 2N population using flow-FISH

The incorporation of PI staining of DNA allows gating on a 2N population of cells. This avoids skewing of the signal by doublets, cells in S phase (synthesising telomere sequences), G_2 or M phases (4N), and also allows exclusion of cells with a sub-2N DNA content (*i.e.* apoptotic cells). 2N cells were therefore gated on first (R1, in FL3 vs. FSC). Analysis of R1 by histogram plot in the FL3 channel confirmed the existence of a single (G_0/G_1) peak (**Figure 3-1**). Despite the denaturation and fixation steps involved in the procedure, the FSC/SSC properties of the 2N cells were remarkably well preserved allowing separate gating on distinct populations (*i.e.* lymphocytes and granulocytes, **Figure 3-2**). Analysis of the FL1 histograms consistently demonstrated a greater heterogeneity in the lymphocyte gate (as shown by the larger CV, or wider histogram plot) as compared to granulocytes. This has been noted previously, and is assumed to be due to the more complex replicative history of the lymphocyte population (Rufer, *et al* 1999). In keeping with this was the increased lymphocyte (but not granulocyte) CV with age (**Figure 3-4**). To confirm the rationale for excluding the putative 4N population, separate gating on the latter gave a telomere signal almost 2-fold greater than the 2N equivalent, as would be expected from the duplicated telomere sequences (**Figure 3-3**).

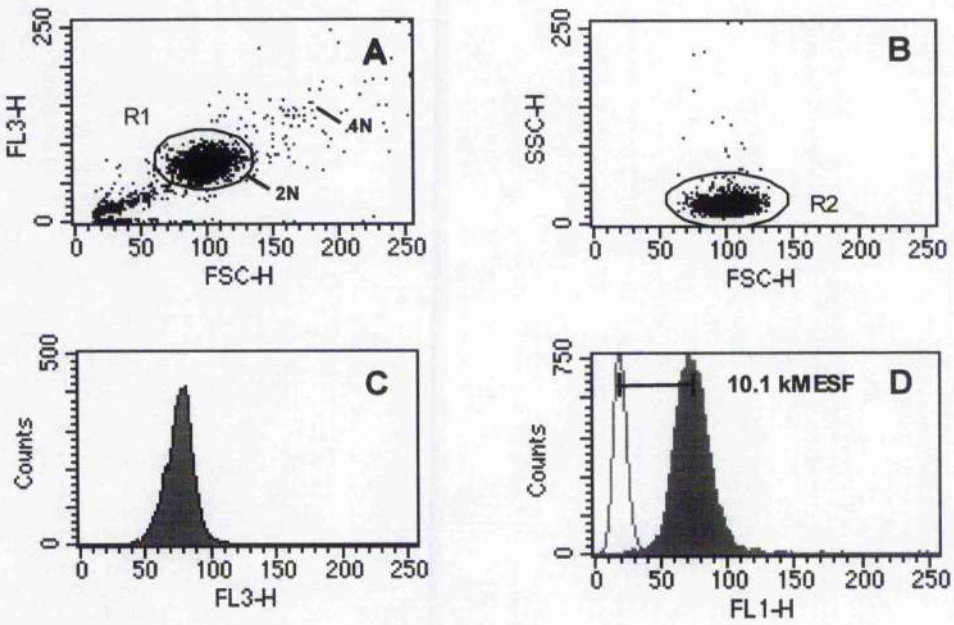


Figure 3-1 Flow-FISH analysis of T-ALL cells

This pure (95%) population of T-ALL blasts served as a control for all flow-FISH experiments. Panels A-D represent the sequential gates used for standard flow-FISH analyses. 2N cells (A and C) in FL-3 (PI) were analysed by FSC/SSC to confirm a monomorphic population (B). The specific telomere fluorescence of cells (Tel_F) was calculated by subtracting the mean background fluorescence of cells hybridised without a PNA probe (T_U, open histogram, panel D) from the mean fluorescence obtained with probe (T_S, shaded histogram, panel D). A correction factor of 4 was applied to the mean FL1 channel number to correct for analysis of FL1 on a scale of 1-1000. Therefore for this example, Tel_F is calculated by $(T_S-T_U) \times 4/G_{cal}$: i.e. (72.3 – 17.5) x 4 / 0.0217 = 10.1kMESF, where G_{cal} is the gradient of the slope obtained from the calibration beads for this experiment (see 2.2.1).

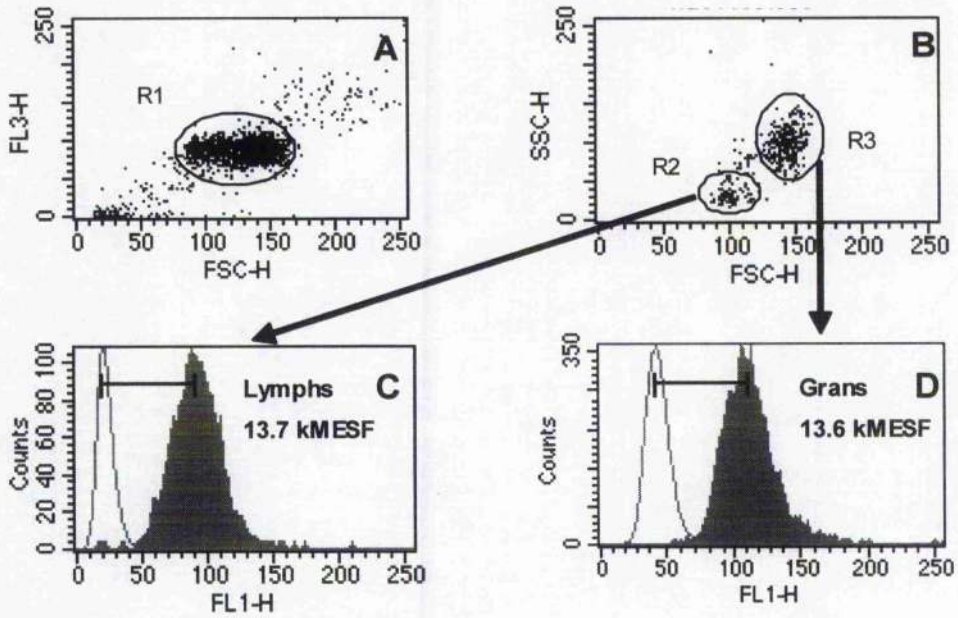


Figure 3-2 Flow-FISH analysis of normal PBL

Nucleated cells (after lysis of red cells) were analysed from a normal 20-year old donor. Analysis of the 2N population (R1, **A**) in FSC/SSC (**B**) allowed resolution of the lymphocyte and granulocyte populations, which could be gated on separately (**C** and **D**). The validity of these gates for their respective populations has been established by Rufer *et al*, using purified lymphocytes and granulocytes (Rufer, *et al* 1999). Note that granulocytes have a higher degree of auto-fluorescence than lymphocytes (a consistently observed feature). To estimate the telomere length in bp, the equation bp = MESF units x 0.02604 x 0.019 x 10^3 was used as previously described (Rufer, *et al* 1999). By this method lymphocytes had a telomere length of 6.8 kbp, and granulocytes 6.7 kbp. The CV for lymphocytes (with probe) was greater than for the granulocyte population (19.4 vs. 15.5 respectively, shaded histograms).

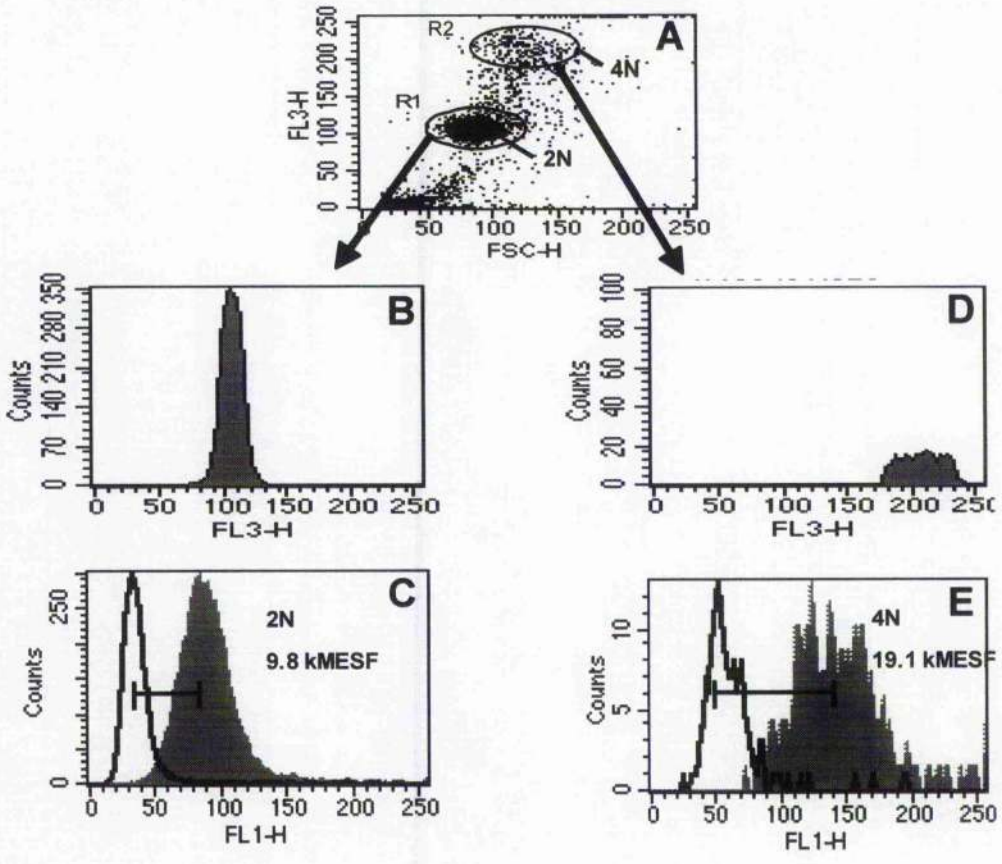


Figure 3-3 Telomere signal in 2N vs. 4N populations.

To further confirm the validity of using a 2N gate an actively dividing population of cells was analysed; in this case *ex-vivo* expanded T-cells at day 6 of culture (see Methods). It has been shown that DNA content is proportional to chromosome (and hence telomere) number (Mandahl, *et al* 1993). The 2N (G0/G1), S and G2/M (4N) populations can be broadly identified from histogram analysis of FL3 and/or FL3 vs. FSC (**A, B** and **D**). Telomere fluorescence for the 4N population was almost double that for 2N cells, as would be expected (**C** and **E**). The slightly lower than expected value is probably due to inclusion of S-phase cells in R2.

3.1.3 Intra- and inter-experimental variation

To further validate the technique in our hands, several aliquots of T-ALL control cells were analysed sequentially in Dr Tim Brűmmendorf's laboratory in Tuebingen, Germany. Their results (mean±SD, 9.7±0.5 kMESF, n=5) compared closely with those obtained in our laboratory (10.5±1.7 kMESF, n=62). The

resulting CV of 17% is in keeping with published results (Brummendorf, *et al* 2000, Rufer, *et al* 1999, Rufer, *et al* 1998). Variation in identical samples stained *within* the same run was greatly reduced (mean 6.9±0.28 kMESF, CV 4%, n=8). In practical terms several steps were taken to reduce the effect of variation on the final data. Firstly, wherever possible, a range of telomere lengths were included in each run, i.e. by incorporating a range of ages (from 0-99) for normal controls, or a range of CML stages (*i.e* CP, AP and BP). Secondly, where direct comparisons were being made between serial telomere measurements within the same patient, a correction factor was applied based on the control cell fluorescence (see Chapter 4). Thirdly, meticulous attention to experimental conditions, reagents etc. was maintained to reduce variation in results. Fourthly, all samples were analysed in duplicate (both stained and unstained) and mean fluorescence used throughout. Fifthly, both our batches of probe (used sequentially over a two-year period) were compared to an aliquot from the German laboratory (the reference probe) to ensure comparable fluorescence intensity.

Probe used	**Mean Lymphs(±SD, kMESF)**	**Mean Grans (±SD, kMESF)**
Reference Probe	11.2±0.7	12.5±0.6
Batch 1 Probe	12.5±0.2	13.8±0.1
Batch 2 Probe	11.9±0.6	13.3±0.7

Table 3-1 Comparison of telomere fluorescence between different batches of PNA probe

To ensure uniformity of fluorescence between batches of PNA probe, we compared telomere fluorescence using PBL from the same individual. This table illustrates a direct comparison of all three lots of probe used within all flow-FISH experiments. These were compared within a single run, each in duplicate. It should be noted that the relationship between the lymphocyte and granulocyte telomere fluorescence remains constant.

3.1.4 Normal age-related telomere loss

A total of 55 normal volunteer samples were collected from discarded umbilical cord blood bottles (UCB, n=12) and otherwise healthy volunteers across a broad age range (median age 39, range 0 – 99 years). Flow-FISH was performed on unfractionated PBL, and granulocyte and lymphocyte telomere fluorescence measured as described above (**Figure 3-4**).

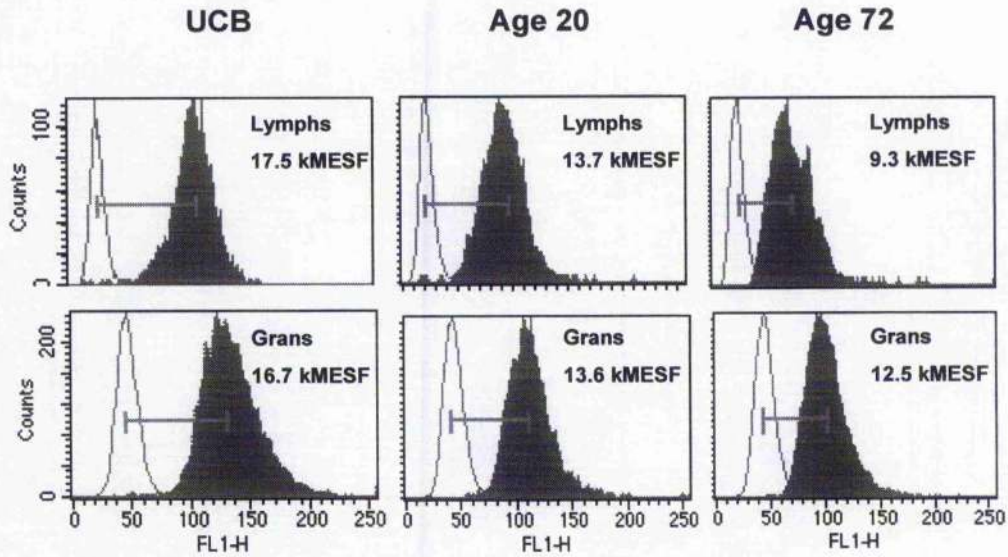


Figure 3-4 Telomere shortening occurs with age

A representative example of telomere fluorescence at extremes of age. As determined by the flow cytometry software there was a progressive increase in CV for lymphocyte fluorescence with age (16.4, 19.4 and 27.1 in this series respectively) which was not evident in the granulocyte population (17.0, 15.5 and 16.5 respectively). This is in keeping with a more diverse replicative history in the lymphocyte population, as a result of repeated clonal expansion.

Simple linear regression analysis gave the equations $y = -0.0686x + 16.398$ ($R^2 = 0.46$, $p<0.0001$) for granulocytes and $y = -0.0969x + 16.93$ ($R^2 = 0.68$, $p<0.0001$) for lymphocytes, giving approximate annual rates of loss of telomere sequence of 34 and 48bps respectively (in keeping with previous estimates (Rufer, *et al* 1999), **Figure 3-5**). Lansdorp's group have recently described a cubic curve as the 'best

fit' for age related telomere length decline in normal PBL (Verfaillie, *et al* 2002). For our data a quartic relationship further improved the fit (albeit slightly). The respective equations were: $y = (-5 \times 10^{-07}) \times x^3 + (0.0003 \times x^2) - (0.0896 \times x) + 16.598$ for granulocytes ($R^2 = 0.46$, $p<0.0001$) and $y = (-9 \times 10^{-06}) \times x^3 + (0.0017 \times x^2) - (0.1804 \times x) + 17.529$ for lymphocytes (R^2 0.7, $p<0.0001$).

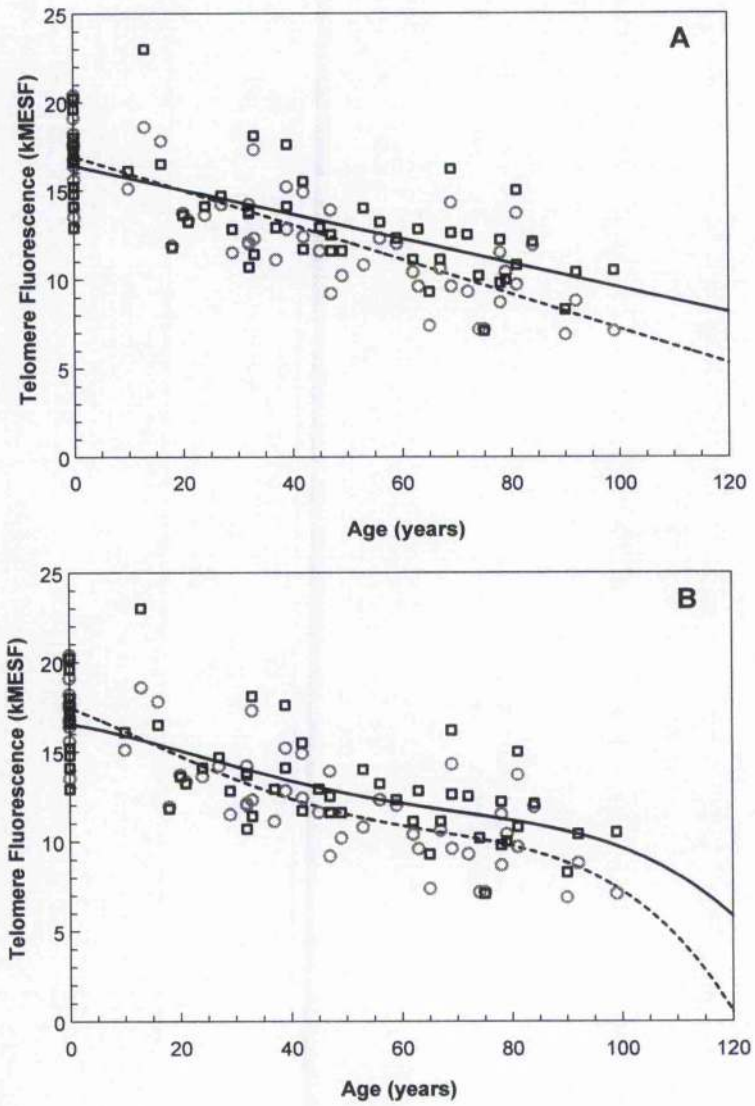


Figure 3-5 Flow-FISH analysis of normal individuals

The top panel (**A**) describes age-related telomere loss in a linear fashion, and the bottom (**B**) using a quartic relationship (see text). Telomere loss in the lymphocyte population (open circles, dotted line) was more rapid with age than that of granulocytes (open squares, solid line). This is most likely due to shorter telomeres in the memory lymphocyte population (as compared to naïve) which predominates with age.

3.1.5 Age-related increase in naïve vs. memory lymphocyte subpopulations accounts for more rapid lymphocyte telomere shortening

To confirm the shift from a predominantly naïve lymphocyte population to that of a memory one (with shorter telomeres (Rufer, *et al* 1998)) we analysed a subgroup of the normal individuals described above for expression of CD45RA vs CD45RO by flow-cytometry. CD45RA is present on $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes and on essentially all B and NK lymphocytes. CD45RA is expressed on naïve T lymphocytes and antigen density rapidly decreases upon activation. Conversely, CD45RO (present on T lymphocytes, monocytes, macrophages and granulocytes) is upregulated upon activation and its presence most likely defines a primed population of memory T lymphocytes. Dual staining of unfractionated PBL (n=46) with CD45RO and CD45RA allowed relative quantitation of memory and naïve cells within the lymphocyte population (**Figure 3-6**). There was a progressive age-related shift from a naïve to a memory phenotype (**Figure 3-7**).

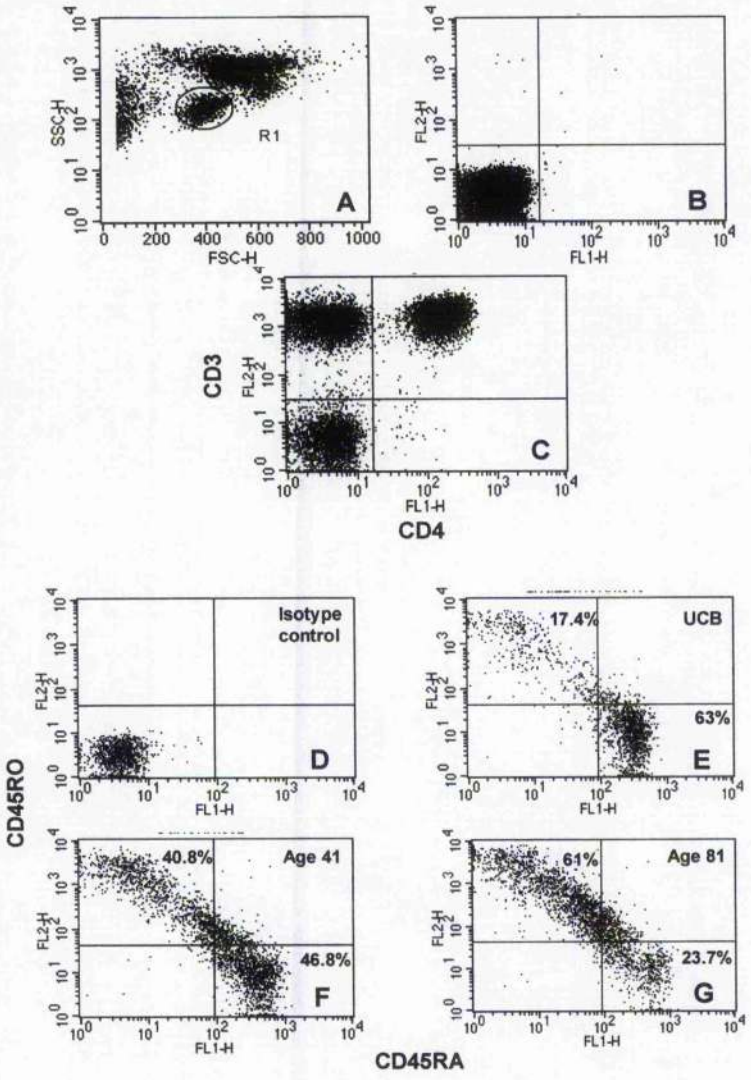


Figure 3-6 FACS analysis of memory vs naïve lymphocytes

The lymphocyte population was identified easily using SSC *vs.* FSC (A). After setting appropriate quadrants using the isotype control (B), CD3-PE and CD4-FITC staining allowed discrimination of the major lymphocyte sub-populations (C) *i.e.* $CD4^+$ T cells (UR), $CD4^-$ T Cells ($CD3^+CD8^+$ cells, UL) and presumed B and NK cells (LL). R1 was therefore used to determine the relative frequencies of CD45RA (FITC) and CD45RO (PE) lymphocyte populations over a range of ages (n=46). A representative series of different ages is shown (E-G) in comparison to the isotype control (D). A shift from a predominantly naïve (CD45RA) to memory (CD45RO) population was observed.

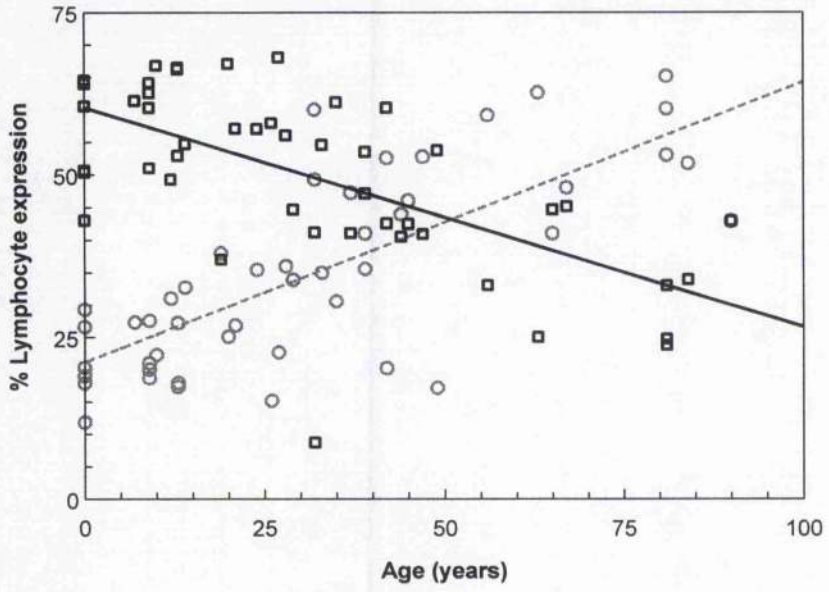


Figure 3-7 A proportional shift from naïve to memory lymphocytes occurs with age

PBL from 46 normal volunteers were studied for relative proportions of naïve (CD45RA, open squares, R^2=0.41, p<0.001) and memory (CD45RO, open circles, R^2=0.58, p<0.001) lymphocytes. A progressive loss of naïve phenotype (with a proportional gain in memory phenotype) was observed with age, as would be expected. The equations of the lines were y = -0.3373x + 60.319 (naïve) and y = 0.4274x + 21.692 (memory).

The proportional shift in naïve to memory phenotype was striking, consistent with the mutually exclusive expression of either CD45RA or CD45RO (but not both together). Taken together, these datasets (*i.e.* the rate of lymphocyte telomere loss in normal individuals with age, and the rate of conversion from a naïve to memory lymphocyte phenotype) allowed us to estimate the degree of telomere loss expected from the observed age-related conversion to a memory phenotype, and allowed comparison to the increased age-related telomere loss measured in normal lymphocytes (approximately 14bps per year, **Figure 3-5**). Such a model relies on several assumptions, as follows; that the previous flow-FISH estimate for memory *vs.* naïve T-cell telomere shortening (2.5kb)(Rufer, *et al* 1998) is a constant and is applicable to our data; and that the T-cell phenotype shift is

relevant to total lymphocyte telomere measurements (*i.e.* exclusion of B and NK cells from the immunophenotyping has little effect on the sample as a whole). Given the predominance of T-cells in the peripheral blood of normal individuals (>80%), the latter assumption would appear reasonable. With these assumptions in mind, a mathematical model of phenotype-shift (i.e. naïve to memory) related telomere loss was derived (**Equation 1).**

1. $A = \dfrac{[N_{t_0}(x+y)] + (M_{t_0}x)}{N_{t_0} + M_{t_0}}$

2. $A = \dfrac{N_{t_0}x + N_{t_0}y + M_{t_0}x}{N_{t_0} + M_{t_0}}$

3. $A = \dfrac{(N_{t_0} + M_{t_0})x + N_{t_0}y}{N_{t_0} + M_{t_0}}$

4. $A = x + \dfrac{N_{t_0}y}{N_{t_0} + M_{t_0}}$

1. $B = \dfrac{[N_{t_1}(x+y)] + (M_{t_1}x)}{N_{t_1} + M_{t_1}}$

2. $B = \dfrac{N_{t_1}x + N_{t_1}y + M_{t_1}x}{N_{t_1} + M_{t_1}}$

3. $B = \dfrac{(N_{t_1} + M_{t_1})x + N_{t_1}y}{N_{t_1} + M_{t_1}}$

4. $B = x + \dfrac{N_{t_1}y}{N_{t_1} + M_{t_1}}$

5. $\dfrac{A - B}{(t_1 - t_0)}$

6. $\dfrac{\dfrac{N_{t_0}y}{N_{t_0} + M_{t_0}} - \dfrac{N_{t_1}y}{N_{t_1} + M_{t_1}}}{(t_1 - t_0)}$

7. $\dfrac{\dfrac{60.3 \times 2.5}{60.3 + 21.7} - \dfrac{30.0 \times 2.5}{30.0 + 60.1}}{(90-0)}$

8. $\dfrac{1.8 - 0.8}{90}$

9. = 0.0111 kbps (11.1 bps) per year

Equation 1 Mathematical modelling of increased telomere loss in lymphocytes as a result of a proportional rise in memory cells

Steps 1-9 of the method used to derive the degree of shortening due to naïve-memory conversion of lymphocytes with age are shown. The mean contribution of naïve (N, the percentage naïve cells at either time point) *vs.* memory (M, the percentage memory cells at either time point) cells to telomere length at age 0 (t_0) is derived as value A, while at age 90 (t_1, the upper age from **Figure 3-7**) it is represented by B. x represents the mean lymphocyte telomere length at the respective time points, with y being the excess telomere length observed in naïve lymphocytes (in this case it is accepted as 2.5 kbps as per Rufer *et al.* (Rufer, *et al* 1998)). The value of x is cancelled out in steps 5/6, and therefore the derived value of immunophenotype-shift mediated telomere loss is independent of starting telomere length.

By these means, we calculated that the progressive shift from naïve to memory lymphocytes accounted for an additional 11.1 bps telomere sequence loss per year. This is extremely close to the measured value of 14bps per year (**Figure 3-5**), and suggests that this excess loss (as compared to granulocytes) is almost entirely due to naïve T-cell expansion and conversion to a memory phenotype (with shorter telomere sequence) rather than extra progenitor cell divisions.

3.1.6 Discussion

We successfully established flow-FISH in our laboratory, and validated it against results obtained in a partner laboratory and against published normal population values for telomere fluorescence. By incorporating an identical aliquot of control cells in every run we obtained a CV of 17% for all our experiments over a time period of 20 months, which is similar to that obtained by other groups. This degree of inter-experimental variation is almost certainly related to the multiple manual steps involved in the technique. In keeping with this was our observation that samples analysed within the same run exhibited considerably less variation in staining intensity. It has been shown recently that hybridisation does not proceed to saturation under the originally published experimental conditions (Baerlocher, *et al* 2002) and this has led Lansdorp's laboratory to further (and meticulously) refine the technique (Baerlocher, *et al* 2002) with particular attention to optimization of the denaturation conditions (15 minutes at 87^0C). This has greatly reduced the inter-experimental variation in staining intensity (Baerlocher, *personal communication*). These modifications are being incorporated into our current protocol and will improve the reproducibility and accuracy of flow-FISH in the future.

The haematopoietic stem cell (HSC) has the daunting task of generating 10^{11} – 10^{12} mature blood cells on a daily basis, and although the mechanisms that control stem-cell fate are far from clear, some intriguing observations concerning telomeres have been made. $CD34^{+}CD38^{lo}$ selected cells in culture lose approximately 40 bps per population doubling and, in keeping with a corresponding lifelong telomere loss *in-vivo*, nucleated cells from foetal liver and UCB have significantly longer telomeres than those from adult BM (Vaziri, *et al* 1994). These data are in keeping with the fact that HSC are not immortal: there is eventual loss of repopulating ability of murine bone marrow following serial transplantation (Harrison and Astle 1982). Such observations support the existence of a telomere checkpoint *in-vivo*, and are consistent with the ability of foetal liver and UCB HSC to generate significantly more progeny that their adult equivalent (Lansdorp, *et al* 1993). HSC and progenitor cell telomere loss is detectable in their peripheral blood leucocyte (PBL) progeny: Rufer *et al.*, in a study of over 500 normal individuals of all ages, detected telomere shortening in PB granulocytes and lymphocytes of 39 and 59 bp per year respectively (Rufer, *et al* 1999). A much greater increase (1000 – 3000 bp/yr) was observed in the first two years of birth. If one assumes that the number of divisions between HSC and mature progeny are identical throughout life, and that telomere loss is replication-induced and constant with each division, then PBL telomere length can be used to measure the replicative history of HSC (Verfaillie, *et al* 2002). These data would predict that the average HSC divides every 1 – 2 years (based on loss of 50 – 100 bps per division) after the age of 2 years or so, which is consistent with data derived from mathematical modelling of the mutation rate in red cells (Vickers, *et al* 2000). Our data also support this, and our mean rates of granulocyte and lymphocyte telomere loss are comparable with those of Rufer *et al*. Interestingly, recent data from Lansdorp's group suggests a further acceleration of telomere loss

after the age of 60 years or so (Verfaillie, *et al* 2002), and our data was also (albeit marginally) best described by a more complex (in this case quartic) regression line, pointing to a late acceleration of telomere loss. It should be emphasised however that our study was not designed to look for age-related shifts in telomere dynamics, and that Lansdorp's sample-set included an excess of individuals at extremes of age. The reasons for more rapid telomere shortening in the elderly are not clear but could be as a result of replicative stress within a diminishing HSC pool, or the consequences of reduced DNA repair efficiency and / or antioxidant defence mechanisms.

From these data it is apparent that telomerase is unable to prevent telomere shortening in the HSC compartment. Telomerase activity is detectable in HSC: it is expressed at low level in steady-state $CD34^+$ cells, is highest in the $CD34^+/38^+$ subpopulation (which contains proliferating progenitor cells), upregulates in response to multiple cytokine stimulation *in-vitro* within 48 hours (peaking at day 7 and declining to baseline after 3-4 weeks) and correlates with cell cycle status (Engelhardt, *et al* 1997). Despite telomerase activity, telomeres shortened by 1 – 2 kbp over the 4 week culture period (albeit at a slower rate during the period of telomerase expression, with an overall loss of 73 bps per division). Telomerase activity was low in the quiescent $CD34^+/38^-$ population, and could also be down modulated with TGF-β. Therefore, despite these cells being telomerase competent, telomere loss still occurs. Thus *in-vitro* telomerase activity *per se* is not adequate to maintain telomere length and is therefore not synonymous with telomere maintenance.

The consequences of this can be detected after BM or HSC transplantation: Notaro *et al* first demonstrated significant telomere shortening, of the order of 79-1446 bps, in the peripheral blood granulocytes of alloSCT recipients as compared

with their donors (Notaro, *et al* 1997). Assuming that telomere shortening is directly proportional to the number of cell divisions, the observed inverse correlation between the number of mononuclear cells received and the degree of telomere shortening was interpreted as reflecting the additional cell divisions required to achieve haemopoietic reconstitution. Brummendorf *et al.* have convincingly demonstrated that the initially rapid telomere loss post transplant (an average of approximately 1kb) occurs over the first year, with a more gradual loss (comparable to the normal rate) thereafter (Brummendorf, *et al* 2001a). The results of studies in children post alloSCT are similar, demonstrating telomere shortening equivalent to approximately 15 years of ageing (Wynn, *et al* 1998).

As to the potential pathological significance of such post transplant telomere shortening in disease, little is known. It must be remembered that late failure of a successful graft is extremely rare, and some individuals in these studies had no demonstrable telomere loss post transplant. Early onset of clonal haemopoietic disorders could be predicted, in an analogous fashion to that of aplastic anaemia, where telomere shortening correlated with chromosomal abnormalities and clonal disorders have been described (Ball, *et al* 1998). Further study, for example on the role of telomere shortening in dysplastic states observed post autologous SCT, is required although clearly there will be many confounding variables such as the mutagenic effects of treatment. Its significance may only eventually be realised with the *ex-vivo* manipulation of stem cells, such as that used in gene therapy or UCB transplant protocols, where a small number of stem cells are required to fully reconstitute an adult marrow. Clearly the passage of time, as well as further study, is needed.

Interestingly, in wild-type mice serially transplanted with transgenic hTERT-expressing BM or HSC (c-Kit^{hi}, Sca-1^{hi}, Thy1.1^{lo}, Lin^{neg}) no telomere shortening

was observed, even after 4 serial transplants (Allsopp, *et al* 2003b). This was in comparison to the wild-type transplanted animals, where they lost approximately 40% of the serially transplanted donor telomeric DNA. However, it was not possible to transplant beyond a fourth attempt; therefore telomere maintenance did not extend replicative capacity. The authors suggested that this may reflect the influence of telomere independent senescence mechanisms, due to repeated HSC handling and cell-stress, akin to the telomere independent 'culture-shock' observed *in-vitro*. This has major potential implications for tissue engineering and *ex-vivo* cell expansion *etc*. Simply over expressing hTERT may not permit unlimited cell expansion, and it is likely that meticulous attention to cell culture conditions etc will be required to avoid inducing cell senescence via other routes.

In addition to HSC determined telomere loss, mature lymphocytes must undergo massive clonal expansion in response to antigenic stimulation, and consequently memory $CD4^+$ and $CD8^+$ cells have significantly shorter telomere sequences than naive (Rufer, *et al* 1998), (Weng, *et al* 1995),. These additional replications have been assumed to explain the more rapid telomere loss in lymphocytes, in parallel with a shift from naive to memory cells with age. Our mathematical model of immunophenotype-related telomere shortening supports this notion, and the calculated increased loss (11.1bps per year) was remarkably close to the measured increase over and above that of granulocytes (14 bps per year). Again this underscores the principle that telomere loss remains significant, even in telomerase competent cells. The potential sequelae of lymphocyte telomere loss, primarily immunosenescence, have been discussed extensively in the literature and are beyond the scope of this section (Globerson and Effros 2000). However such a mechanism could explain the inefficiency of immune surveillance post BM- or HSC-transplant (as suggested by the progressive increase in risk of solid

tumours) as well as loss of immune function in the elderly. Again these *in-vivo* effects may be exaggerated by future *ex-vivo* manipulation of such cells.

In summary, telomere length measurement can offer unique insights into cell turnover and ageing, and flow-FISH is particularly applicable to the study of haematological cell types. We therefore studied, in detail, the telomere dynamics of the archetypal HSC malignancy CML.

4. Results 2

4.1 Telomere dynamics at diagnosis and during progression of chronic myeloid leukaemia

4.1.1 Introduction

CML offers an interesting and valuable model in which to study telomere dynamics during tumour progression, as the degree of telomere shortening is likely to reflect Ph^+ HSC turnover. Although there is evidence of lymphohaemopoietic progenitor involvement, circulating T lymphocytes are not typically involved in the malignant clone (Brummendorf, *et al* 2000, Haferlach, *et al* 1997, Jonas, *et al* 1992).

Several reports have documented increased PBL telomere shortening in CML (**Table 4-1**), and have attributed varying degrees of prognostic significance to the degree of telomere loss observed. In 1997 Iwama *et al.* reported that increased telomere loss in CP CML (as compared to normal controls) predicted for increased likelihood of BP within the study time period and also that those patients with telomeres within the normal range had a higher incidence of cytogenetic responses to interferon-alpha (IFN) (Iwama, *et al* 1998). Boultwood *et al.* demonstrated that increased age-adjusted telomere loss was predictive for time to AP (but not BP) (Boultwood, *et al* 2000) and more recently Brűmmendorf has shown that CP patients developing BP within 2 years of sampling had significantly shorter PBL telomeres than those who progressed after 2 years (Brummendorf, *et al* 2000). The degree of telomere shortening at diagnosis therefore warrants further investigation as a prognostic indicator. What was consistently observed within these studies was that mean telomere length in CP CML was shorter than in

normal age-matched individuals, and that further telomere shortening was observed with disease progression.

Study	Technique Used	Normal Controls	CP CML	AP or BP CML	Reference
1	Southern	8.7kb, n=34	6.4kb, n=34	4.1kb*, n=12, BP	a
2	Southern	9.7kb, n=75	6.0kb, n=59	ND	b
3	Southern	10.0kb, n=33	6.1kb, n=33	4.5kb*, n=21, BP	c
4	Southern	Not given	19 pts in normal range (mean 7.8kb), 25 patients below (mean 5.2kb)	ND	d
5	Flow-FISH	13.2kMESF, n=147	11.0kMESF, n=96	9.5kMESF*, n=14, AP&BP	e
6	Southern	7.8kb, n=108	5.6kb, n=7	4.7kb, n=12, BP	f

Table 4-1 Telomere length in CML.

This table summarises the data presented in 6 studies of telomere length published to date. All demonstrated significantly shorter telomeres in CP CML as compared to the normal control cohort. Additionally, 3 out of 4 (studies 1, 3 & 5) showed further (and significant) shortening with disease progression. The prognostic relevance of these data within their respective study is described in the text. *, significantly shorter than CP; a, (Boultwood, *et al* 1999); b, (Boultwood, *et al* 2000); c, (Ohyashiki, *et al* 1997b); d, (Iwama, *et al* 1998); e, (Brummendorf, *et al* 2000); f, (Engelhardt, *et al* 2000).

These observations have led Lansdorp's group to suggest that telomere attrition, eventually leading to genetic instability and clonal evolution, could play a role in progression of CML (**Figure 4-1**).

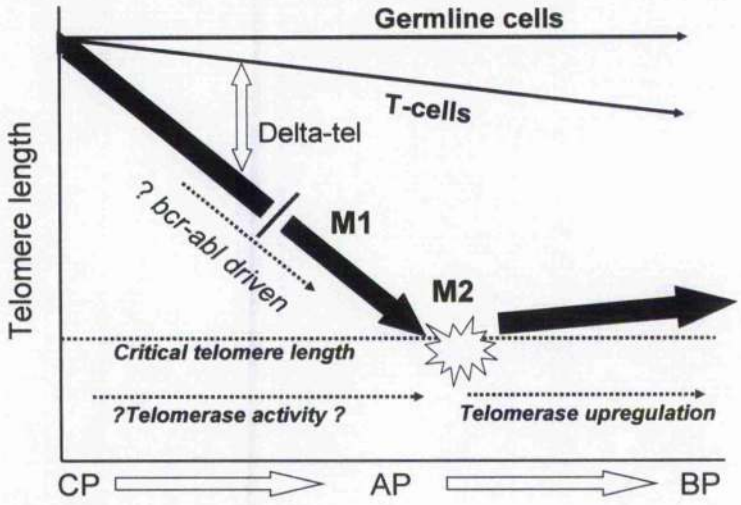


Figure 4-1 A model of telomere and telomerase dynamics during progression of CML

Clonal expansion subsequent to acquisition of *BCR-ABL* in the HSC compartment results in progressive telomere loss, detectable in myeloid progeny in the peripheral blood. The malignant population is driven through the 'Hayflick Limit' (M1) and proceeds to early cell crisis (M2) and clonal evolution. Telomerase cannot prevent telomere loss during CP, and its regulation during this period remains poorly characterised. In contrast, BP exhibits upregulated telomerase and short (but stable) telomere length. Using Ph^- T cells as an internal control for somatic cell telomere length, the degree of telomere loss can be measured on an individual basis ('Delta-Tel') thereby correcting for genetic and age related variation in telomere sequence length.

In this chapter we aimed to further define the prognostic significance of telomere shortening at diagnosis, measure rates of telomere attrition and to determine the dynamics of telomere length changes over time in responding and non-responding imatinib mesylate treated groups.

4.1.2 Telomere length in CML PBL is representative of the BCR-ABL$^+$ CD34$^+$ compartment

In the studies described in **Table 4-1** it was assumed that telomere length in CML PBL was representative of the Ph^+ HSC compartment. To establish that this was a valid assumption we performed flow-FISH upon freshly selected cell populations (**Figure 4-2**) and unfractionated PBL from 2 patients at time of diagnosis of CML

(**Figure 4-3**). To confirm that the $CD34^+$ cells were part of the CML clone, FISH was performed for *BCR-ABL* (98% and 95.6% *BCR-ABL* positive for patients A and B respectively).

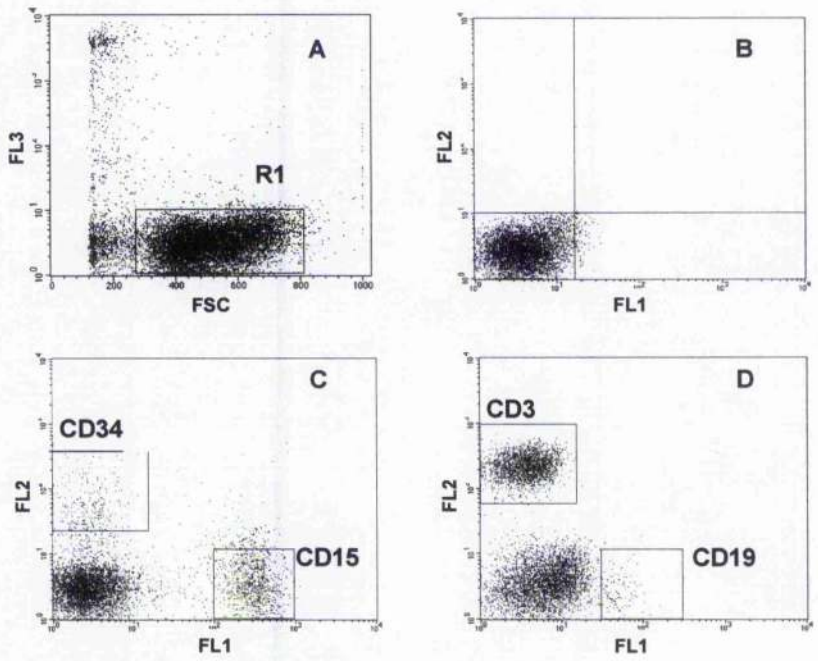


Figure 4-2 FACS sorting of selected populations of cells from CML PBL

$CD34^+$ (PE), $CD15^+$ (FITC), $CD3^+$ (PE) and $CD19^+$ (FITC) labelled cells were selected at high purity (>95%) from leucapheresis material. PI staining with analysis in FL3 was used to gate on live cells (**A**, R1), and gates were then set on the populations of interest (**C** and **D**) with reference to the isotype control (**B**). Flow-FISH was performed immediately on the freshly selected cells.

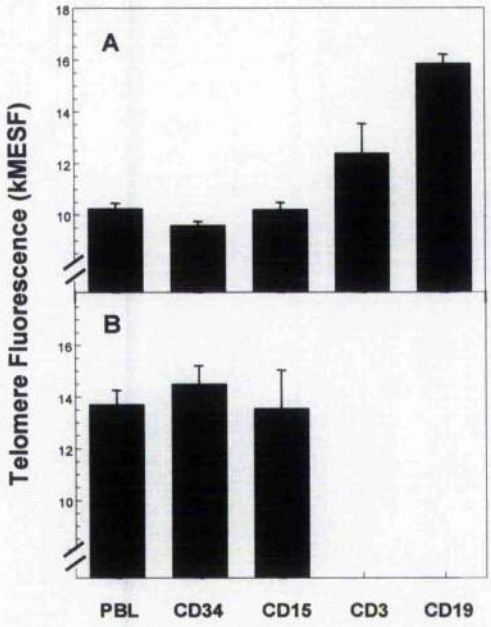


Figure 4-3 Flow-FISH measurement of selected subpopulations from CML patients at diagnosis

The mature B cell ($CD19^+$) population displayed by far the longest telomeres (approximately 2.0 kb longer than circulating T cells), consistent with a recent observation (Martens, *et al* 2002). Samples were analysed in duplicate and mean values are displayed with SD bars. In patient B insufficient cells were obtained from the CD3 / CD19 sort for analysis. Note the broken y-axes.

Telomere fluorescence of $CD34^+$ cells was directly comparable to that of whole PBL or the $CD15^+$ mature myeloid population in both patients A and B, in keeping with a previous study which also reported no significant difference between the populations (4.8kb and 4.4kb for BM $CD34^+$ cells and PB MNCs respectively in CML) (Engelhardt, *et al* 2000).

4.1.3 Telomere shortening occurs during progression of CML

PBL telomeres at all stages of CML were significantly shorter than that of a cohort of normal individuals approximately matched for age (**Table 4-2** and **Figure 4-4**). Additionally, BP telomeres were significantly shorter than those of early CP. The latter observation was confirmed after correction for age related loss: Age adjusted differences in telomere length (CML PBL, blast or granulocyte$_{tel}$ – calculated

normal granulocyte$_{tel}$) were calculated for CML patients at different stages of disease using the expected normal granulocyte values as a reference (from the equation of the quartic regression line, **Figure 3-5B**). To establish whether any residual age-related influence on telomere length remained in CML, telomere fluorescence was plotted against age for each of the CML cohorts (**Figure 4-5**). Interestingly, preservation of age related decline was observed for all stages (although this did not reach statistical significance for early CP or AP), which was most marked with myeloid BP. The relatively limited age-range of the patients (as compared to the normal cohort) and the fact that this corresponded to the 'flat' part of the quartic regression line for the normal population inevitably hampered the ability to detect statistically significant correlations for these groups. The strong correlation for the myeloid BP samples was unexpected, and may point to inefficient telomere maintenance mechanisms in advanced age when faced with significant replicative stress.

Cohort	n	Median age in years (range)	Median duration of CML in months (range)	PBL Telomere length kMESF (±SD)	Age-related degree of telomere shortening kMESF (±SD)	P values vs N adults / vs CP CML < 2yrs
UCB	12	0	-	16.4 (±2.3)	-	-
N adults*	35	57 (24 -81)	-	12.5 (±2.3)	-	-
CP < 2 years	54	54 (24 – 77)	0 (0 – 22)	10.5 (±3.1)	-2.2 (±3.0)	<0.01 / -
CP > 2 years	12	55.5 (45 – 70)	50 (25 – 84)	10.2 (±2.3)	-2.3 (±2.6)	< 0.01 / NS
AP	15	63 (41 – 75)	63 (41 – 75)	9.3 (±2.5)	-2.8 (±2.4)	<0.001 / NS
BP	17	61 (37 – 75)	61 (37 – 75)	6.1 (±2.9)	-6.2 (±2.9)	<0.0001 / <0.0001

Table 4-2 Flow-FISH results from cohorts of normal individuals and CML patients

*35 patients from the total cohort of 55 normal individuals were selected to mirror the age range and distribution of the CML cohorts. N adults, normal adults.

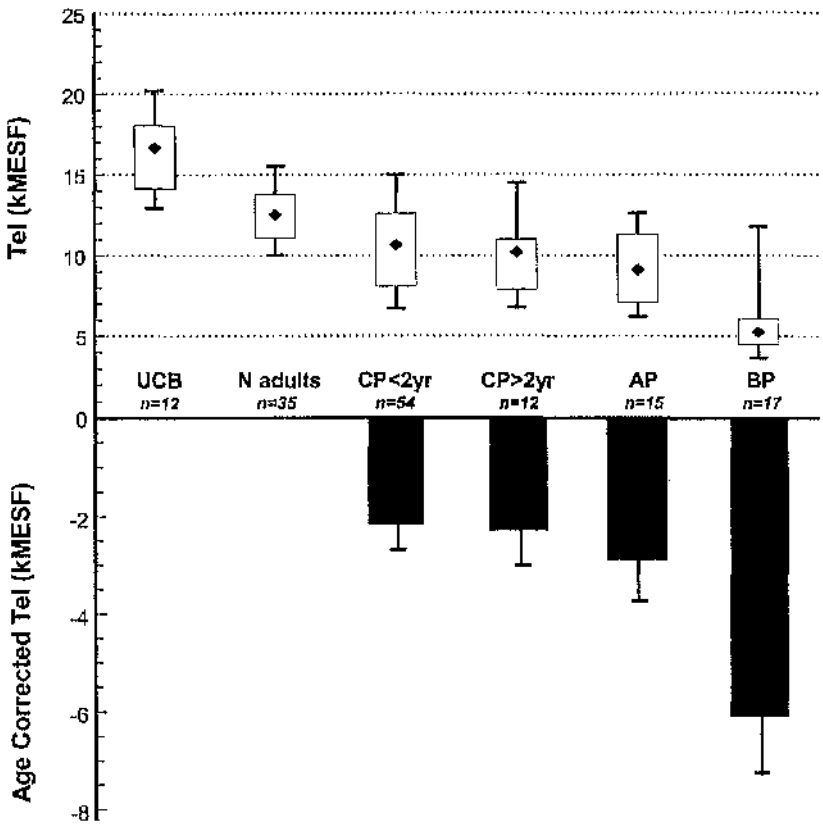


Figure 4-4 Telomere length and age-related degree of telomere shortening in normal volunteers and at different stages of CML

The top section of the chart shows PBL telomere length with median and percentile ranges shown (black bars 10 – 90%, open box 25 – 75%). The lower section illustrates mean age corrected differences in telomere length from that of granulocytes in the normal population (bars represent standard error). 35 patients from the total cohort of 55 normal individuals were selected to mirror the age range and distribution of the CML cohorts. All stages of CML had significantly shorter telomeres than the normal adult group (see text and **Table 4-2**). In addition BP demonstrated further shortening in comparison to CP of duration less than 2 years ($p < 0.0001$). As in previous studies a wide range of telomere lengths were noted, limiting the ability to define small but significant differences between groups. N adults, normal adults (age matched); UCB, umbilical cord blood; Tel, telomere length.

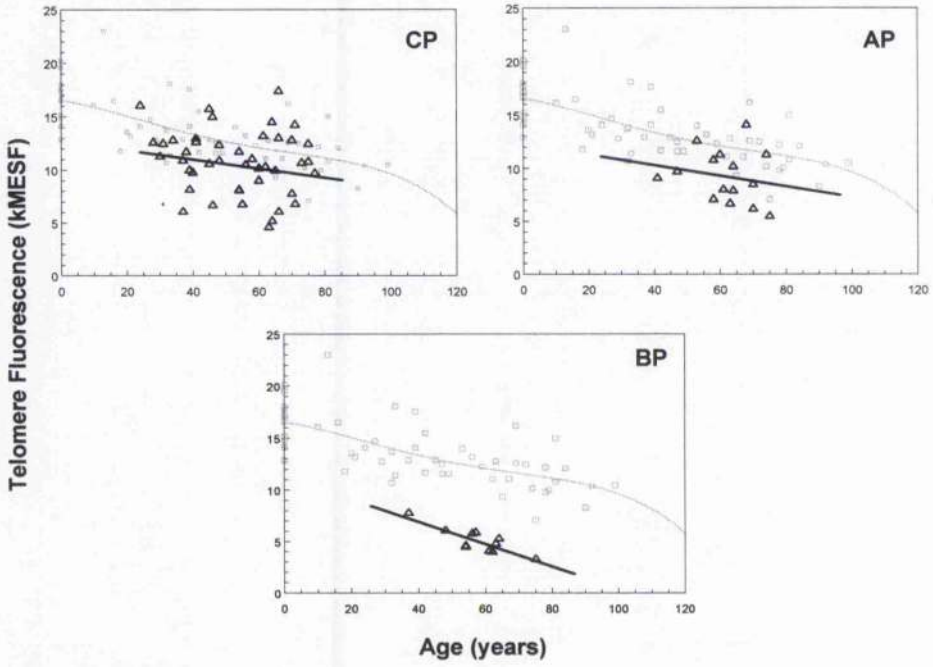


Figure 4-5 Age-related telomere fluorescence from different stages of CML

Telomere fluorescence from early CP, AP and myeloid BP (only 4 lymphoid BP samples were studied and are therefore not shown) from the cohorts described in **Table 4-2** were plotted against age (open triangles, CML samples; open squares, normal granulocytes). A trend towards an age related decline in telomere length was observed with early CP and AP (R^2=0.05, p=0.1 and R^2=0.04, p=0.5 respectively), but was highly significant and most marked in myeloid BP (R^2=0.68, p<0.01).

As in previous studies, there was a large variation in telomere length between patients, particularly in CP. This is almost certainly attributable to a combination of genetic factors, disease heterogeneity and technical variation, indicating that large numbers of patients would be required to demonstrate small differences between groups (*i.e.* different disease stages and prognostic sub-groups at diagnosis). To allow us to interpret such inter-individual differences we used expanded T-cells as an internal control for normal (*i.e.* Ph^-) telomere length (**Figure 4-6**). Delta-tel was calculated from (T $Cell_{tel}$ – PBL or $granulocyte_{tel}$) in kMESF, and provided a measure of the CML-related degree of telomere shortening within an individual patient. Although lymphocytes and granulocytes shorten at different rates (a potential confounding factor for interpretation of delta-tel values), the relationship

between the two populations is relatively constant over the age range of our CML cohort (**Figure 3-5 B**). A trend towards shorter telomeres in AP patients compared to CP in the cohort group was confirmed by measurement of delta-tel in 9 CP and 7 AP patients (0.8 ± 1.8 and 3.4 ± 1.9 kMESF respectively, mean ± SD, $p<0.05$, **Figure 4-7**), which demonstrated significantly greater shortening in the AP group. T cell expansion in adequate numbers from representative BP samples was only successful in one case (shown separately in **Figure 4-8**), presumably because of the advanced nature of the leukaemic process with either established or incipient bone marrow failure.

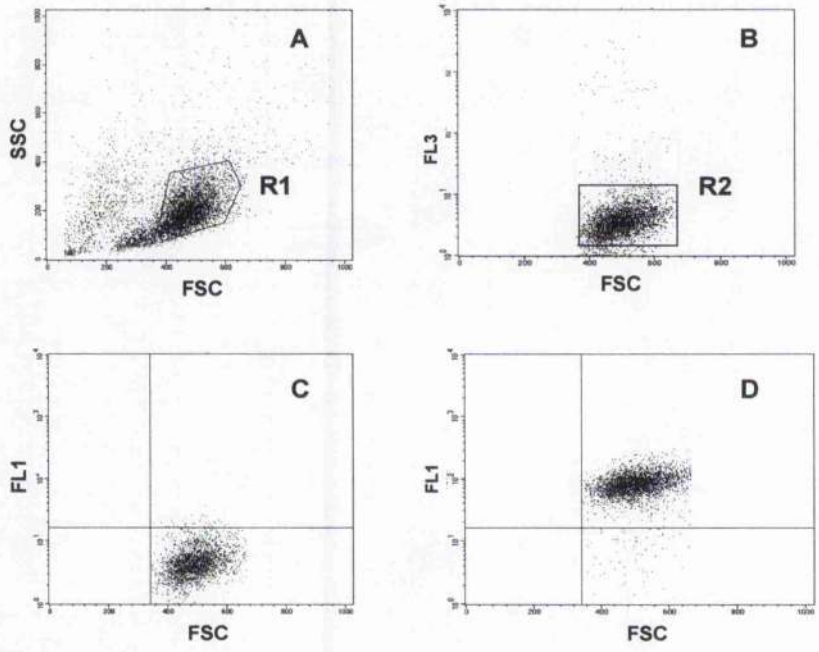


Figure 4-6 *Ex-vivo* expansion of T-cells.

Whole PBL from CML patients were cultured for 7-10 days in the presence of IL-2 and PHA. When adequate numbers of T-cell clones were visible, the culture was harvested and analysed by FACS for T-cell purity. Viable cells were identified by PI staining (R2, **B**), and CD3-FITC negative (**C,** isotype control) and positive (**D**) stained populations used to determine T-cell purity. In this representative example 93% of the viable cells were T-cells.

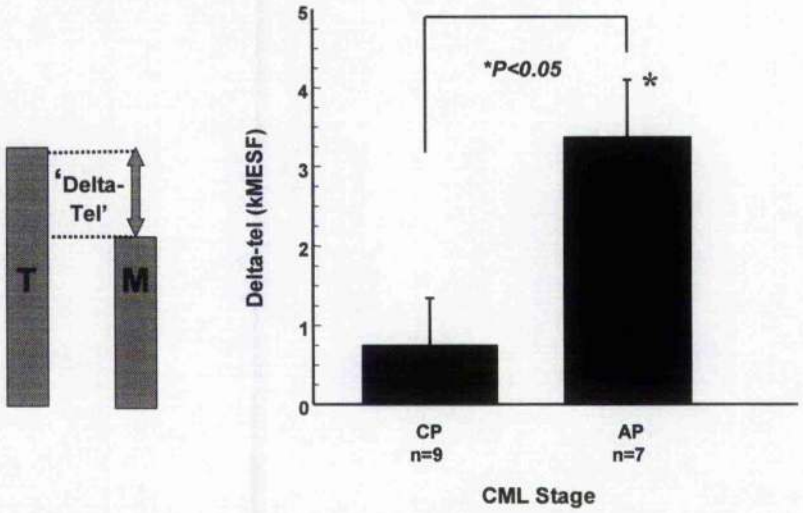


Figure 4-7 Degree of telomere shortening in CP and AP CML using patient T cells as an internal control of Ph⁻ telomere length

To correct for age related and genetic variation in telomere length, delta-tel was calculated (see text). Significantly shorter telomeres were detected in the myeloid compartment of AP patients (mean values plus SE bars) as compared to early CP ($p<0.05$, see text). T, T cell compartment telomere length; M, myeloid compartment telomere length.

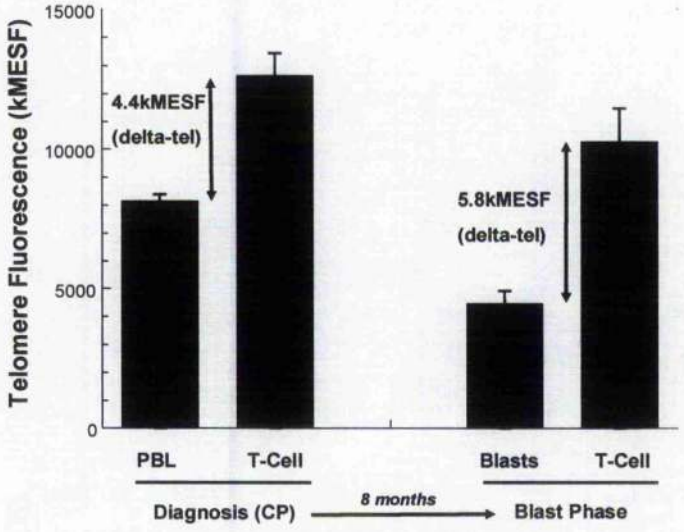


Figure 4-8 Serial measurement of delta-tel in patient progressing from CP to BP

This patient demonstrated an additional 1.4kMESF telomere loss on progression to BP: in comparison to the normal rate of granulocyte telomere loss this represents an approximately 20-fold increase in rate.

To determine if individual patients undergo telomere shortening during progression of their disease from CP to BP we analysed paired samples from 7 individuals. All patients had the CP sample taken at diagnosis (apart from 1 who was sampled after 22 months of CP), with a median time to repeat sampling at onset of BP of 49 months (range 6 – 75). Where possible (in the case of frozen paired samples, n=5) these samples were analysed within the same staining run, otherwise a correction factor was applied (n=2) derived from the control cell fluorescence (**Equation 2**).

Corrected sample$_{tel}$= [Mean control T-ALL cell$_{tel}$/measured control cell$_{tel}$] x sample$_{tel}$

Where tel is telomere fluorescence (kMESF)

Equation 2 Correction for variation in fluorescence intensity between experiments

Mean telomere length of CP and BP samples was 9.1 ± 1.9 and 6.0 ± 2.0 kMESF respectively, and paired analysis of samples demonstrated significantly shorter telomere sequence in BP samples ($p<0.05$). The mean degree of shortening was therefore calculated as 3.1 ± 2.8 kMESF, or 0.75 kMESF per year, approximately 10 x the rate of normal.

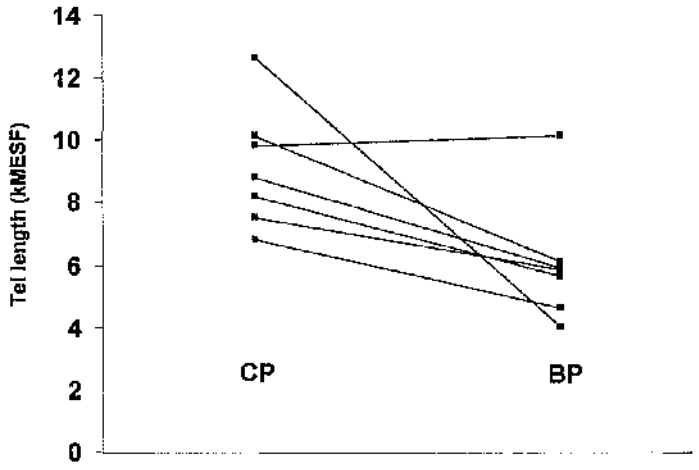


Figure 4-9 Telomere shortening in paired CP and BP samples from individual patients

Telomere shortening was observed in BP as compared to their respective CP sample in 6 out of 7 samples analysed. A mean decrease of 3.1±2.8 kMESF was observed with disease progression ($p<0.05$).

4.1.4 The degree of telomere shortening at diagnosis correlates with prognostic score

Brümmendorf *et al.* demonstrated that short telomeres in CP predicted for time to disease progression. To investigate this relationship further we prospectively measured telomere length in 32 newly diagnosed patients and correlated PBL telomere length with their Hasford prognostic score. There was no significant difference in PBL telomere length between the high-risk (HR), intermediate-risk (IR) and low-risk (LR) prognostic groupings (Kruskall-Wallace test, $p=0.38$), although there was a trend to shorter telomeres with increased risk score (LR 10.1±2.5, IR 9.2±3.8, HR 8.4±2.5 kMESF, mean±SD, **Figure 4-10**). Delta-tel was calculated for these patients using *ex-vivo* expanded T-cells from 32 consecutive newly diagnosed patients. The mean delta-tel for all patients was 2.1 ± 2.8 kMESF. High-risk Hasford score (n=12) patients had significantly greater mean delta-tel values (3.4 ± 2.8 kMESF, mean ± SD) than low-risk score (n=8) patients

(0.6 ± 2.2 kMESF, $p<0.05$), with intermediate risk (n=12) patients having values falling between these extremes (1.3 ± 1.4 kMESF, **Figure 4-11**). There was no significant difference in T-cell telomere length between the prognostic groups after a correction for age-related shortening was made (p=0.5, Kruskall-Wallace test).

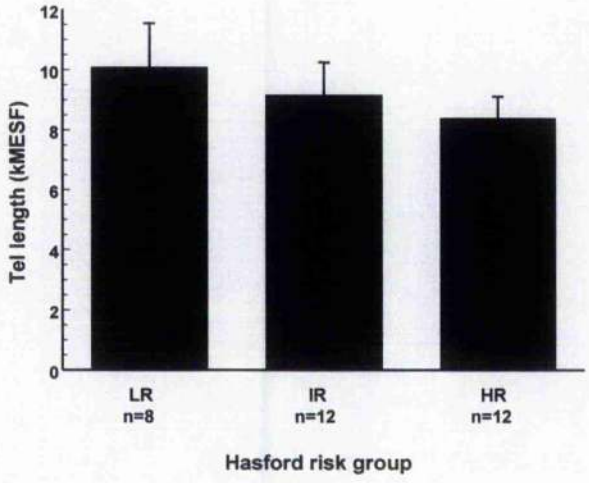


Figure 4-10 Mean PBL telomere fluorescence for patients at diagnosis

Telomere length was measured on whole PBL from 32 CML patients at diagnosis. Although there was a trend to shorter telomere for the high-risk (HR) group, this was not statistically significant (Kruskall-Wallace test p=0.38). IR, intermediate-risk; LR, low-risk. Histogram bars represent the mean for each group, with standard error bars.

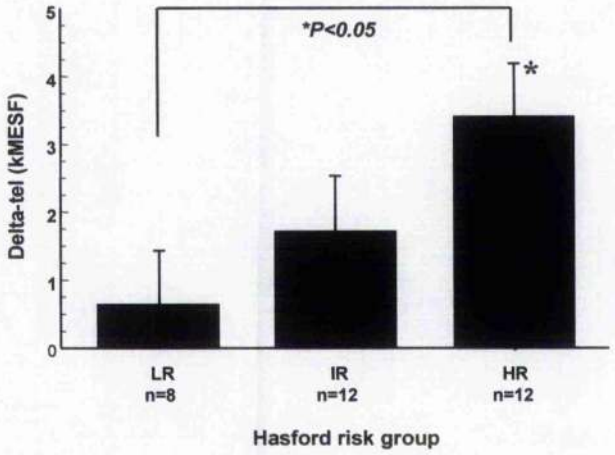


Figure 4-11 Delta – tel values at diagnosis correlate with prognostic score in CML

32 consecutively diagnosed CML patients had delta-tel measured and correlated against conventional Hasford prognostic risk groups (mean values plus SE bars). High-risk patients had significantly greater telomere shortening at diagnosis than low-risk ($p<0.05$). LR, low-risk; IR, intermediate- risk; HR, high-risk.

4.1.5 Telomere dynamics in CCR vs non-responders to imatinib therapy

To determine the rate of telomere shortening in individual patients, it was necessary to select those who, despite treatment, remained 100% Ph^+ as determined by BM cytogenetics. This then allowed for the valid assumption that all myeloid PBL were clonal in origin. The spectacular success of imatinib therapy in inducing complete cytogenetic responses (CCR, *i.e.* 100%Ph^-) allowed us to directly compare the telomere dynamics between persistently 100% Ph^+ patients and those attaining 100% Ph^- status over time. All patients who obtained a CCR

were in CP (n=10, median duration of disease 11.5 months, range 0 – 54) and of those who remained 100% Ph^+ (n=11, median duration of disease 40 months, range 0 – 84) 8 were in CP and 3 in AP. CP and AP patients were treated with 400mg and 600mg / day of imatinib mesylate respectively, as part of the Novartis UK Expanded Access Programme, at our institution. Follow-up sampling on CCR and non-responders was performed after a median period of 6 months (range 4 – 9) and 8 months (range 2 - 14) respectively. To correct for variation in staining between experiments separated by time in the same patients, a correction factor was applied based on the control cell kMESF values (**Equation 2**). Those achieving a CCR demonstrated a mean increase in telomere signal of 2.4 ± 2.0 kMESF, consistent with the re-establishment of primarily Ph^- haemopoiesis (**Figure 4-12**). After correction for age, patients went from 2.3 ± 1.6 kMESF below to 0.6 ± 1.2 kMESF above that expected for normal granulocytes. In contrast, those patients who remained 100% Ph^+ underwent a mean telomere loss of 0.9 ± 2.3 kMESF, a rate of loss that is approximately 20 times that of the granulocyte compartment in cross – sectional data from normal individuals.

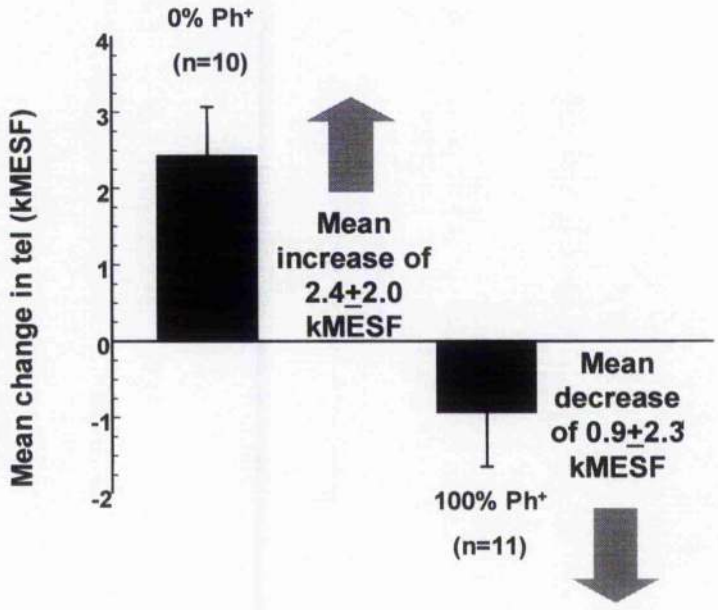


Figure 4-12 Telomere dynamics over time in cytogenetic responders and non-responders to imatinib therapy

To calculate relative rates of telomere loss, 11 patients who remained 100% Ph+ despite imatinib therapy had follow -up telomere measurements performed (see text). These patients (100% Ph^+) showed a mean rate of telomere shortening of approximately 20 x the rate expected from cross-sectional data from normal volunteers. Conversely, and as expected, the restoration of polyclonal haemopoiesis (0% Ph^+) resulted in an increase in mean telomere length. Histogram bars represent the mean change, with standard error bars shown. Tel, telomere length.

4.1.6 Discussion

In this chapter we have determined the degree, rate and potential prognostic significance of telomere shortening in CML. Using several methods (comparison of cohorts at different disease stages, age-corrected comparisons, measurement of delta-tel incorporating a benign control population of presumably normal telomere length, and repeat sampling of responding, non-responding and progressing patients) we have established that the degree of telomere shortening has

prognostic significance at diagnosis, and proceeds rapidly with progression of disease. Models of telomere dynamics in CML suggest that significant telomere loss at diagnosis could lead to early progression of disease, as a result of telomere dysfunction and genetic instability (cell crisis, **Figure 4-1)**. Consistent with this, our data have shown that delta-tel correlates to a significant degree with the Hasford risk score. It should be emphasised that risk definition is not simply a reflection of an early or late diagnosis: in an important study involving 83 patients with pre-diagnostic blood counts (up to 36 months before CML was diagnosed), the length of the diagnostic latency was independent of the calculated prognostic score (ICSG CML 1987). Such scores would seem therefore to be true indicators of disease 'aggressiveness', rather than simply reflecting the time taken to diagnose the illness, and consequently the remaining survival.

The lack of any age-corrected difference in expanded T-cell telomere length between the prognostic groups argues against any predisposing influence of normal telomere length in disease evolution. Features of high-risk disease in the Hasford scoring system (*i.e.* large spleen size, high blast, basophil, eosinophil and platelet count, and advanced age(Hasford, *et al* 1998)) may reflect the size and/or rate of growth of the Ph^+ clone with greater degrees of telomere loss arising as a consequence. One drawback of this score is that it was based on cohorts of patients treated with IFN, and treatment options for CML have now changed. The introduction of the selective BCR-ABL tyrosine-kinase inhibitor imatinib mesylate (IM) has resulted in high cytogenetic response rates in CP disease (Druker, *et al* 2001b), but much less so in AP and BP (Druker, *et al* 2001a) where additional mutations co-exist with *BCR-ABL*. Delta-tel could be of value as a surrogate marker for advanced disease and could be predictive of response to IM. Unfortunately the variety of treatments administered to our cohort of newly

diagnosed patients did not permit this analysis. As recently shown, cytogenetic and molecular responses achieved with IM therapy are associated with a normalization of previously shortened telomere length, arguing against a pre-existing telomere length deficit in normal hemopoietic cells from patients with CML at the time of malignant transformation (Brummendorf, *et al* 2003). Our current data support this view, with a return to 0.6 kMESF above baseline for age in patients achieving CCR. Sequential measurements to establish the rate of telomeric attrition in individual patients may further increase the value of this assay, as rates of shortening appear to vary widely between patients, and allow estimation of time to crisis and disease progression. With recent improvements to the technique by Lansdorp's group increasing reproducibility (Baerlocher, *et al* 2002) we would advocate validation of delta-tel measurements as a prognostic indicator within a large prospective clinical trial.

Telomeric attrition in PBL is generally assumed to result almost entirely from replicative loss, and as such may be used as a marker for stem cell turnover. That the Ph^+ HSC compartment is cycling more rapidly than their Ph^- counterparts, thereby explaining the greater telomere loss, is suggested by two main observations. Firstly, ^{3}H-thymidine suicide assays (specifically toxic for S-phase cells) demonstrate normal PB and BM colony forming cells (CFCs) to be largely quiescent, whereas those from CML are predominantly in cycle (Eaves and Eaves 1987). Secondly, cell cycle analysis of $CD34^+$ cells from normal donors and CML patients has shown a greater percentage actively cycling in the latter group (Holyoake, *et al* 1999a). Our data from normal volunteers are in keeping with normal HSC dividing every 1-2 years (assuming a loss of 50 – 100bps per division) (Rufer, *et al* 1999). We have also confirmed previous estimates of a mean 1kb loss at time of presentation of CML (Brummendorf, *et al* 2000),

suggestive of an extra 10-20 HSC divisions at the time of presentation. With our additional data, a more detailed model of telomere dynamics at diagnosis and during progression of CML is proposed (**Figure 4-13**).

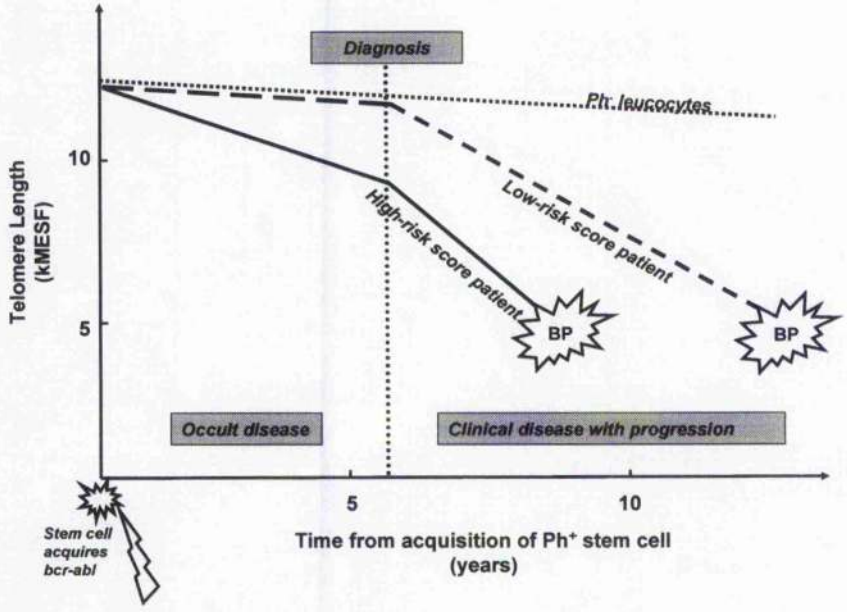


Figure 4-13 Updated model of telomere dynamics over time in CML

Our data allow the proposal of a more detailed model of telomere shortening, allowing for certain assumptions (see text). Based on data from the atomic bomb survivors in Hiroshima, there is a mean latent period of 6-7 years from (presumed) acquisition of *BCR-ABL* before clinical presentation (Ichimaru, *et al* 1981). Although low-risk patients display a rate of telomere loss during this period only slightly greater than that of normal (approximately 300bps), high-risk patients have lost a mean of 3.4 kMESF (approximately 1.7kb) at the time of diagnosis (giving a rate of loss some 14-fold greater than normal). Our data also indicate telomeric shortening after diagnosis at a rate of 10 - 20 times that of normal PBL, pointing to a biphasic or accelerating rate of loss before onset of BP. The latter is characterised by loss of some 40% of telomere sequence compared with that at diagnosis.

BP patients exhibit an age-corrected mean telomere loss of 6.2kMESF (3.1kb), indicating at least 30 – 60 additional CML HSC doublings. However, all such models require a number of assumptions, as follows: the only mechanism of telomere loss is replicative; the amount lost remains constant with each cell division and is similar in CML and normal individuals; the number of divisions

required to produce the progeny (in which telomere length is measured) remains constant; and finally, and possibly most importantly, HSC and progenitor telomerase activity remains unchanged throughout the course of the disease and is similar in CML patients and in normal individuals. A paucity of data on mechanisms of telomere loss in CML and telomerase expression in the HSC compartment currently allows such assumptions to be made. Alternative mechanisms of telomere loss require to be considered however. It is known that telomere repeat sequences are highly susceptible to damage from reactive oxygen species (ROS) (Oikawa and Kawanishi 1999), which can cause telomere shortening in human diploid fibroblasts *in vitro* (von Zglinicki, *et al* 2000). Sattler et al have recently demonstrated a marked increase in ROS activity in a tetracycline dependent *BCR-ABL* expressing cell line (Sattler, *et al* 2000). These data, together with the recently described *BCR-ABL* mediated down-regulation of the DNA repair complex DNA-PKcs (Deutsch, *et al* 2001), may predict a mechanism of telomeric DNA damage by ROS, ineffective repair and potential sequence loss. Thus telomeric sequence loss in CML could be a surrogate indicator of DNA damage and repair inefficiency. It is also possible that telomere attrition could be facilitated by telomerase dysfunction. Such a mechanism is most likely responsible for the bone marrow failure and increased incidence of cancer observed in the human disorder DKC (**1.6.6**), where acquisition of oncogenic mutations are thought to be secondary to critical telomere loss. It is therefore of importance to characterise the expression of the major telomere components in CML, given the rapid rate of telomere attrition.

5. Results 3

5.1 Altered hTERT expression during the progression of CML

5.1.1 Introduction

If one is to accept the hypothesis that telomere shortening has evolved primarily as a tumour suppressor mechanism, logic dictates that tumours must circumvent this obstacle to achieve immortality. The majority (>90%) do so by expressing telomerase at levels well above those of normal somatic tissue (Kim, *et al* 1994). In addition, one may also expect telomerase to be upregulated relatively late in tumour development, when selection pressure for telomerase expressing cells (*i.e.* critically short telomeres) is exerted. Whether this involves a selective outgrowth of high-telomerase activity cells (such as a malignant stem-cell compartment) that has been present from early tumourigenesis, or involves telomerase upregulation (via additional mutations) in a telomerase competent population, is unknown. CML is relatively easy to study in this regard; early and late serial samples from evolving disease are easily obtainable, and clinical and pathological staging indices are well established.

Broccoli *et al.*, in the first study to use the TRAP assay to study CML, failed to demonstrate any increase in telomerase activity in samples from CP or early AP CML when compared to that of normal PBL (Broccoli, *et al* 1995). Many of the CML samples (BM MNCs) actually had lower activity than the respective purified normal cell sub-populations (granulocytes, lymphocytes and monocytes). Indeed prior to these data, normal PBL were considered to be telomerase-negative, and it was only following improvements in TRAP sensitivity that such activity could be

demonstrated. Interestingly, the telomerase activity levels from *de-novo* acute leukaemias were significantly higher than from CP CML; however numbers in this early study were small, and conclusions were therefore limited. Progression from CP to BP disease was associated with a statistically significant (5-6 fold) increase in telomerase activity in a small study which also demonstrated a huge variation in BP telomerase activity in BM MNCs (Ohyashiki, *et al* 1997c). Highly variable telomerase activity in BP has also been confirmed recently, although only a small non-significant increase in BP BM (as compared to CP) was demonstrated (Verstovsek, *et al* 2003a). The authors did however suggest that relatively 'high' levels in CP were associated with shorter survival, although no comparative data from normal BM were presented. A 2-fold increase in telomerase activity was observed by Engelhardt *et al.* on progression from CP CML to AP or BP, in $CD34^+$ selected populations of cells (Engelhardt, *et al* 2000). This study was flawed however, in not confirming that the CP $CD34^+$ cells were *BCR-ABL* positive (Ph^- $CD34^+$ cells are commonly found in diagnostic, particularly BM, specimens). From these studies it is apparent that there is considerable heterogeneity in telomerase activity in CML (particularly in BP disease), with a possible trend to increased levels with disease progression. As the major determinant of telomerase is thought to be hTERT, it is therefore of considerable interest to quantitate the expression profile of this gene in CML, including its alternatively spliced isoforms. Additionally, the dramatic rates of telomere shortening described in the previous chapter raise several questions about the progression of CML;

1. As hTERT expression is a major determinant for telomerase activity, is it upregulated during the progression of CML?

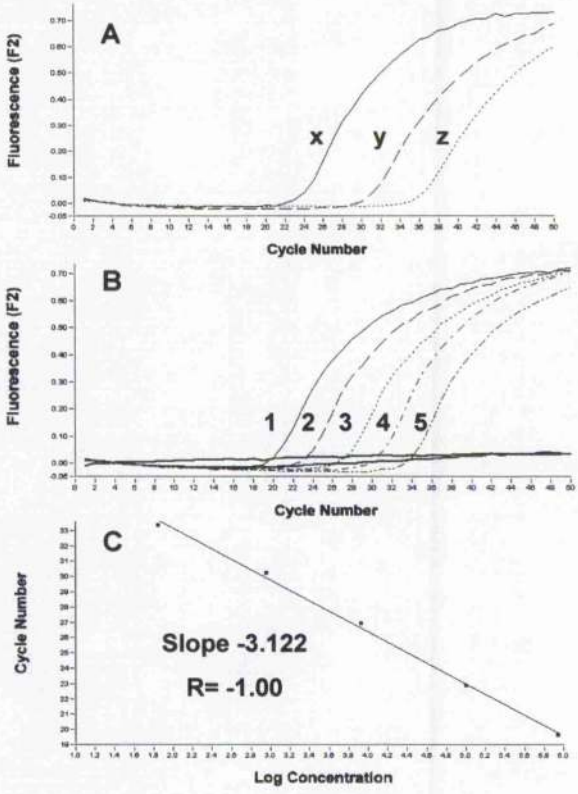
2. Do splicing patterns of hTERT mRNA alter with disease progression?

3. Can quantitation of hTERT expression in CML PBL be used as a marker to define disease progression?

These questions will be addressed in this chapter.

5.1.2 Quantitation of hTERT mRNA using the LightCycler™ instrument

A commercially available kit was used to quantitate hTERT from total RNA, which utilised PBGD as a housekeeping gene to correct for RNA loading (**Figure 5-1**). It should be emphasised that the probes and primers in the LightCycler™ hTERT kit are designed so as not to detect the β-deleted altered splice variant (Manufacturer's Data Sheet, Roche Diagnostics). Although the α-deleted form may still be detected, this forms only a very small part of the overall hTERT mRNA splice-pattern (typically less than 1-2% and often zero, see later), and the assay may be considered as detecting full-length transcripts (*i.e.* +α+β) only.



Sample	hTERT transcripts (crossing point)	PBGD transcripts (crossing point)	hTERT/PBGD (%)
x KY01cells	7.03×10^4 (23.51)	3.46×10^5 (21.16)	**20.3%**
y CML CD34 cells	6.13×10^2 (30.5)	1.68×10^4 (25.62)	**3.6%**
z Normal PBL	1.73×10 (35.75)	3.81×10^3 (27.81)	**0.45%**

D

Figure 5-1 Quantitation of hTERT mRNA using the LightCycler™

A representative set of data for 3 samples (x, y and z) obtained from a single Q-RT-PCR run is shown. Panel **A** shows the sigmoid detection curves generated for hTERT. A series of RNA standards were run in parallel (**B**), allowing generation of a standard curve (**C**). The crossing points (cycle number at which fluorescence is above background) obtained from the samples in **A** were used to determine the transcript concentration from **C** and **D**. These steps were performed by the system software. Simultaneous measurement of PBGD allowed normalisation for RNA loading, and calculation of hTERT expression as a percentage of PBGD expression was performed as in **D**.

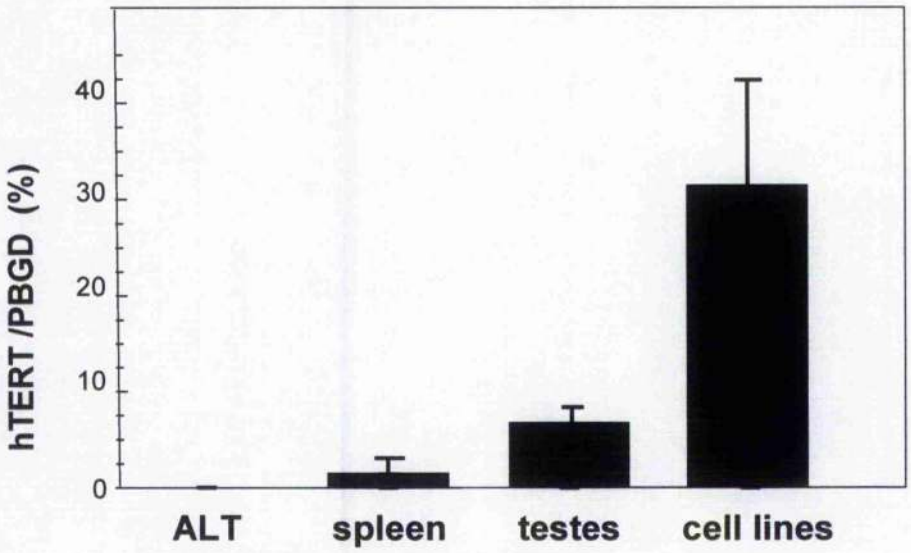


Figure 5-2 hTERT mRNA expression in a range of cell lines and normal tissues.

Expression of hTERT mRNA from ALT and CML cell lines and cDNA from normal spleen and testes was measured. cDNA can be easily used in the assay in place of RNA by omitting RT (and correspondingly increasing the H_20) in the master mix. A range of CML lines was analysed, and the other samples were done in duplicate. The values obtained were: ALT, 0.04% and 0.06%; spleen 1.1% and 1.3%; testes 6.2% and 6.7%; KY01 33.3%, LAMA84 62.7%, K562 21.9%, EM2 22.1%, KU812 17.1%, BV173 34.7%, TOM1 20.2%, ALL-MIK 33.8%. Mean values are shown with standard error bars.

To assess the dynamic range of the assay we measured hTERT expression in a range of samples (ALT cell lines, which do not express telomerase, normal spleen tissue, normal testes and a range of CML cell lines, **Figure 5-2**). As predicted hTERT mRNA was low / undetectable in the ALT cell lines, with the highest mean activity (approximately 30%) in the CML cell lines. Testes, a telomerase-high normal tissue had a mean hTERT/PBGD ratio of 6.4%. The inter- and intra-experimental reproducibility of the assay was studied by analysing duplicate samples (**Table 5-1**).

A

Sample	Result 1 (hTERT/PBGD)%	Result 2 (hTERT/PBGD)%	Mean	SD	CV (%)
1	23.3	20.3	21.8	2.1	9.7
2	4.0	5.7	4.8	1.2	24.8
3	4.6	6.1	5.4	1.1	19.8
4	3.2	2.3	2.8	0.6	23.1
5	11.5	8.2	9.8	2.3	23.7
6	3.4	3.6	3.5	0.1	4.0
7	6.8	4.8	5.8	1.4	24.4
8	2.8	2.0	2.4	0.6	23.6
9	7.3	7.8	7.6	0.4	4.7
mean				***1.1***	***17.5***

B

Sample	Result 1 (hTERT/PBGD)%	Result 2 (hTERT/PBGD)%	Mean	SD	CV (%)
1	43.1	43.8	43.4	0.5	1.1
2	12.1	12.3	12.2	0.1	1.2
3	3.3	3.5	3.4	0.1	4.2
mean				***0.3***	***2.2***

Table 5-1 Reproducibility of Q-RT-PCR for hTERT over a range of values

Intra-run variation in paired analyses of identical samples (**B**) was considerably less than that observed between runs (**A**). The relatively low dynamic range of results is inevitable, due to the use of a ubiquitous and highly expressed housekeeping gene (PBGD) with which to compare a relatively rarely expressed transcript (hTERT). Although inter-run variation was within acceptable limits, wherever possible a range of different cohorts or, when required, paired samples, were run in each PCR run to minimise this effect. The highly reproducible intra-run data meant that analysis of samples in duplicate was not required.

5.1.3 PBL hTERT expression is increased with advanced CML

To determine whether hTERT was upregulated in CML PBL (thereby validating Q-RT-PCR for hTERT mRNA as a marker of disease progression) we analysed total RNA extracted from over 70 samples, including normal individuals and CML patients at various stages of disease (see **Section 2.1.8, Table 5-2** and **Figure 5-3**. The data from all CML patients is tabulated in Appendix 1). Whole PBL were used both to avoid enriching for lymphocytes via a Ficoll-Hypaque step (thereby

confounding the analysis with telomerase expressing normal cells), and to determine the possible clinical utility of the assay (where minimal sample handling and high throughput are important). Normal allogeneic donors were included because of their high 'left-shifted' (immature) granulocyte count (n=3, total WCC was between 20-30 x 10^9/L after 5 days of rHu-G-CSF), thereby bearing a resemblance to the mixed immature / mature myeloid picture typical of CP CML. Transcripts were detected in all samples apart from 6 (3 CML at diagnosis, and 3 in established CP). There was no significant increase in hTERT transcripts in CP CML as compared to normal PBL. AP and BP demonstrated mean hTERT transcript levels significantly above those of normal PBL (but not, interestingly, CP CML at diagnosis); however the range of expression was extremely wide (particularly when BP was considered as a group). The >100 fold variation in hTERT expression in BP was not simply due to sampling differences (blast counts varied from 30%- 80%), although these may have had a minor influence. Indeed 7 of the BP specimens had hTERT levels on or below the mean level for normal PBL. All of these 7 samples were of myeloid lineage and, interestingly, all 3 BP samples that had hTERT levels similar to those of cell lines (*i.e.* >20%) were lymphoid on immunophenotype analysis (data from routine clinical analysis, and therefore not shown).

These data prompted separate analysis of the BP subgroups, with lymphoid BP exhibiting significantly greater hTERT expression (**Figure 5-4**). Only a small number of paired CP and BP RNA samples were available for analysis: recovery of good quality RNA suitable for Q-RT-PCR was rarely achieved from archived PBL CP samples due to the poor recovery of neutrophils from frozen (with release of nucleases etc). The dramatic increase in hTERT expression on progression from CP or AP to BP in both lymphoid BP samples (as compared to myeloid,

Figure 5-5) show that increased hTERT expression is not an inherent property of these samples during CP, and is a genuine characteristic of the BP phenotype.

Category	n	Mean	Median	SD	Range	P value vs Normal PBL/CML Diag
			(hTERT/PBGD)%			
Normal PBL	8	1.3	1.1	1.2	0.3 - 3.8	NA / NA
Allo Don	3	0.4	0.5	0.3	0.1 - 0.7	NA / NA
CML Diagnosis	18	3.4	3	2.6	0 - 7.5	0.1 / NA
CML estCP	7	1.4	0.2	2	0 - 5.2	0.4 / 0.07
CML AP	13	6	5.4	4.5	0.7 - 16	0.009* / 0.09
CML BP (all)	21	7	3.3	9.1	0.2 - 34.4	0.03* / 0.4
CML BP (Myeloid)	15	3.4	1.9	3.3	0.2 - 10.5	0.17 / 1.0
CML BP (Lymphoid)	6	17.4	17.4	12.6	4 - 34.4	0.002* / 0.009*

Table 5-2 Summary statistics for hTERT expression in normal and CML PBL

Descriptive statistics and p-values (Mann-Whitney U-test) are shown for each of the cohorts studied. A wide range of values was apparent, most marked for BP samples. Considerable overlap with normal values was seen for all CML cohorts apart from lymphoid BP. Allo Don, allogeneic donor; estCP, established CP; SD, standard deviation; * statistically significant p-value.

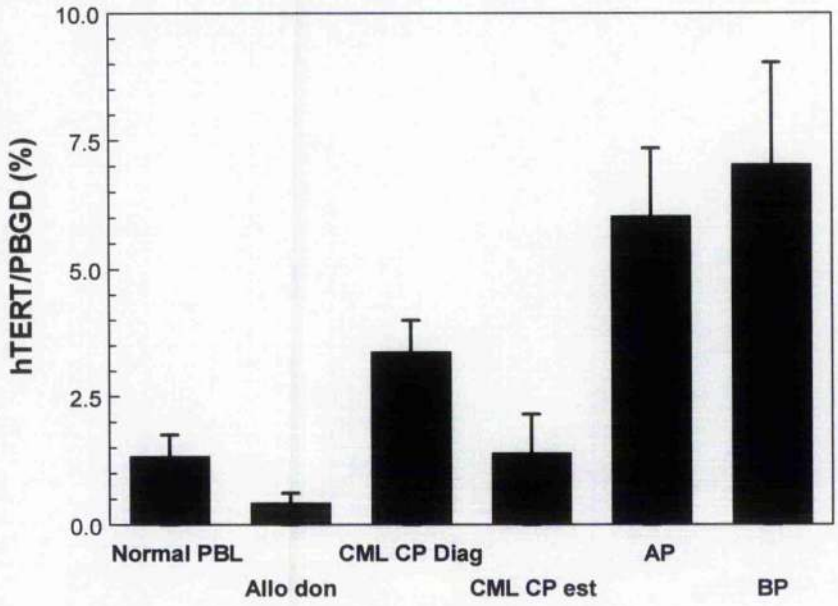


Figure 5-3 hTERT expression in normal and CML PBL

A histogram depiction of the data in **Table 5-2**. Histogram bars represent mean values with standard error bars. PBGD, porphobilinogen deaminase.

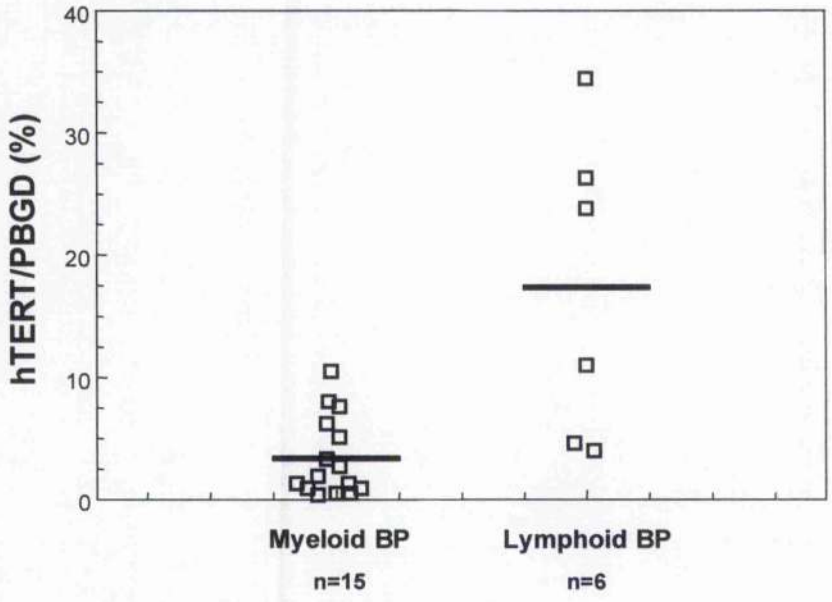


Figure 5-4 hTERT expression in myeloid vs lymphoid BP.

hTERT expression was significantly increased in lymphoid BP ($p<0.01$). Bars represent mean values.

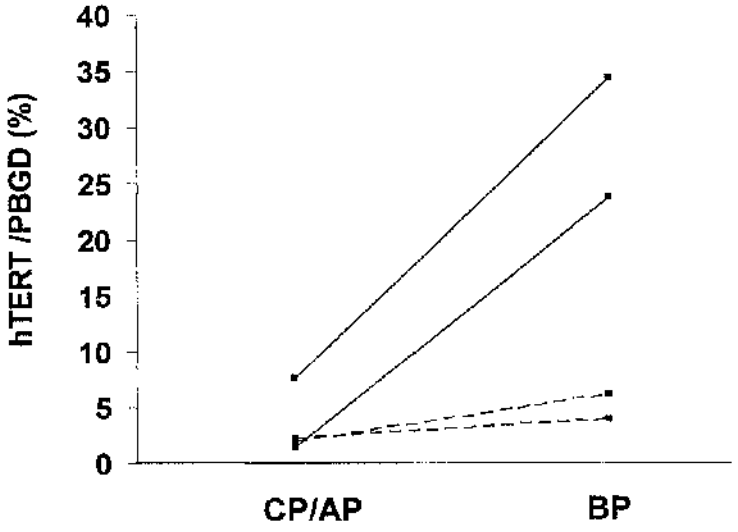


Figure 5-5 hTERT expression in paired CP or AP and BP specimens

On progression to BP, all 4 samples showed an increase in hTERT expression. The myeloid BP samples (broken lines) increased from 1.9% and 2.2% (both CP) to 6.2% and 4.0% respectively. The lymphoid BP specimens (solid lines) increased from 1.5% and 7.6% (both AP) to 23.8% and 34.4% respectively. The latter levels were similar to those detected in CML BP cell lines (**Figure 5-2**).

5.1.4 Resolution of alternatively spliced hTERT mRNA by RT-PCR

Altered splicing of hTERT mRNA is a recently described post-transcriptional regulatory mechanism of telomerase activity (Section **1.6.9.3.3**). As the Q-RT-PCR assay only detected full-length hTERT mRNA, we performed RT-PCR with a single set of primers to detect the commonly expressed alternatively spliced mRNA species (**Figure 5-6**).

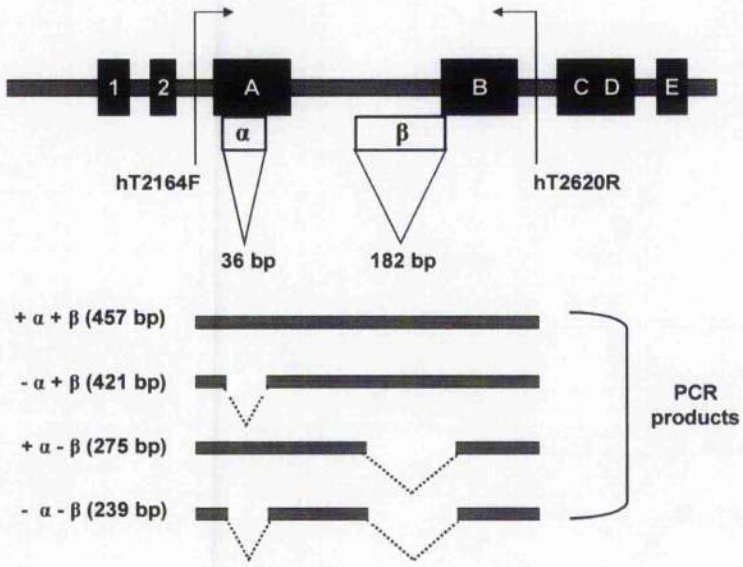


Figure 5-6 The reverse transcriptase domain of hTERT mRNA with alternative splicing sites and respective PCR products

1, 2 and A-E represent the conserved RT motifs. The position of deletions α and β are illustrated (as originally described by Kilian *et al* (Kilian, *et al* 1997)), flanked by primers hT2164F and hT2620R. The potential PCR products from the alternative splice combinations are shown, with the full-length product (+α+β) extending from base 2164 to 2620 (457 bp). Loss of the α domain (-α) removes a 36 bp sequence from the A motif, with the translated protein appearing to confer dominant negative hTERT activity (Colgin, *et al* 2000). Splicing of the β site causes a 182 bp deletion, resulting in translation of a non-functional truncated protein (Ulaner, *et al* 2000). Domains 1 and 2 are hTERT specific and A-D are common to all reverse transcriptases (Lingner, *et al* 1997).

By utilising a single PCR and single primer set, the relative quantities of each of the mRNA species could be measured. This was achieved by resolving the PCR products on a DNA 1000 chip in the Agilent Bioanalyser and expressing the relative amounts of each of the 4 potential mRNA variants as a percentage of total transcripts (**Figure 5.7**).

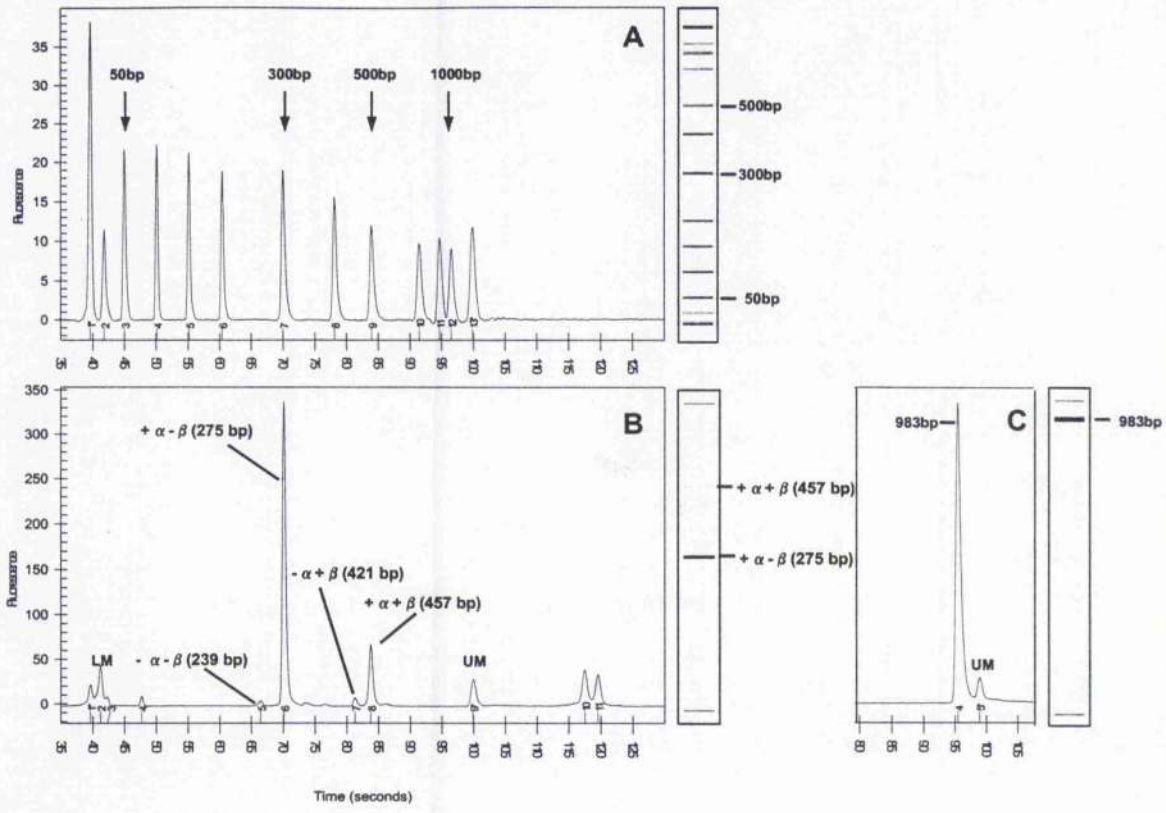


Figure 5-7 Electropherogram analysis of alternatively spliced hTERT mRNA RT-PCR products.

A single set of primers was utilised to amplify the 4 potential alternatively spliced hTERT mRNA products. These were analysed on the Agilent Bioanalyzer instrument, and quantitated in $\mu g/\mu l$. Incorporation of a DNA ladder (**A**) facilitates automatic sizing of each peak, the concentration of which is then determined automatically. The relative percentages of each product were then calculated. A CML $CD34^+$ selected sample exhibiting all 4 potential splice products is illustrated (**B**), with concentrations (and relative percentages of all transcripts) as follows; $+\alpha+\beta$ 4.0 $\mu g/\mu l$ (16.5%), $-\alpha+\beta$ 0.5 $\mu g/\mu l$ (2.1%), $+\alpha-\beta$ 19.3 $\mu g/\mu l$ (79.8%) and $-\alpha-\beta$ 0.4 $\mu g/\mu l$ (1.7%). RT-PCR for the housekeeping gene GAPDH (22.3 $\mu g/\mu l$) is shown in (**C**). Sizes (in bps) of each of the relevant peaks are indicated and a 'gel' like image is shown for comparison in each of the right-hand panels. LM, lower marker; UM upper marker.

As a control for cDNA quality and quantity, GAPDH RT-PCR was performed on all samples that underwent splice-variant PCR (**Figure 5.7**). A satisfactory control PCR was determined by a clearly visible GAPDH band of 983bp, with a concentration of >5.0 $\mu g/\mu l$.

5.1.5 Only full-length (+α+β) hTERT mRNA is associated with telomerase activity

Previous reports have determined that telomerase activity is generally only detected in the presence of full length (+α+β) transcripts. We sought to confirm these observations in our CML samples. Several samples (all PBL), found to be negative for +α+β transcripts were selected for TRAP analysis, and compared to those positive for +α+β (PBL and $CD34^+$ CML cells). All 4 samples which were negative on TRAP assay failed to express +α+β by RT-PCR, while the 4 samples which did express +α+β were positive by TRAP (**Table 5-3** and **Figure 5-8**). The TRAP-negative samples were also low / negative for +α+β as quantitated by the LightCycler (0%, 0.03%, 1.2% and 2.5% respectively). This illustrates the greater sensitivity of the LightCycler platform, and illustrates that low-level expression of +α+β hTERT does not necessarily translate into TRAP activity as detected by this assay. This observation may also imply post-translational mechanisms of telomerase control in this cell population.

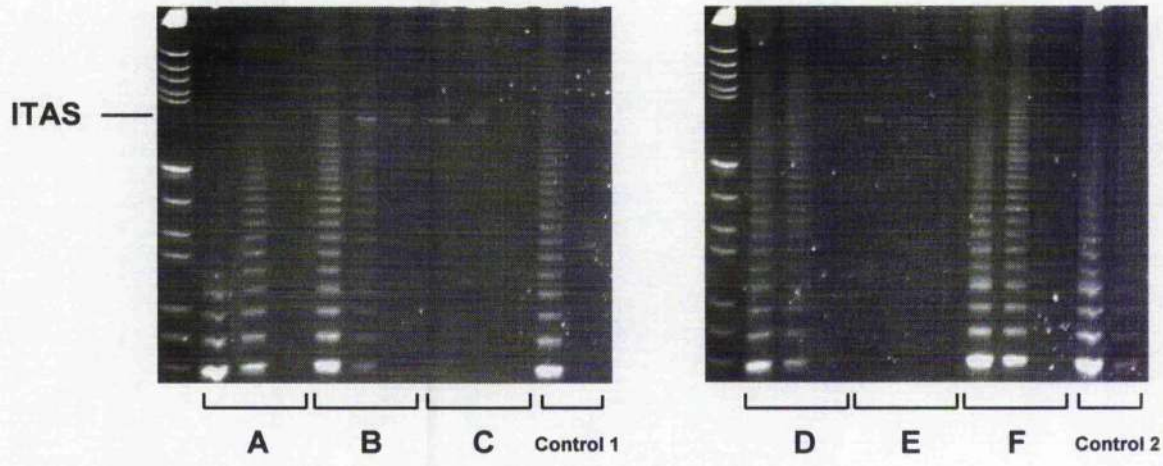


Figure 5-8 TRAP analysis of selected CML samples.

TRAP was performed as described and 5µl of product was run on a 12% non-denaturing polyacrylamide gel. 6 samples and respective controls are illustrated. 3 lanes were run for each sample (1µg and 0.1µg protein lysate, and 0.1µg heat inactivated lysate respectively, from left to right). Control 1 represents 1000 GLC4 cell equivalent preparation with a heat treated control, and Control 2 is a 1000 and 100 GLC4 cell equivalent preparation from left to right respectively). CML samples A-F are described in the table below (**Table 5-3**). The ITAS band (at approximately 150bp) is illustrated. It can be seen that in lane 1 of sample A no ITAS is visible, and the processivity of the enzyme appears to be weak. Lane 2 of sample A however (at a 1:10 dilution) has resulted in a diluting out of the TRAP inhibitors, and a distinct ITAS band. Thus the processivity of the assay was indeed being masked in lane 1. Conversely sample C has no telomeric repeat laddering, but the ITAS control is clearly visible. This is a true negative result. It is harder to visualise the ITAS in the strongly positive samples (i.e. D and F) because the telomeric repeats have sequestered the reaction mix reagents and there is less available for ITAS amplification.

Sample	Type	UPN	hTERT/PBGD (%)	% +α+β	TRAP
A	34^+	-	3.3	17.8	+
B	34^+	-	3.6	24.1	+
C	PBL	13	2.5	0	-
D	34^+	-	3.6	25.4	+
E	PBL	6	0	0	-
F	34^+	-	16	36.4	+
G	PBL	3	4.9	ND	+
H	PBL	12	0.03	0	-
I	PBL	9	1.2	0	-

Table 5-3 Comparison of TRAP vs. hTERT RT- and Q-RT-PCR in selected CML $CD34^+$ and PBL samples

9 CML samples were selected based on the presence or absence of +α+β as determined by RT-PCR and LightCycler and analysed for telomerase activity by TRAP. There was a clear correlation between presence / absence of +α+β transcripts as measured by RT-PCR and TRAP activity. For this illustration samples are independently named A-I, and the relevant UPN is given for data shown in **Appendix 1**. The $CD34^+$ data is described in full in the next chapter, the samples having been used as +α+β positive controls in this case.

5.1.6 Patterns of alternatively spliced hTERT mRNA in CML PBL

To determine if splicing patterns were disease-stage specific, and as an initial step in investigating the potential significance of alternative splicing for hTERT regulation in CML, we performed RT-PCR for splice variants on 53 PBL samples from all stages of CML (**Appendix 1**). Striking heterogeneity of expression was evident, particularly in the BP samples and especially with regard to full-length (+α+β) functional hTERT expression. The non-functional, β-deleted (+α-β) variant was the most frequently expressed, and to the greatest degree in all but 1/16 diagnostic samples and 3/17 BP samples which expressed greater levels of +α+β

than +α-β). This is in keeping with published results for normal tissues, solid tumours and cell lines ((Ulaner, *et al* 2000, Yi, *et al* 2001)), where the β-deleted (and non-functional) variant is most commonly expressed. In comparison to a range of cell lines (described in **Figure 5-2**), 7/8 (87.5%) of which expressed all transcripts, only 12.5% and 29% of diagnostic CP and BP samples respectively expressed all. No estCP or AP samples expressed the full range of transcripts. The percentage of samples expressing full-length +α+β transcripts, and the degree to which they expressed it (as a percentage of total hTERT transcripts) is illustrated in **Figure 5-9** and **Figure 5-10** respectively. It can be seen that BP is associated with a greater proportion of samples expressing a greater percentage of unspliced full length +α+β hTERT mRNA.

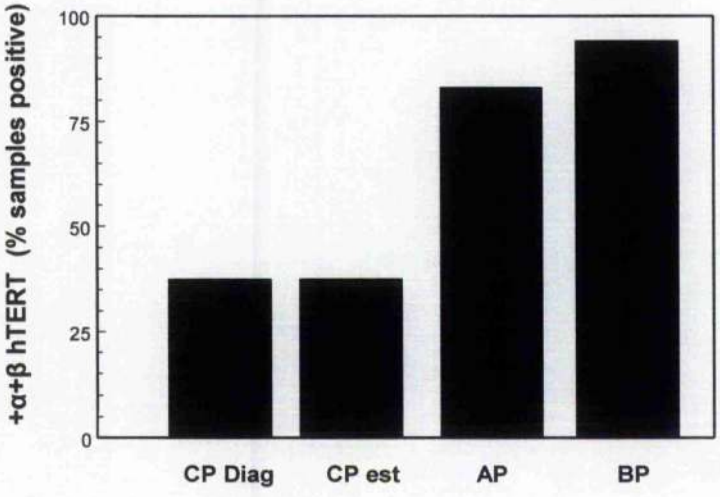


Figure 5-9 Proportion of samples expressing +α+β transcripts at different disease stages

Disease progression was associated with near universal expression of full-length (+α+β) transcripts, in addition to 100% of the cell lines described in **Figure 5-2.**

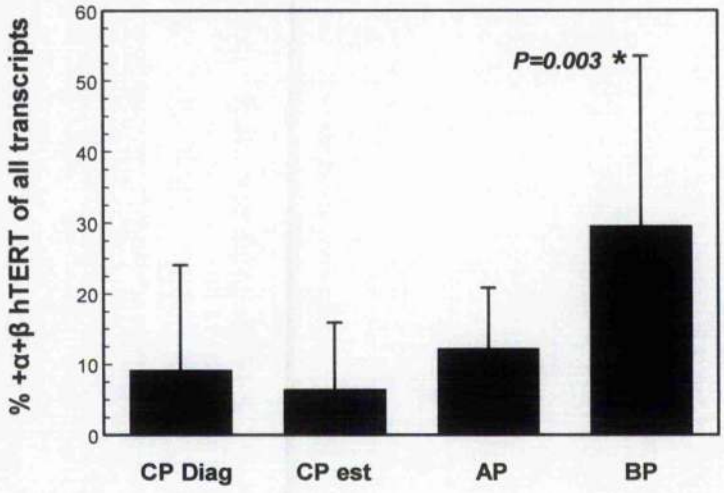


Figure 5-10 Percentage of total hTERT transcripts which are full-length (+α+β)

BP disease was associated with an approximately 3-fold (statistically significant) increase in the mean percentage of full-length transcripts. However, as illustrated by the SD bars, the range of expression was large. The relevant results for the leukaemia cell lines were 18.8±6.8 (mean±SD). The increase in BP full-length transcript expression was at the expense of β-deleted variant (+α-β), which underwent a significant fall in mean expression levels (from 82.4±19.0 to 63.6±24.2%, p=0.02). Complete expression data was only available for 4 lymphoid BP samples, therefore a valid comparison between myeloid and lymphoid BP was not possible.

Two possible ways in which an absolute increase of full-length (+α+β) functional hTERT mRNA may occur are via a proportional increase in the total number of all hTERT transcripts, or via a shift in splicing patterns leading to an increase in the overall proportion of full-length (+α+β) functional hTERT mRNA. While the former mechanism does not necessitate a shift in splicing patterns, the latter clearly does. To determine whether a shift away from splicing (thereby increasing levels of full-length hTERT) influenced overall +α+β hTERT levels (as determined by LightCycler), we correlated the percentage of unspliced hTERT transcripts with the LightCycler data (**Figure 5-11**). Somewhat surprisingly (given the heterogeneous nature of PBL specimens, and the inherent difficulty in comparing different PCR techniques) a significant correlation was seen, at least suggesting that shifting splicing patterns may influence overall hTERT activity in this setting.

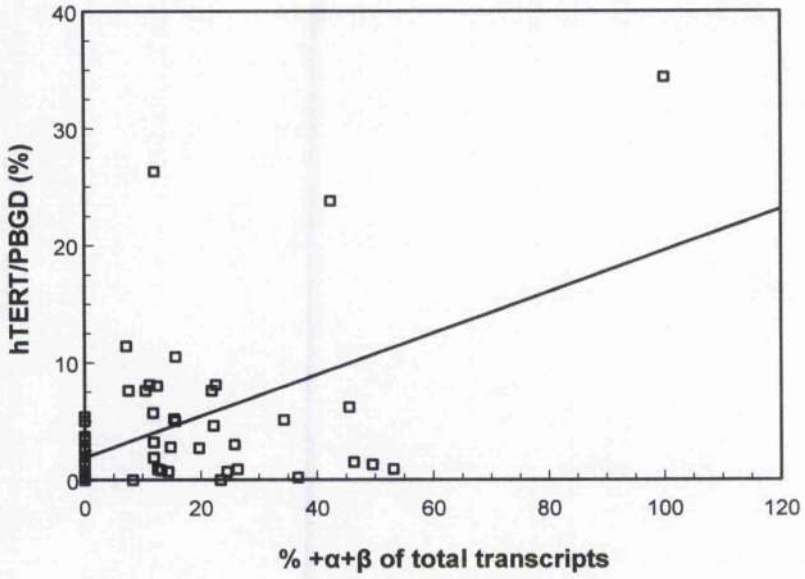


Figure 5-11 Correlation of % +α+β transcripts (by RT-PCR) with Q-RT-PCR measurement in all CML stages

A significant correlation was observed between these two parameters (R^2=0.25, p=0.0002), implying that splicing control may play a part in regulating overall functional hTERT levels in CML.

So far, these data do not reveal any differences in *total* (*i.e.* all potential splice variants) quantity of hTERT transcripts detectable at any given stage of disease, or in cell lines for that matter. By combining the two data sets for any given sample (LightCycler quantitation and RT-PCR for spliced mRNA) a complete picture of hTERT gene expression can be estimated by the equation:

Total Transcripts (as % of PBGD) = {100 / % +α+β of all transcripts} x {(+α+β / PBGD) x 100}

Equation 3 Estimation of total hTERT transcript expression

The limitations of this approach (other than the results are an estimate) are that samples failing to express full-length transcripts (either by RT-PCR and / or Q-RT-PCR) cannot be included in the analyses, thereby potentially skewing the data. With these reservations in mind however, the comparison of total transcript quantities between the disease stages is of interest

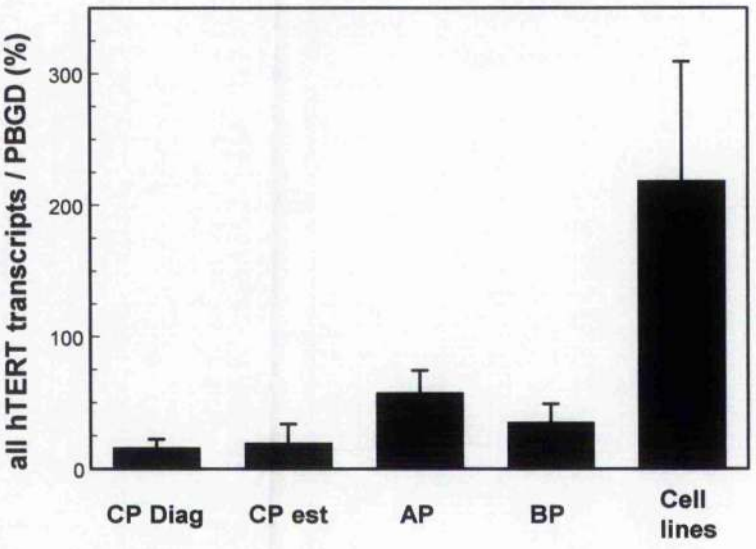


Figure 5-12 Estimated total hTERT transcript expression

Using Q-RT-PCR and RT-PCR data (defining the quantity of +α+β hTERT relative to PBGD and the percentage +α+β hTERT of total transcripts respectively), the total expression level of all hTERT mRNA variants was estimated as a percentage of PBGD expression. Due to the limitations of the calculation (see text), only 4 diagnostic CP and 2 est CP samples could be included, and are illustrated for completeness. There were no significant differences in hTERT expression between diag CP, AP or BP. The cell lines exhibited greatly increased expression however ($p=0.009$ and 0.0008 *vs.* AP and BP respectively).

Total hTERT transcripts were in significantly greater abundance in cell-lines than primary human CML samples. Indeed there were no statistically significant differences between any of the material from the different disease stages, where numbers permitted adequate analysis (namely CP at diagnosis, AP and BP).

5.1.7 Discussion

We have demonstrated Q-RT-PCR for hTERT to be a reproducible technique, which is considerably more sensitive than RT-PCR. A wide dynamic range of hTERT expression was evident, and this correlated broadly with the published range of telomerase activity (by TRAP) within these samples; namely high in tumour or leukaemia cell lines, moderate in HSC populations and testes, low or undetectable in PBL and absent in ALT cell lines. Additionally, only full length hTERT transcripts (+α+β) were detected by Q-RT-PCR, and we have established that such transcripts are a prerequisite for detectable TRAP activity.

Several aspects of +α+β hTERT expression in CML samples from different disease stages are of interest. Firstly, CML PBL samples at diagnosis had comparable expression levels to normal PBL. This is not surprising: despite their clonal nature CML PBL cells are in various stages of myeloid terminal differentiation and such cell types are not typically associated with significant telomerase activity. Secondly, despite consisting of at least 30% blasts (by definition), the BP samples (as a whole) failed to show a significant mean increase in hTERT expression over CP PBL, and demonstrated a large range of values. However, LBP samples had significantly greater hTERT expression than MBP. This observation suggests that hTERT 'competent' cell types (*i.e.* B-lymphoid cells in this case) have an inherent ability to upregulate hTERT expression in a similar fashion to their normal counterparts, and that this may facilitate high expression during disease progression. Thirdly, despite no significant increase in mean absolute +α+β hTERT levels, disease progression was associated with a shift in splicing patterns; BP samples exhibited significantly less splicing (primarily of the +α-β variety), with a proportional increase in unspliced full-length +α+β transcripts. Indeed a weak (but statistically significant on ANOVA analysis) correlation was

demonstrated between overall +α+β hTERT levels detected by the LightCycler, and the proportion of +α+β transcripts of total. This raises the intriguing possibility that subtle changes in splicing of hTERT mRNA may be involved in determining functional +α+β transcript levels, and hence telomerase activity.

Accumulation of a particular mRNA species is a function of 3 cellular processes; namely transcription rate, mRNA half-life and alternate splicing activity. If one assumes that hTERT mRNA half-life remains constant during disease progression, then our attempt to quantify expression of all hTERT transcripts suggests that transcriptional rate does not change during CML progression, and that even BP samples have much lower (approximately 7-fold) expression than equivalent cell lines. However, as described above, within this picture of 'unchanging' expression there exists a shift towards preferential expression of +α+ β transcripts in the BP samples.

For the reasons outlined in Section 5.1.3, these experiments were performed upon PBL from various stages of CML. From our data it is clear that hTERT measurement in PBL *per se* would not be helpful in determining disease progression: overlap between disease stages is considerable and it appears to offer no additional advantage beyond conventional classification of disease stage. However it remains possible that a rising hTERT level (from baseline at diagnosis) may herald disease progression. The rarity of paired samples in our study made this difficult to determine however. Almost certainly, the major confounding factor with utilising PBL material is the cell mix involved, with changes in hTERT expression reflecting alterations in relative proportions of cell types. Thus BP samples are likely to express more hTERT as a consequence of them containing large numbers of $CD34^+$ cells, rather than as a consequence of a more subtle shift at the molecular level causing a change in gene expression. Indeed the marked

heterogeneity of hTERT expression patterns illustrated in Appendix 1 (particularly in BP) are likely to be due, in large part, to heterogeneity of cell content. Additionally there is marked disease heterogeneity from both clinical and laboratory points of view. Although recent advances have been made in determining the molecular nature of this (particularly with deletions of the derivative chromosome 9 (Huntly, *et al* 2001)) much still needs to be learned. From this aspect it is therefore not surprising that inconsistent patterns of telomerase expression are detected; it is their significance that needs to be determined. To further define the nature of such changes, CP and BP $CD34^{+}$ cells require to be studied.

Our data would indicate that upregulated expression of hTERT is a rare event during progression of CML (the implications of this will be covered in the final discussion) that is largely dependent on cell-background (*i.e.* lymphoid or myeloid) and is consistent with published data showing non-significant increases in TRAP activity upon entry into BP (Verstovsek, *et al* 2003a). This is despite the detection of marked telomere shortening in BP samples (see previous chapter). As far as we are aware, no previous attempts have been made to sub classify BP samples with regards TRAP activity, and it is possible that the findings of such studies reflect different proportions of BP phenotypes (*i.e.* MBP, LBP or mixed lineage). This observation also raises questions regarding telomere maintenance during CP disease and upon progression to BP. Many studies performed upon solid tumour material demonstrate increased TRAP activity in the specimens, in comparison to surrounding normal tissue and earlier stage of tumourigenesis, and this is generally accompanied by increased hTERT expression.

In summary, despite the considerable telomere shortening observed during progression of CML from CP to BP, increased expression of functional hTERT

transcripts (at least compared to CP PBL) is apparently not a prerequisite for MBP disease. This raises the intriguing possibility that CML is primarily a disorder of telomere shortening, and does not conform to conventional models of telomere/telomerase dynamics in tumourigenesis. To answer this question more definitively it is necessary to perform more detailed studies of telomerase component expression on $CD34^+$-selected cell populations.

6. Results 4

6.1 Expression of telomerase components and telomerase activity in CP CML and non-CML CD34$^+$ cells

The finding, albeit inconsistently, of elevated *in-vitro* telomerase activity in CP CML CD34$^+$ cells (Engelhardt, *et al* 2000, Ohyashiki, *et al* 1997c) presents something of a paradox: the rapid rate of telomere loss observed by ourselves and others would suggest that *in-vivo* it is unable to prevent telomere loss. If one accepts that the conventional TRAP assay is representative of telomerase activity *in-vivo* it is therefore difficult to explain the rapidity of telomere loss observed at this early stage of the disease.

Detection of TRAP activity however does not always reflect ability to maintain telomeres (Ouellette, *et al* 1999) and this is corroborated *in-vivo* with the observation that telomerase-competent HSC continually lose telomere length. Indeed mTR haploinsufficient mice (mTR$^{-/+}$) have defective telomere elongation yet fail to demonstrate any reduction in telomerase activity by TRAP (Hathcock, *et al* 2002). Additionally, mTERT$^{-/+}$ and mTERT$^{-/+}$ ES cells have no detectable differences by TRAP (Liu, *et al* 2000b), despite greatly increased telomere loss in the former. These authors suggested that differences in dosage of the main telomerase components, while limiting *in-vivo*, were not reflected *in-vitro* by TRAP.

It is therefore conceivable that undetected telomerase dysfunction in the CML HSC compartment could contribute to the observed rate of telomere loss. The precedent for such a mechanism has already been set, in the form of the inherited disorder dyskeratosis congenita (see section **1.6.6**). Not only is the phenotype of

these individuals similar to $mTR^{-/-}$ mice (in that it affects primarily tissues with a high turnover) (Blasco, *et al* 1997, Lee, *et al* 1998), but it is associated with dramatic telomere shortening and an increase in cancer incidence. The latter is thought to be due to the genetic instability resulting from critical telomere shortening and thus provides direct support for the hypothesis that telomeres, while normally limiting for tumourigenesis *in-vivo*, may promote clonal evolution and tumourigenesis when dysfunctional. One crucial aspect of this model remains unexplained however; namely the mechanism of telomere maintenance in tumour cells with constitutionally impaired telomerase function. The most obvious explanation is that these tumours depend upon the ALT mechanism; however there is, to our knowledge little evidence as yet to support this model. It is also conceivable that such cells (given the intense selection pressure exerted by short telomeres plus a genetically unstable background) may acquire additional means with which to upregulate telomerase. Admittedly DKC is rare: however the association of short telomeres and genetic instability is not, and has been observed in many solid tumours and leukaemias. It therefore is an intriguing prospect that clonal evolution, at least in some tumours, may be propagated by the telomere shortening induced by early telomerase dysfunction.

The aim of this chapter was therefore to comprehensively characterise and quantitate expression of the major telomerase components and telomerase activity in purified normal and *BCR-ABL*-positive candidate stem ($CD34^+$) cells.

6.1.1 Peripheral blood BCR-ABL$^+$ CD34$^+$ cells demonstrate increased cell cycle activity

CP CML is associated with marked telomere shortening detectable in PBL, therefore we measured relative cell-cycle activity in a pure progenitor cell/HSC (CD34$^+$ selected) population. Additionally, as TRAP activity is reduced or absent in G_0 populations, such data also allow correlation of differences in TRAP activity with the cell cycle status of the cell sample. Because of the requirement to resolve the G_0 population we utilised the high-resolution cell cycle analysis method as described by Jordan *et al.* (Jordan, *et al* 1996). Resolution of G_0 *vs.* G_1 and $S/G_2/M$ (**Figure 6-1**) demonstrated significantly fewer CML CD34$^+$ cells in stage G_0 and correspondingly greater numbers in $S/G_2/M$ (**Table 6-1**), as compared to non-CML. This is in keeping with the increased cell cycling observed in CML progenitors using other methodologies (reviewed in (Holyoake, *et al* 2002)).

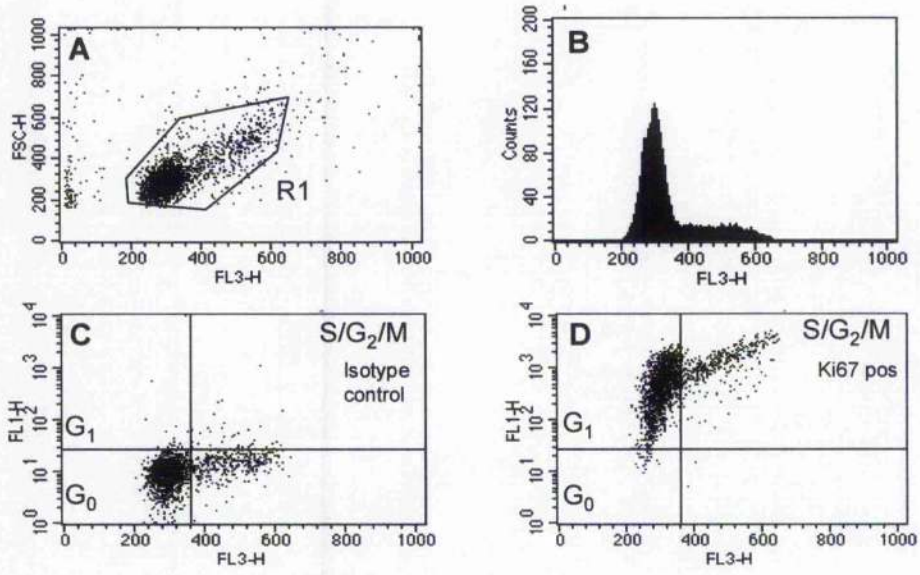


Figure 6-1 High-resolution cell-cycle analysis of CD34$^+$ cells

A representative CML CD34$^+$ sample is shown. The addition of FITC labelled Ki67 antibody (a cell cycle activation antigen that is expressed in early G_1) to 7-AAD (detected in channel FL3) allowed discrimination of G_0 *vs.* G_1. Gating on the 2N/4N intact cell population (**A**) does not allow discrimination of G_0/G_1 on FL-3 analysis alone (**B**). Ki67 expression (channel Fl-1) resolved these two populations however (**D**), as compared to the isotype control (**C**).

Samples (number)	% CD34 (median, range)	% *BCR-ABL pos* (median, range)	% in G_0 (mean±SD)	% in G_1 (mean±SD)	% in $S/G_2/M$ (mean±SD)
CML *(16)*	94.1 (88.9-98.1)	96.3 (86.5-99.4)	5.8±4.4*	79.6±5.4	11.8±4.4**
Non-CML *(14)*	94.1 (88.0-99.0%)	NA	15.0±8.1*	74.9±8.7	5.1±4.2**

Table 6-1 CD34$^+$ cell purity and cell-cycle status

High purity CD34$^+$ cells were selected and subsets used for all the experiments in this chapter. CML patients were chosen with high percentages of *BCR-ABL*-positive cells in the CD34$^+$ population (one patient was excluded because this was low at 26%). Significant differences in percentages of cells were observed between the G_0 and $S/G_2/M$ compartments for the two populations (but not between G_1). * and **, $p<0.05$; SD, standard deviation; NA, not applicable.

6.1.2 In-vitro telomerase activity, as measured by TRAP, is increased in the BCR-ABL$^+$ CD34$^+$ compartment and correlates with cell-cycle profile

It is conventional to assess telomerase activity semi-quantitatively by using the TRAP assay. The methods used for the quantification step are numerous; we utilised the Agilent Bioanalyser with a DNA-500 Chip to resolve the 6-bp incremental peaks (**Figure 6-2**). For all samples the first 6 such peaks were added together (in ng/µl) to generate an 'area under the curve' as previously described (Hamad, *et al* 2002). This was then expressed as a percentage of the activity observed from a 10,000 GLC-4 equivalent cell preparation.

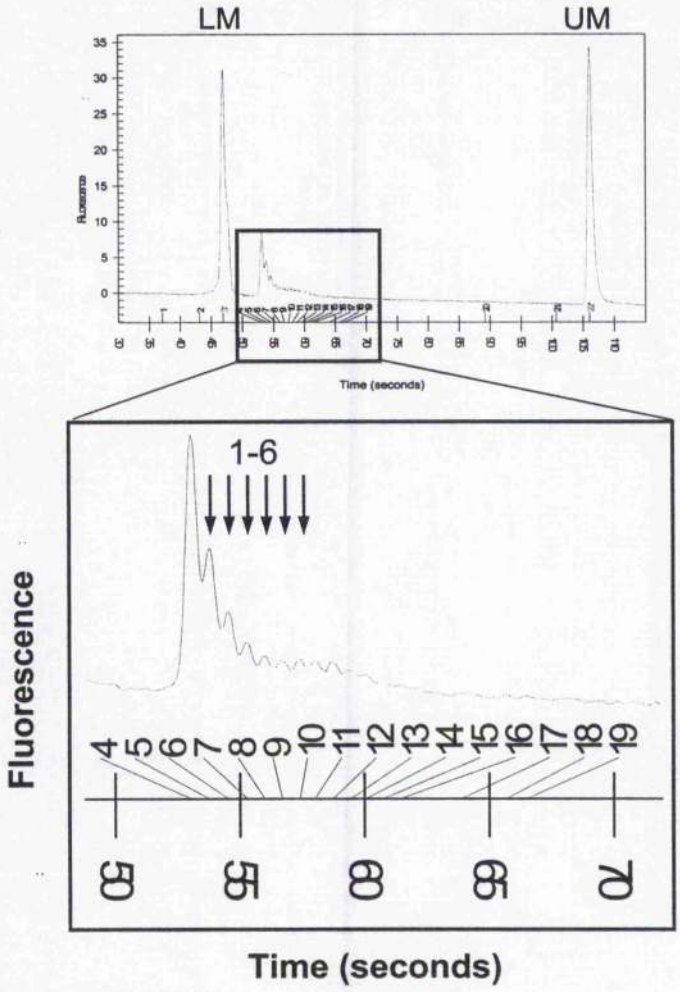


Figure 6-2 Quantitation of TRAP activity using the Agilent Bioanalyzer

Analysis of TRAP products was performed on the Agilent as described in the text (a non-CML $CD34^+$ sample is illustrated). The first peak, which consisted of the primers plus one telomere repeat, was excluded (**Figure 2-3**). The next 6 peaks (as shown) had sizes of 56, 62, 68, 74, 80 and 86 bps respectively, and the respective concentrations were 0.13, 0.07, 0.03, 0.02, 0.03 and 0.03 ng/μl. The sum of these products (0.31 ng/μl) was expressed as a percentage of the sum of 10^4 GLC4 cell activity (0.91 ng/μl), which in this case was 34.0%.

All $CD34^+$ samples expressed TRAP activity, with higher levels (some 1.5-fold) detectable in CML (n=16) as compared to non-CML (n=8) cell populations (94.8 ± 22.1% vs. 64.6 ± 21.3% respectively, mean percentage activity of 10^4 GLC4 cells ± SD, p=0.004). This activity correlated significantly with the cell-cycle profile of the samples however, with samples containing a greater proportion of G_0 cells (*i.e.* the non-CML samples) having the lowest TRAP activity. Conversely there was a trend

towards samples with greater numbers in $S/G_2/M$ (i.e. primarily CML) having greater TRAP activity. This would suggest that the relatively small differences in TRAP between these populations are primarily a reflection of cell-cycle status, and may not reflect any genuine cell-to-cell increase in telomerase activity in CML. However only studies looking at sorted cell-cycle stage specific populations would be able to resolve this issue.

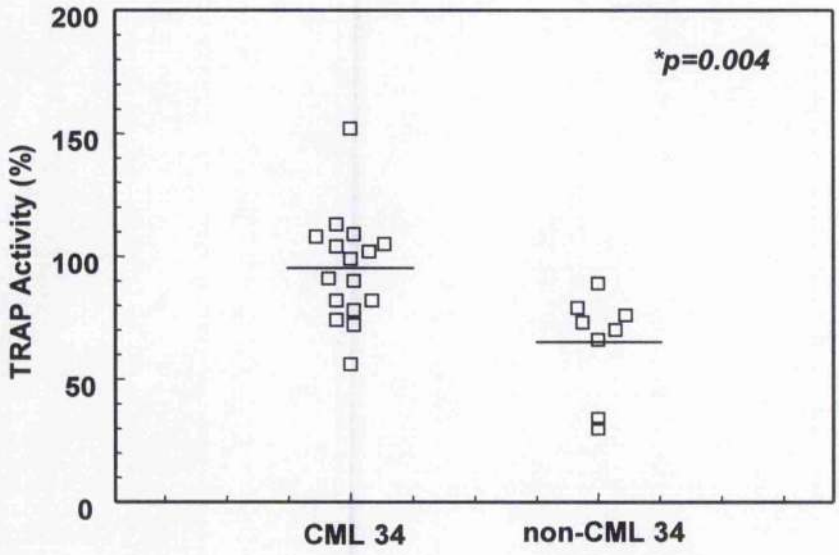


Figure 6-3 TRAP activity in CML *vs.* non-CML CD34$^+$ cells

A small (1.5-fold) but statistically significant increase in TRAP activity was observed for the CML samples. Activity was expressed as a percentage of that seen in 10,000 GLC-4 cells.

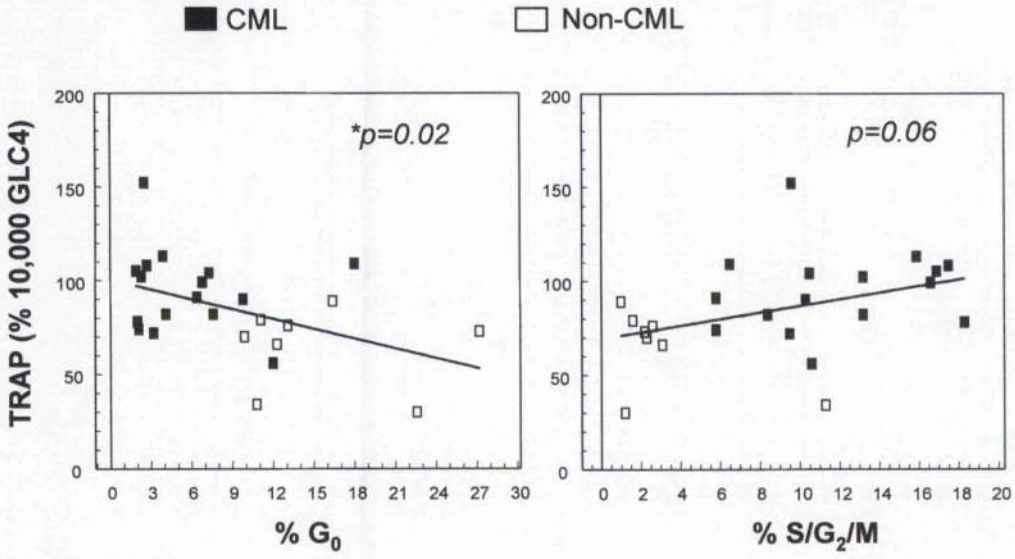


Figure 6-4 TRAP activity correlates with cell-cycle profile of CD34$^+$ cells

TRAP activity was seen to correlate inversely with the percentage of CD34$^+$ cells in G_0, as might have been expected (adjusted r^2 0.17, p=0.02). This relationship was only observed when all samples (CML and non-CML) were analysed together, presumably as this increased the dynamic range of measurements. Conversely a trend towards higher TRAP levels was observed with increasing numbers of cell in $S/G_2/M$ (adjusted r^2 0.11, p=0.06). No correlation was observed with G_1 (adjusted r^2 0.005, p=0.3). ■, CML samples; □, non-CML samples.

6.1.3 Full length functional (+α+β) hTERT mRNA expression is not increased in BCR-ABL$^+$ CD34$^+$ cells

Interestingly, despite their leukaemic origin, increased cycling status and TRAP activity, the CML CD34$^+$ cells did not demonstrate a significant increase in +α+β hTERT expression (**Figure 6-5,** p=0.08) as measured by Q-RT-PCR. A trend towards higher levels in the CML samples was largely the result of two samples which exhibited markedly higher +α+β hTERT expression than the remainder; however there were no obvious differences in their clinical parameters (*e.g.* on review of their diagnostic data they were definitely in CP). Comparison of the median level for both groups confirmed this; at 3.6% and 3.2% for CML and non-CML respectively. No correlation (either positive or negative) was observed between +α+β hTERT expression and TRAP activity or percentage of cells in each

of the cell cycle stages for CML, non-CML or combined samples. However, Q-RT-PCR on the LightCycler quantitated full-length (+α+β) hTERT mRNA only; it is known that alternative splicing may play a role in regulation of hTERT expression and that other hTERT transcripts may be present, some of which may have a regulatory role.

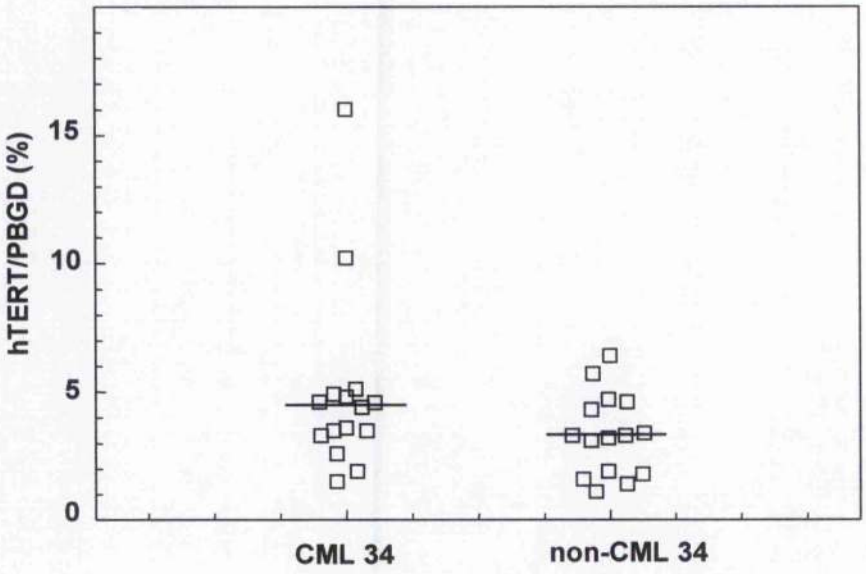


Figure 6-5 +α+β hTERT mRNA expression by Q-RT-PCR in primary CD34$^+$ cells

Total RNA from CML *vs.* non-CML CD34$^+$ cells was analysed for expression of +α+β (full-length functional) hTERT. Although there was a trend towards higher levels of +α+β hTERT in the CML samples, this did not reach statistical significance (p=0.08) and was skewed by two samples exhibiting high levels; median values for the two groups were similar (see text).

6.1.4 Altered hTERT mRNA splicing profiles in BCR-ABL-positive and negative CD34$^+$ cells

Two possible ways in which an absolute increase of full-length (+α+β) functional hTERT mRNA may occur are via a proportional increase in the total number of all hTERT transcripts, or via a shift in splicing patterns leading to an increase in the

overall proportion of full-length (+α+β) functional hTERT mRNA. While the former mechanism does not necessitate a shift in splicing patterns, the latter clearly does.

As described previously, RT-PCR for hTERT splice variant expression was analysed on the Agilent, and the percentage of each transcript (of total) calculated. By combining these data with quantitative (LightCycler) analysis of full-length +α+β hTERT expression, a complete expression profile of the hTERT gene can be derived **Figure 6-6**. As +α+β hTERT mRNA is quantitated against the PBGD gene with the LightCycler system, and its percentage (of total transcripts) is determined by splice-variant RT-PCR, a quantitative estimate of total hTERT transcripts (*i.e.* all spliced isoforms) can be made (as illustrated by histogram height in **Figure 6-6**). This was comparable for both CML and non-CML cells. As has been previously described, the +α-β variant (β-deleted) was expressed to the greatest degree by far, although all samples also expressed the functional +α+β transcript. The percentage of all transcripts which were full-length (+α+β) was significantly greater for the CML samples ($p=0.001$), at the expense of the β-deleted (+α-β), which was significantly reduced ($p=0.004$). Interestingly, the percentage of +α+β hTERT (of all transcripts) as determined by splice variant RT-PCR displayed a highly significant correlation on a sample-to-sample basis with the absolute levels (as measured by Q-RT-PCR) for both CML and non-CML samples (**Figure 5**). This suggests that subtle shifts in splicing patterns determine overall +α+β hTERT expression in the $CD34^+$ population, rather than the simple on/off +α+β expression demonstrated in some previous studies.

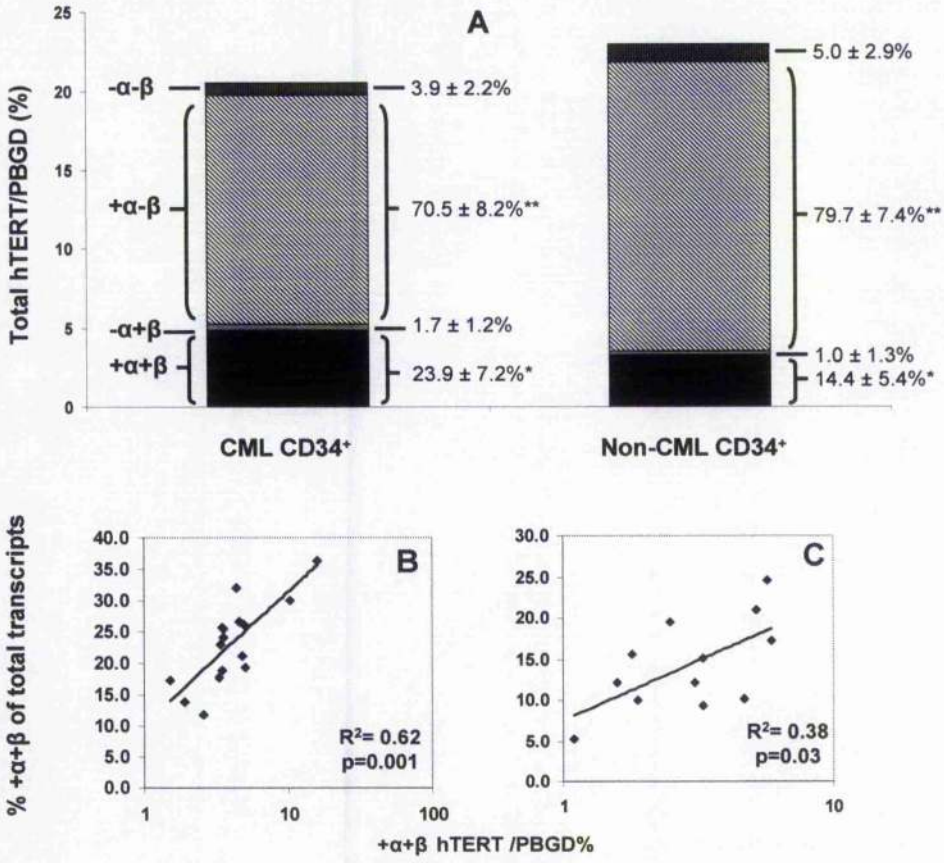


Figure 6-6 Total hTERT expression in CML and non-CML CD34$^+$ cells

By combining data from +α+β hTERT Q-RT-PCR and splice variant RT-PCR (see text), a complete picture of steady state hTERT expression could be constructed (**A**). Total transcript levels (as indicated by histogram height) were similar for CML and non-CML CD34$^+$ cells, however full length +α+β transcripts constituted a significantly grater proportion of these (as indicated by numbers to the right of the bars) in the CML samples (p=0.001). When results generated by the two PCR techniques were compared, a significant correlation was observed between +α+β hTERT/PBGD and the percentage of +α+β hTERT mRNA as determined by splice variant RT-PCR (**B** & **C**).

6.1.5 5-fold lower levels of hTR expression are observed in CP BCR-ABL$^+$ CD34$^+$ cells

The other major component of telomerase, hTR, is also tightly regulated in the haemopoietic system (Weng, *et al* 1997), and many studies have shown it to be upregulated in tumours (Soder, *et al* 1998, Soder, *et al* 1997a). We performed Q-RT-PCR for hTR on CML *vs.* non-CML CD34$^+$ selected cells (**Figure 6-7**).

Interestingly, the mean hTR level in CML $CD34^+$ cells was some five-fold lower than that for non-CML. Together, hTERT and hTR are the minimal requirements for reconstituting TA activity *in-vitro*; it may therefore, be of functional relevance if the ratio between these molecules is altered significantly. The use of the same housekeeping gene for quantitation as that for hTERT (*i.e.* PBGD, with the same primers, probes and PCR conditions), allowed direct comparison of the relative (mean) hTERT/hTR expression ratio. This was approximately $1:10^2$ for hTERT: hTR in *BCR-ABL*$^+$ $CD34^+$ cells (1:179), and $1:10^3$ for non-CML cells (1:991, **Figure 6-13**). A $1:10^4$ hTERT:hTR ratio has previously been described for tumour cell lines (Yi, *et al* 2001). No correlation (either positive or negative) was observed between hTR expression and TRAP activity or percentage of cells in each of the cell cycle stages for CML, non-CML or combined samples

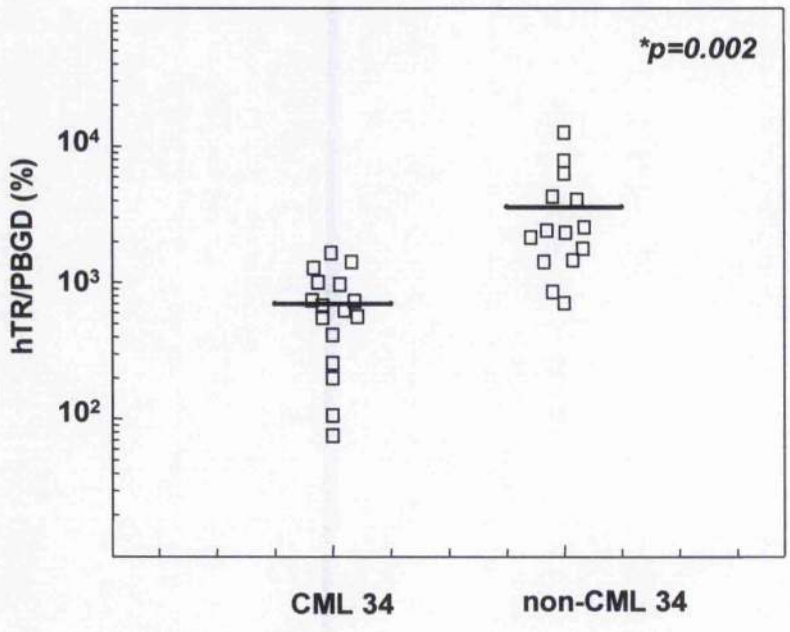


Figure 6-7 Reduced hTR expression in CML $CD34^+$ cells

Q-RT-PCR was performed for hTR, using identical PCR conditions (and the same housekeeping gene) as for hTERT. A significant (p=0.002) reduction in hTR expression was observed for the CML samples. Note that the y-axis is on a logarithmic scale.

6.1.6 Dysregulated expression of telomerase components following cell-cycle activation of BCR-ABL$^+$ CD34$^+$ cells

To assess whether expression of hTERT and hTR was altered under conditions of increased replicative stress, we cultured CD34$^+$ cells in a defined medium with a 5-growth factor cocktail. Cells were harvested at day 3 for two reasons: firstly a previous study (looking at TA by TRAP) demonstrated increased TA in CD34$^+$ cells beginning at day 3 (Engelhardt, *et al* 2000), and secondly pilot studies in our laboratory with this cocktail showed rapid loss of CD34-positivity after this time point concomitant with differentiation. In general the CD34$^+$ percentage at time of harvest was adequate for direct analysis (1 non-CML sample had a CD34$^+$ count of 52% however and required a further MACS purification step to increase this to >85%). The median CD34$^+$ cell purity at analysis at day 3 was 88.9% (range 84-90.7%) and 74.3% (69-87.2%) for CML and non-CML specimens respectively. Cell-cycle profiles showed a similar degree of cell-cycle activation at day 3 of culture (**Table 6.2**), although there was considerably greater expansion of CML CD34$^+$ cells as might be expected. Conversely non-CML cells, again unsurprisingly, had not expanded by day 3, and indeed had actually decreased in number. This was almost certainly due to differentiation and early death of non-viable cells. Despite these differences CML CD34$^+$ cells showed a mean 30% reduction in hTERT levels while the non-CML samples increased by 117% ($p=0.02$). Interestingly, at day 3, the mean percentage of unspliced +α+β hTERT in the CML samples fell by a similar degree (27.7%) in the CML samples (from 27.2±4.9% to 19.6±2.5%, mean±SD) but showed little change in the non-CML cells (from 19.5±1.6% to 18.2±0.8%, mean±SD). The latter observation indicates that the splicing pattern is not simply PCR artefact related to absolute +α+β hTERT levels (which increased in the non-CML samples on culture), and is further evidence of a regulatory role for splicing. The increase inCD34$^+$ cells, although it

cannot be proven with these data, most likely represented increased hTERT transcription.

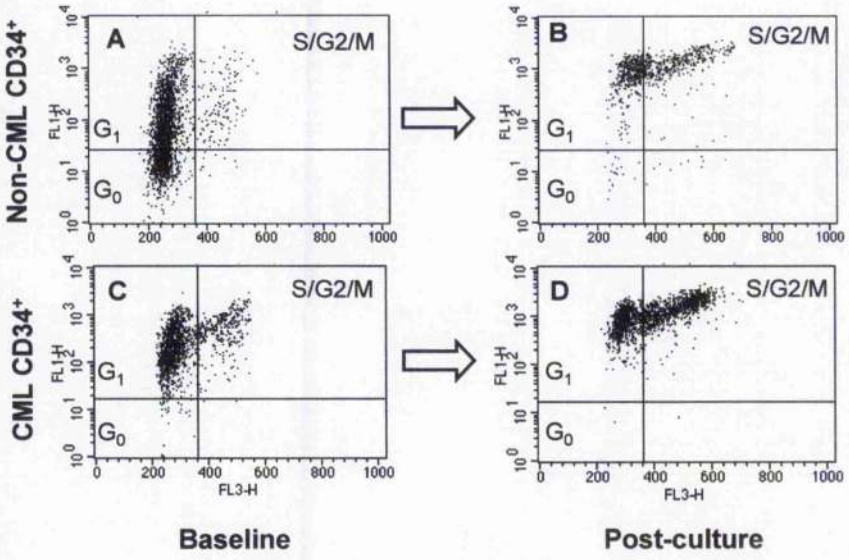


Figure 6-8 Increased cycling of CD34+ cells subsequent to short term cell culture

At least 2 x 10^6 $CD34^+$ cells were cultured in defined serum-free medium for three days. To confirm comparable cell cycle activation profiles between CML and non-CML samples cell cycle analysis was performed at day 0 and day 3. Almost all G_0 cell had entered the cell cycle by day 3.

	$CD34^+$ day 3 cell culture results			Cell Cycle Analysis		
	Fold-expansion (total cells)	Fold-expansion ($CD34^+$ cells)	$\%CD34^+$ of total (mean±SD)	$\%G_0$ (mean±SD)	$\%G_1$ (mean±SD)	$\%G_2$/S/M (mean±SD)
CML (n=4)	2.6±0.7*	2.8±1.0*	87.8±2.9*	0.4±0.2*	50.9±6.7	46.5±6.9
Non-CML (n=4)	0.7±0.2	0.6±0.2	68.3±9.5	1.1±0.4	36.7±12.0	52.0±5.7

Table 6-2 $CD34^+$ cell expansion and cell cycle status at day 3 of culture

	hTERT expression			hTR expression		
	Day 0 hTERT/PBGD% (mean±SD)	Day 3 hTERT/PBGD% (mean±SD)	% Change (mean±SD)	Day 0 hTR/PBGD% (mean±SD)	Day 3 hTR/PBGD% (mean±SD)	% Change (mean±SD)
CML (n=4)	5.8±3.6	2.5±1.6	-30±44.6	870±320	386.5±89.2	-48.1±30.5
Non-CML (n=4)	4.6±5.1	7.9±3.6	+117.1±76	2192±1539.3	1689.0±1690.3	-36.5±30.4

Table 6-3 Expression of telomerase components at baseline and day 3 of culture

6.1.7 Patterns of telomerase component expression with progression to BP

Chapter 5 detailed the changes in expression of telomerase components in PBL during CML progression. Interpretation of these data were however limited by the degree to which the PBL reflected changes at the HSC/progenitor CML cell level. To address this issue we compared expression of hTERT and hTR in $CD34^+$ selected *BCR-ABL*$^+$ CP cells, BP PBL (with a median of 55% blasts) and a limited number of BP $CD34^+$ selected samples (n=3). Myeloid BP samples were seen to have significantly lower $+\alpha+\beta$ hTERT levels (by Q-RT-PCR) than CP $CD34^+$ cells ($p=0.01$), while the opposite was true for lymphoid BP ($p=0.02$, **Figure 6-9**).

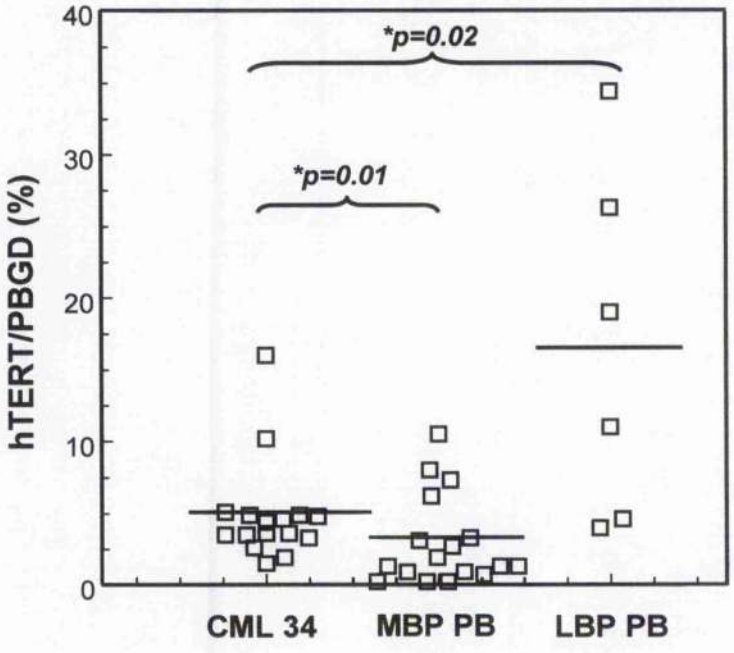


Figure 6-9 Comparison of CP CML CD34 cells with BP PBL of myeloid and lymphoid lineages

These data confirm the impression from Chapter 5 of significant lineage dependent differences in hTERT expression, in this case emphasised by comparison with CP $CD34^+$ cells. MBP, myeloid BP; LBP, lymphoid BP.

These differences were also apparent on comparing splice variant distribution and estimated quantities of total hTERT transcripts (*i.e.* all differentially spliced

species) as previously outlined in Figure 6-6 (**Figure 6-10**). Only 4 LBP samples were available for splice variant PCR, however the greater proportion of full-length (+α+β) hTERT observed was in keeping with the LightCycler data from Figure 6-9. These data suggest a greater than 2-fold increase of all hTERT transcripts in LBP samples as compared to CP CD34$^+$ cells, with a 2-fold reduction seen in MBP samples.

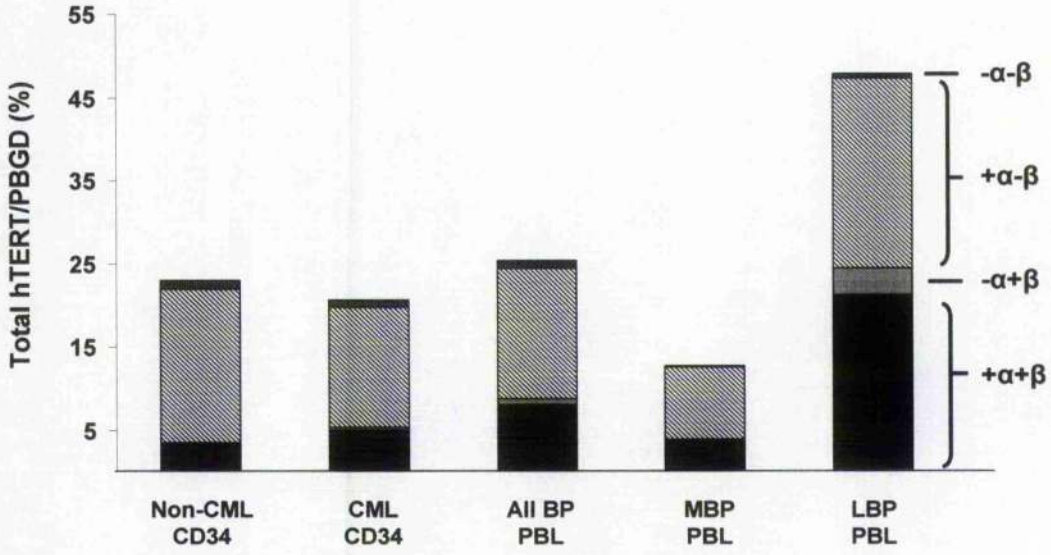


Figure 6-10 Total hTERT expression in CML and non-CML CD34$^+$ cells in comparison to BP samples

By combining data from +α+β hTERT Q-RT-PCR and splice variant RT-PCR, a complete picture of steady state hTERT expression could be estimated for samples with complete data available. Total transcript levels (as indicated by histogram height) were reduced in MBP samples (n=12) as compared to CP CD34$^+$ cells, although they contained a similar mean proportion of +α+β transcripts (27% vs 24% respectively, as indicated by the height of the solid black bar). On the other hand (although numbers were small) LBP samples displayed a greater full-length (+α+β) transcript proportion (n=4, mean 42%).

Limited availability of primary BP material of sufficient quality and quantity unfortunately precluded an extensive analysis of CD34$^+$ selected cells from all stages. Given the presence of mature (hTERT mRNA^{lo}) cells in the BP samples, it is possible that these could 'dilute-out' the hTERT positive CD34$^+$ cells in MBP. In answer to this possibility we analysed CD34$^+$ -selected 3 MBP samples on which

material was available and in which hTERT expression was low in whole PBL (**Figure 6.12**). Although no statistically-supported conclusions can be drawn from these data TRAP activity and +α+β hTERT expression was indeed confirmed to be low in these specimens. The PB and $CD34^+$ selected data from all 3 MBP samples is given in **Table 6-4.**

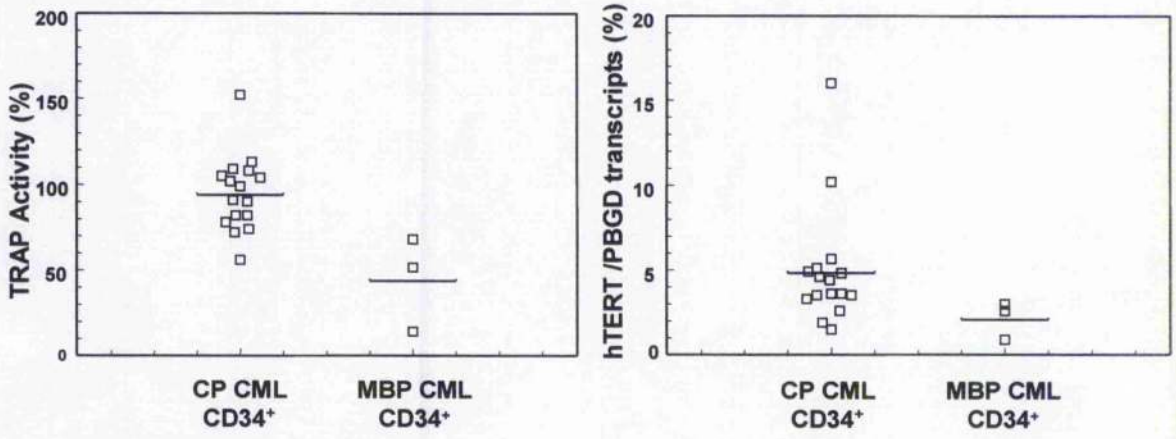


Figure 6-11 Comparison of TRAP activity and full-length (+α+β) hTERT expression in CD34+ selected CP and MBP samples

CD34 selected MBP samples (89, 91.6 and 93.8% pure respectively) exhibited low TRAP activity and full-length (+α+β) hTERT expression in comparison to CP samples. Statistical analysis was not performed due to the small sample size.

	CD34$^+$ +α+β hTERT/PBGD% (+α+β % of all transcripts)	PBL +α+β hTERT/PBGD% (+α+β % of all transcripts)
MBP 1	0.9 (17.3)	-
MBP 2	2.6 (17.4)	0.9 (12.1)
MBP 3	3.0 (16.5)	1.1 (0)

Table 6-4 +α+β hTERT expression data from CD34$^+$ selected MBP samples with their respective unselected PBL values.

It can be seen that all MBP CD34$^+$ samples have levels of hTERT expression below the median for CP CD34$^+$ cells (3.6%). The hTERT expression is clearly enriched upon CD34$^+$ selection, as compared to the respective PBL values. This demonstrates that cell mix is indeed a factor in determining overall hTERT levels in BP, with the more mature cells that are present in PBL BP samples diluting the relevant (*i.e.* blast) population.

Thus low-level +α+β hTERT expression in MBP samples would appear to be a genuine finding, reproducible at the CD34$^+$ cell level.

If hTR is indeed limiting for telomere maintenance in CML CD34$^+$ cells during CP disease, it may be predicted that progression to the aggressive acute leukaemia-like BP stage would be accompanied by an increase in hTR to permit telomere maintenance. In keeping with this, the mean hTR level from 9 BP PBL samples was 1645% (±1900%, mean±SD), compared to 698% for CP CML CD34$^+$ cells (p=0.07, **Figure 6-12**). The data concerning hTR: hTERT ratios referred to in Section **6.1.5** are illustrated in **Figure 6-13** plus the corresponding data from 7 MBP PBL samples on which complete data was available. Non-CML CD34$^+$ samples had a significantly greater ratio than CML CP CD34$^+$ cells (p=0.0001). BP samples had a significantly greater hTR: hTERT ratio than CP cells (p=0.03),

however there was no difference as compared to the non-CML population (p=0.84).

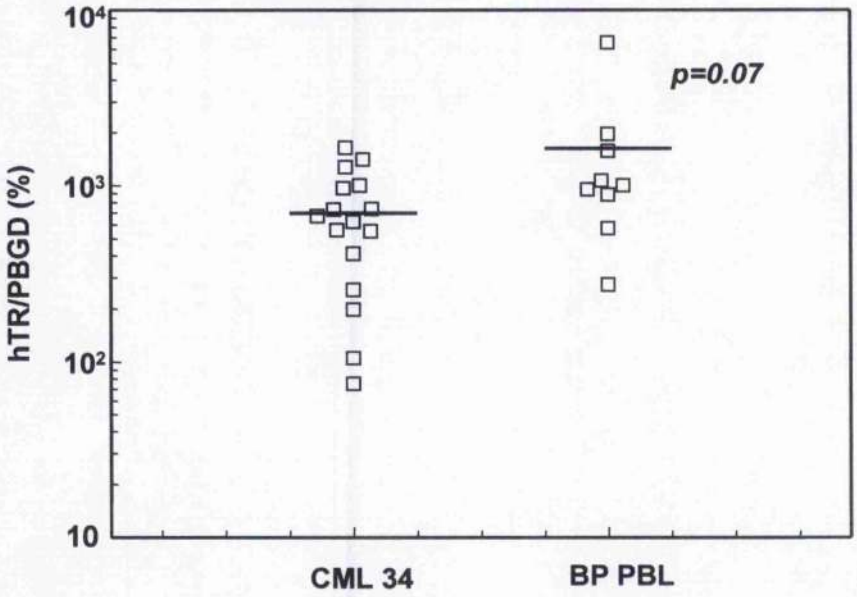


Figure 6-12 Trend to increased hTR expression with progressive (BP) disease

Material from BP samples was unfortunately limited. However 9 PB samples (7 MBP and 2 LBP) from BP patients exhibited a trend towards increased hTR levels as compared to CP $CD34^+$ cells. The PBL specimens, by their nature, have many mature (*i.e* hTR low) cells and this may actually be an underestimate of levels in the blast ($CD34^+$) population. Numbers did not permit separate analysis of LBP and MBP samples.

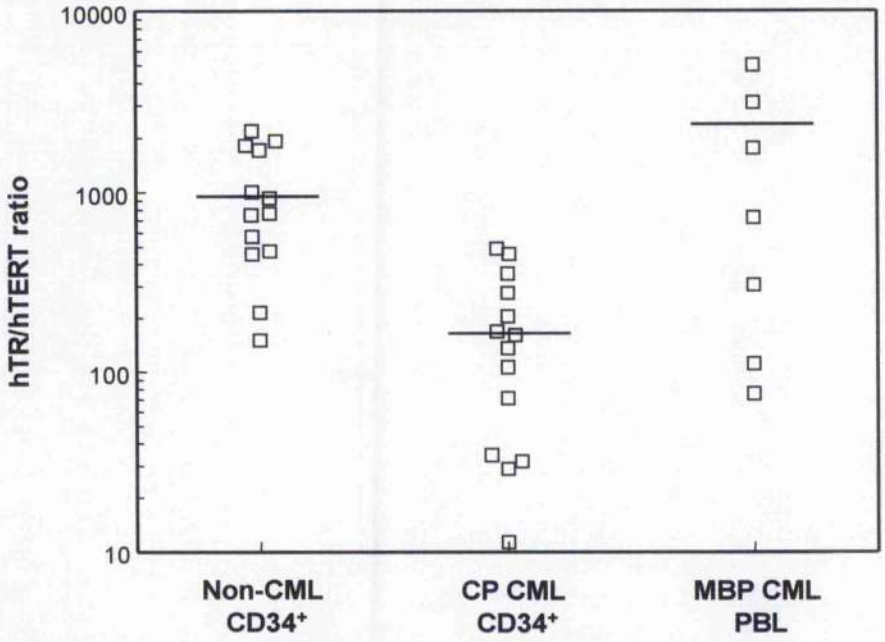


Figure 6-13 Altered hTR;hTERT ratios in CP and BP CML

Comparison of $CD34^+$ selected CML and non-CML samples in comparison to MBP PBL expression.

Thus, on progression to BP CML, there is a significant (partly) lineage-dependent alteration in expression of the major telomerase components. What is not apparent from these data is how this is related to telomere length; indeed the low-level TRAP activity and hTERT expression in the myeloid BP specimens raises the possibility of alternative mechanisms of telomere maintenance being involved. We therefore analysed the BP flow-FISH data with respect to evidence for ALT.

6.1.8 No evidence for ALT as a mechanism of telomere maintenance in BP CML

It is well established that the ALT mechanism of telomere maintenance is associated with an enormously wide range of telomere length, resulting in

extremely long TRF fragments on Southern blotting (Bryan, *et al* 1995). Flow-FISH provides similar telomere length distribution data, with the fluorescence profile in FL-1 indicative of the range of telomere length (as determined by the CV). We reappraised all BP flow-FISH profiles (17 MBP and 4 LBP) for any suggestion of an ALT-like (i.e. excessively wide) distribution; however none was observed (**Figure 6-14**).

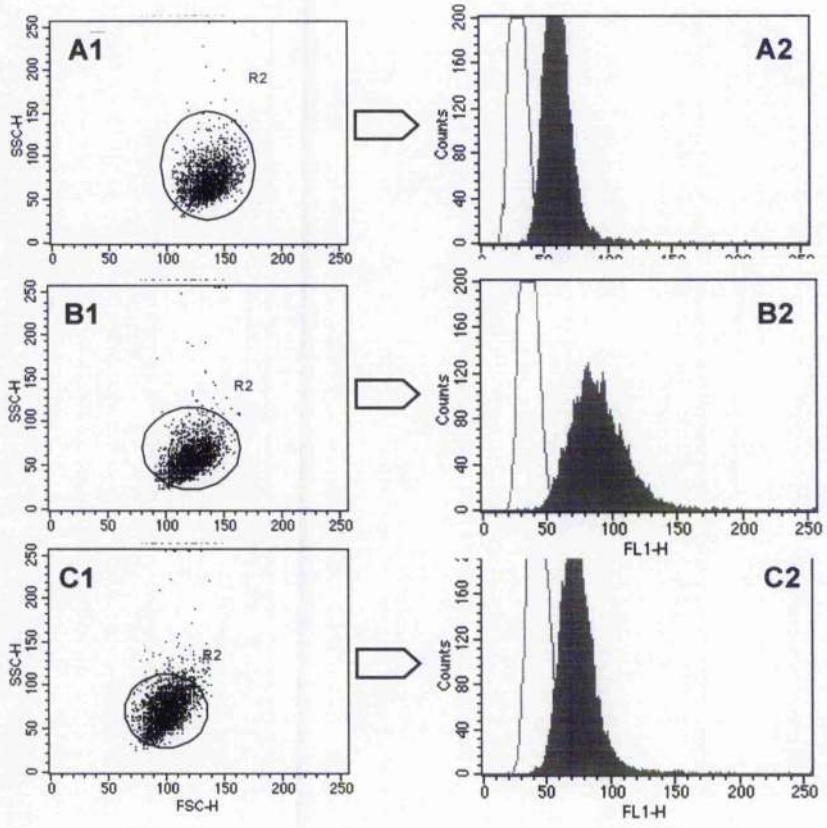


Figure 6-14 No evidence for an ALT phenotype in myeloid BP samples

Flow-FISH profiles of 3 representative myeloid BP samples, A B and C. After gating on the 2N population (as described in Chapter 3) FSC/SSC analysis (Panel A1-C1) revealed a moderately homogenous population of cells. These consisted of a mix of (largely) blasts, myeloid lineages in various sates of maturation and occasional lymphocytes and monocytes (based upon standard morphological indices obtained during routine clinical assessment). Despite this the CVs of telomere probe fluorescence (solid grey histogram, unstained open histogram panels A2-C2) were comparable to those of normal cells (CV values for stained population 16.9, 22.3 and 18.9 respectively). Near-normal telomere-length distribution profiles (see **Figure 3-4**) argue against a significant role for ALT in myeloid BP telomere maintenance.

The Interaction between telomere shortening and telomerase activity is thought to be a dynamic one: progressive telomere sequence loss may result in ever greater selection pressure for telomerase expressing cells to predominate. This mechanism would predict for late upregulation of telomerase activity. Alternatively, it may be an early event in some cancers. At what stage this happens in tumour evolution is not clear, therefore we correlated BP telomere length with expression of hTERT and hTR.

6.1.9 hTERT expression is associated with critically short telomeres in MBP

In an attempt to clarify whether expression of telomere components are related to telomere length, we correlated levels of the major telomerase components with telomere length as measured by flow-FISH. 14 MBP samples had complete data for analysis. Conversely only 4 LBP samples were complete (all incidentally having high hTERT expression and telomere lengths of 4.0, 5.2, 9.2 and 14.4 kMESF), which prevented a similar analysis for this lineage. The MBP samples showed a striking (and highly statistically significant) inverse correlation of full-length (+α+β) hTERT mRNA expression with telomere length. This is consistent with a model of late hTERT expression: *i.e.* at or near critical telomere lengths (approx 5 kMESF, or 2.5 kb, according to this model).

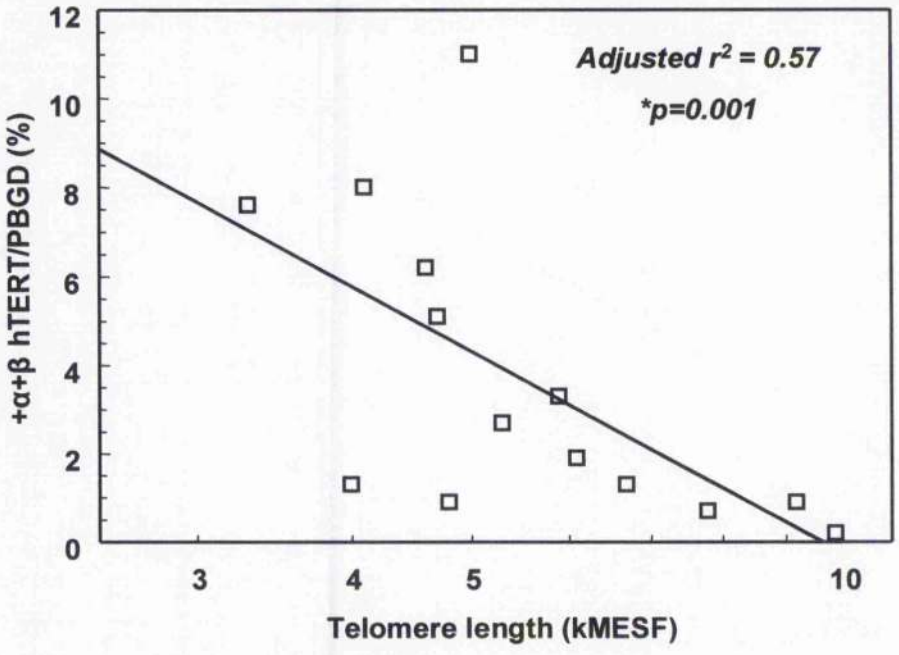


Figure 6-15 Increased +α+β hTERT expression is associated with short telomere length in MBP samples

A significant inverse correlation was seen between telomere length and +α+β hTERT

Only 7 MBP samples had complete hTR and flow-FISH data available, so correlation was not performed (although there was of course a trend towards increased expression of hTR in myeloid BP **Figure 6-13**).

6.1.10 Discussion

In summary we have, for the first time, completely characterised the expression of hTERT (and its splice-variants), hTR and TRAP activity in a purified leukaemic progenitor / stem-cell population. By incorporating non-malignant $CD34^+$ cells as a normal control, we have established the threshold expression of telomerase components in equivalent polyclonal cell types with which to compare leukaemic cells.

Our data explain, at least in the context of CML, the paradox of increased TRAP activity in association with greatly reduced telomere length: an increased proportion of cycling $CD34^+$ cells in CML results in greater detectable TRAP activity. Our demonstration that peripheral blood CML $CD34^+$ cells display increased cycling activity is not particularly surprising in itself, and is in agreement with a previous study (Holyoake, *et al* 1999a).

Many other investigators have demonstrated a clear pattern of TRAP activity in proliferating tissues such as oral mucosa (Kannan, *et al* 1997) and endometrium (Kyo, *et al* 1997). Our detection of low TRAP activity within G_0-rich samples is in agreement with previous studies demonstrating a clear correlation with TRAP and cell cycle activity in tumours (Belair, *et al* 1997) and with downregulation of activity on entry into a quiescent state (Holt, *et al* 1997). In the haemopoietic system a similar relationship between proliferation and TRAP status exists: activity is repressed in $CD34^+CD38^-$ (quiescent) cells, with upregulation upon proliferation and expansion in the $CD34^+CD38^+$ compartment prior to down regulation upon differentiation (Engelhardt, *et al* 1997). Thus it seems that in normal tissues, primary solid tumours and leukaemias the overriding determinant of TRAP status is the percentage of cells in the cycling compartment, as previously hypothesised (Ohyashiki, *et al* 2002). One way of avoiding this, while allowing detection of genuine cell-to-cell and sample-to-sample differences in telomerase activity, is to compare cell-cycle stage specific (*i.e.* S-phase) selected samples, and these studies are currently underway in our laboratory.

Given the problems in interpreting TRAP data, one alternative is to focus on expression levels of the major telomerase components hTERT and hTR which are the key components that are required for active telomerase. In this regard we showed no significant correlations between cell cycle status and expression levels

of hTERT or hTR, and in addition there was no obvious relationship between these parameters and TRAP activity in quantitative terms. At first sight this may appear somewhat surprising given the oft-quoted role of hTERT as the major determinant of TRAP activity. Many of these studies have however expressed this relationship simply in qualitative terms: *i.e.* presence or absence of full-length (+α+β) hTERT correlates with the presence or absence of TRAP activity (demonstrated in Section **5.1.5**), and in this regard, we demonstrate a 100% correlation. Post-translational telomerase regulatory mechanisms may of-course preclude any attempt to correlate the relationship between hTERT and TRAP in quantitative terms.

Comparable median levels of full-length +α+β hTERT mRNA transcripts (as measured by Q-RT-PCR) were detected in CML $CD34^+$ cells vs. non-CML. By combining two PCR techniques and analysing on a sample-to-sample basis, we demonstrated a close correlation between absolute full-length +α+β transcript levels and the percentage of all hTERT transcripts (by splicing pattern RT-PCR) that these represented. This is not simply a mathematical inevitability; an alternative scenario would be that splicing patterns remained static and transcriptional control solely determined absolute hTERT transcript levels. Thus, on a sample-to-sample basis there would appear to be a regulatory role for splicing in determining functional +α+β hTERT levels: in other words by reducing hTERT mRNA splicing activity a cell can increase the proportion of full-length +α+β transcripts, an increase which is detectable in absolute terms. As far as we are aware this is the first time such a quantitative relationship has been detected; previously, a qualitative (*i.e.* on-off) relationship has been observed in foetal development (Ulaner, *et al* 1998), with a remarkably consistent splicing pattern observed in tumour cell-lines (Yi, *et al* 2001). Cell lines are of course, by nature, a highly selected cell population and patterns of telomerase expression in such

samples cannot be extrapolated to primary material. Certainly the use of a highly pure fraction of primary cells facilitates such analysis.

A primary aim of this Section was to determine if there were any CML-related differences in expression levels of hTERT or hTR. The 5-fold reduction in mean levels of the latter was of particular interest given that it has now been shown to be limiting for telomere homeostasis *in-vivo* (Mitchell, *et al* 1999b). Additionally, several studies have detailed low hTR levels resulting in telomere shortening with no detectable fall in TRAP activity (Hathcock, *et al* 2002, Liu, *et al* 2000b). Such data would support a model in which CML cells expressing sub-normal levels of hTR (but not obviously deficient in TRAP activity) undergo progressive telomere shortening as a result. hTR may only become limiting transiently, as a cell requires maximal telomerase function (*i.e.* during S phase). Thus it may have no detectable effect on baseline TRAP activity, or the latter may be insufficiently sensitive to detect it. Whether the ratio of hTR to hTERT is significant in this regard is entirely speculative, but is an intriguing possibility. Additionally, the mechanism of reduced hTR should be clarified; for example is it a direct effect of the BCR-ABL kinase function, perhaps via transcriptional alteration, is a stabilising factor (*e.g.* dyskerin) altered in its expression or activity, or are epigenetic events (*e.g.* promoter methylation) involved? All these mechanisms can be tested on primary cells. Differential regulation of telomerase components was also apparent on stimulation of CML $CD34^+$ cells *in-vitro*. While (in retrospect) it may have been more meaningful to look at a 24 hr time-point re gene expression, the fact that hTERT was still elevated at 3 days in the non-CML cells (by over 100%) as compared to CML (a 30% fall) despite near identical degrees of cell-cycle activation is at least suggestive of telomerase dysregulation in CML. An alternative explanation may be that differentiation was further advanced in the CML population by that stage, and that hTERT was being downregulated as a consequence. The fall in hTR levels

(by approximately 30%) may have been for similar reasons. The fact that >80% of cells analysed at day 3 were $CD34^+$ however argues against this as a mechanism.

By establishing a 'baseline' of hTERT and hTR expression in CP CML $CD34^+$ cells, we were able to fully interpret levels of these components in the BP samples described in Chapter 5. We confirmed that myeloid BP samples expressed significantly lower levels of hTERT (and probably TRAP activity) than the CP progenitor population, and that lymphoid samples were significantly higher in this regard. Thus lineage determined hTERT expression would appear to be a genuine phenomenon in CML. All lymphoid BP samples were immunophenotyped as B-cell in nature. In this regard it is interesting to note that in Ohyashiki's recent review of TRAP activity in haematological malignancy the B-cell malignancies consistently have the greatest proportion of high TRAP activity tumours (namely acute lymphoblastic leukaemia and non-Hodgkin's lymphoma) (Ohyashiki, *et al* 2002). These cells may have the fewest barriers in place to hTERT upregulation by the nature of their phenotype. The low level hTERT detectable in myeloid BP samples prompted a review of flow-FISH profiles for patterns suggestive of an ALT mechanism; however there was no evidence for this. What was apparent was that myeloid BP samples appeared to upregulate hTERT only in association with very short telomeres.

Thus a picture emerges whereby lymphoid BP emerges as a B-cell lineage telomerase-competent high-hTERT expressing clone (perhaps with longer telomeres) contrasting with myeloid BP, a telomerase incompetent lineage. Further telomere shortening in the latter would appear to select for hTERT expression. Intriguingly we also demonstrated elevated hTR levels (albeit not with statistical significance) in the BP samples as compared to CP. If hTR is indeed

limiting for telomere maintenance during CP CML this may be a prerequisite for BP disease.

7. Discussion and Conclusions

To establish the validity of telomere dynamics and telomerase expression as meaningful prognostic indices or as therapeutic targets in leukaemia, they must be extensively characterised in primary material, both normal and malignant. We have studied almost 200 samples form normal individuals and those with CML, a disorder that lends itself to such study.

We and others have demonstrated that telomere shortening correlates significantly with prognosis and conventional risk-score systems, which supports (but does not prove) the notion that telomere length could be an index of the process that drives disease progression. Demonstrating that telomere shortening actually contributes to the genomic instability of the disorder is a much more difficult proposition however; long-term animal models containing primary CML HSC are extremely difficult to generate, and inevitably of limited life-span. Candidate *BCR-ABL*$^{+}$ HSC rapidly differentiate *in-vitro*, and are impossible to propagate in significant numbers for any duration beyond a few days in culture. Furthermore the established CML cell-lines are all from BP disease and display extremely high telomerase activity (with additional mutations) and are not representative of the CP disorder. The recent rapid progress in identifying stem-cell genes involved in self-renewal, such as bmi-1 (Park, *et al* 2003), may however make it possible to manipulate leukaemic HSC for extended periods in culture or in murine models, thereby facilitating study of telomere dynamics and disease evolution.

One argument against critical telomere erosion being a major factor in the clonal evolution of CML is the overwhelming predominance of non-random karyotypic abnormalities seen during evolution of the disease. Catastrophic telomere loss usually results in end-to-end fusion of chromosomes and ring forms, abnormalities

which should be readily visible within a disorder that is probably the best characterised (in a karyotypic sense) human malignancy. It may of course be the case that different cell types vary in their response to telomere shortening or loss (Gonzalez-Suarez, *et al* 2000).

Additionally, we and other investigators have also shown that TRAP and hTERT expression are only elevated in a proportion of BP cases. While this may be partly dependent upon cell-lineage (*i.e.* myeloid *vs.* lymphoid) there are clearly some cases of advanced disease with no demonstrable upregulation of telomerase activity or its components (in fact we observed low levels of hTERT in myeloid BP). Having determined that ALT is an unlikely mechanism of telomere maintenance in these cases, either such levels are adequate to maintain telomere length or there has been no selection pressure exerted to express high telomerase activity at this stage of the disease. The former argument is possible; paradoxically BP disease would appear to have significantly slower cell cycle kinetics than CP MNCs (Handa, *et al* 1997). It may be the case that maturation block, perhaps in conjunction with upregulated anti-apoptotic mechanisms, is adequate for large numbers of blast cells to accumulate at a rapid rate. There may therefore be no real requirement for high telomerase activity to facilitate telomere maintenance in what is a relatively low-turnover population (and as we have shown telomerase activity correlates with cell-cycle activity of the sample). In MBP, increased hTERT strongly correlated with very short telomere lengths; thus some cases of CP CML have progressed to BP (via clonal evolution) without the need for any obvious increase in their telomere maintenance. Only at extreme degrees of telomere shortening (an average of approximately 2.0 - 2.5kb, **Figure 6-15**) would hTERT expression appear to be increased. It is therefore possible that hTERT measurement could be of prognostic value in this scenario; however the prognosis of BP CML is universally poor and it would be of more value to test such a

hypothesis in a similar condition such as AML where prognostic indices would be of genuine value for patient management. Several small studies have looked at TRAP activity in AML, with most demonstrating high values to be a poor prognostic indicator (Li, *et al* 2000b, Ohyashiki, *et al* 1997a) (although the most recent suggested just the opposite (Verstovsek, *et al* 2003b) in adult disease). To further clarify this we are currently quantifying hTERT expression in several hundred samples from the MRC AML trials sample bank.

As compared to solid tumours, leukaemias do not require the development of a blood supply, the capacity for anchorage independent growth, the ability to invade or metastasise. Indeed Gilliland's 'two-hit' hypothesis, in relation to AML, suggests a minimum number of genetic events are involved in its pathogenesis. In this model acute leukaemias arise from the consequence of a collaboration between one class of mutations or gene rearrangements that confer a proliferative and/or survival advantage to haematopoietic progenitors, and a second class that primarily act to impair differentiation and subsequent apoptosis of cells (Kelly and Gilliland 2002). Solid tumours potentially require many more cell divisions to propagate and stabilise the many mutations needed for the full malignant phenotype, thereby accruing greater telomere attrition and random karyotypic abnormalities in the process.

As far as CML is concerned, it is likely that the rapid rate of telomere loss observed is due to several mechanisms. Telomerase dysfunction (via reduced hTR expression), ROS mediated damage, reduced DNA repair (via DNA-PKc down regulation) and increased HSC turnover potentially contribute to the process of telomere attrition, a process that may act as a surrogate marker of genomic instability as well as contribute to it (**Figure 7-1**). A very recent paper defined an intriguing link between localisation of hTERT and cellular oxidative stress; in a cell-

line model both transfected and endogenous hTERT were seen to be excluded from the nucleus subsequent to ROS generation and /or exogenous oxidative stress (Haendeler, *et al* 2003). This was corroborated functionally by a marked reduction in nuclear TRAP activity with a proportional rise in that detectable in the cytosol. Furthermore, *overall* cellular TRAP activity remained unchanged despite this. It is conceivable that such a mechanism operates in *BCR-ABL*$^{+}$ cells where ROS generation is significantly increased. Loss of hTERT protein from the nucleus may indirectly reduce hTR levels, due to reduced binding / stabilisation of this critical component, thereby compromising telomere homeostasis. Thus intracellular compartmentalisation of telomerase may affect telomere maintenance in the face of unchanged whole-cell telomerase activity. Sub-cellular localisation and function of telomerase require to be defined in future studies.

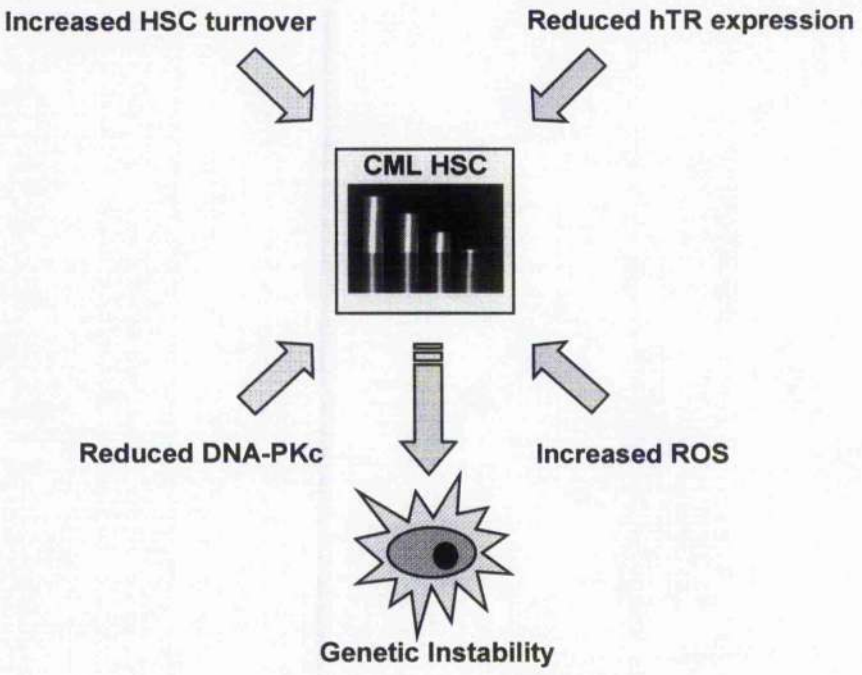


Figure 7-1 Potential effectors of telomere shortening during progression of CML

The fact that telomerase upregulation is not consistently observed in BP CML does not mean that this disorder is unsuitable for treatment with telomerase inhibitors. The consistent presence of short telomeres (at least in myeloid BP) would allow leukaemia selectivity to such agent to be exploited. At least in theory, it may not matter whether the clone expresses a lot of telomerase or not. Indeed less activity may facilitate a greater degree of inhibition. Promoter driven therapies however (as developed by W. N. Keith (Plumb, *et al* 2001)) may be less applicable. In contrast, treatment of CP disease may raise some concerns. A recent paper on arsenic induced ROS and telomere damage encapsulated potential problems with this strategy; arsenic, chronically administered in low-moderate doses (from environmental contamination) is likely to be tumourigenic via this mechanism (Liu, *et al* 2003). However, give in pharmacological doses it has a clear anti-tumour effect, apparently via a similar route. These findings complement the tumour suppressor / tumour facilitator paradox of telomere biology, and suggest that caution must be exercised in treating CP CML with telomerase inhibitors as clonal evolution may be precipitated. Preferential selection of an ALT dependent clone could be imagined as the worst case scenario in this case.

There is now a weight of evidence to suggest that telomere length is of prognostic significance in CML. Since May 2000, when this project was commenced, treatment options for CML have however changed radically. The introduction of the orally administered selective tyrosine-kinase inhibitor imatinib mesylate has revolutionised not only the treatment of CML, but the approach to cancer therapy in general. As a selective inhibitor of the BCR-ABL tyrosine kinase, imatinib is able to block downstream phosphorylation events and ultimately kill the cell which would seem to be dependent on kinase activity for its survival (Druker, *et al* 1996). This has been translated into spectacular clinical success, with a high incidence of

complete cytogenetic remissions (76% at 18 months (O'Brien, *et al* 2003)), and levels of disease only detectable by sensitive nested or Q-RT-PCR (Lin, F W, Drummond MW et al, Blood, *in press*) in CP patients treated with imatinib monotherapy. It is too early as yet to detect a survival advantage; however the likelihood of disease progression with imatinib (as compared to IFN, the previous gold-standard non-transplant therapy) is significantly less (O'Brien, *et al* 2003). It now appears likely that treatment-specific prognostic indices (e.g. whether or not a complete cytogenetic response will be obtained) will assume more importance than leukaemia specific indices (generally based on diagnostic findings). The Hasford score would appear not to be predictive of response to imatinib as yet, although these studies will take many years to complete. It is however clear that imatinib is much less efficacious in AP and in particular BP disease (Kantarjian, *et al* 2002), thus identifying how 'far down the line' a patient is at diagnosis may predict their response to the drug. In this context telomere length is worthy of assessment, and we would advocate prospective analysis of telomere length within a randomised clinical trial involving imatinib therapy. By identifying those patients unlikely to respond to the drug and progress quickly, alloSCT could be planned at the outset.

The complexity of the telomerase holoenzyme will almost certainly frustrate attempts to achieve a rapid understanding of its biology and regulatory mechanisms. Essentially two 'extremes' of investigative techniques have been used by our own, and most other, groups. Firstly the TRAP assay, which gives a crude whole cell lysate based semi-quantitative measurement of telomerase activity, and secondly the highly specific quantitation of expression of individual telomerase components. Crucial studies, such as post translational regulation (including localisation studies) need to be performed on relevant primary cell populations. For example next to nothing is known about how (and where) the

enzyme is assembled and delivered to its site of action. These studies are to be performed on human embryonic stem cells and primary HSC in our laboratory in the near future, and will allow comparison with leukaemic cells. Successfully targeting telomerase enzyme activity in this population remains a highly desirable goal.

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9. Appendix 1

Sample Number	Stage	hTERT mRNA (+α+β) (hTERT/PBDG%)	% +α+β mRNA	% -α+β mRNA	% +α-β mRNA	% -α-β mRNA
1	Diag CP	1.5	46.3	3.9	41.9	7.9
2	Diag CP	5.0	15.6	0	81.9	2.5
3	Diag CP	4.8	ND	ND	ND	ND
4	Diag CP	5.4	0	18.1	81.9	0
5	Diag CP	3.5	0	0	100	0
6	Diag CP	0.0	0	0	59.3	40.7
7	Diag CP	2.8	14.8	0	85.2	0
8	Diag CP	0.0	0	0	74.1	25.9
9	Diag CP	1.25	0	0	100	0
10	Diag CP	3.0	25.8	0	67.2	7.0
11	Diag CP	0.0	0	0	83.8	16.2
12	Diag CP	0.1	0	0	100	0
13	Diag CP	2.5	0	0	100	0
14	Diag CP	2.8	ND	ND	ND	ND
15	Diag CP	4.4	ND	ND	ND	ND
16	Diag CP	0.0	8.3	0	91.7	0
17	Diag CP	3.4	0	0	100	0
18	Diag CP	0.5	0	0	100	0
19	Diag CP	ND	36.6	1.7	51.1	10.6
20	estCP[1]	1.5	0	0	100	0
21	estCP	2.7	0	0	100	0
22	estCP	0.2	ND	ND	ND	ND
23	estCP	0	0	0	0	100
24	estCP[2]	ND	0	0	100	0
25	estCP	0.8	13.2	0	86.8	0
26	estCP	0	0	0	100	0
27	estCP	5.2	15.4	0	84.6	0
28	estCP	0	23.4	0	76.6	0
29	AP	3.7	0	0	100	0
30	AP	0.9	26.3	0	53.9	19.7
31	AP	8.1	11.2	0	82.0	6.7
32	AP	5.0	0	0	100	0
33	AP	0.7	24.5	0	75.5	0
34	AP	5.7	11.8	0	88.2	0
35	AP	11.4	7.2	0	92.8	0
36	AP	ND	13.5	0	86.5	0
37	AP	7.6	10.5	0	89.5	0
38	AP	27.5	ND	ND	ND	ND
39	AP	8.1	22.6	0	77.4	0
40	AP	3.2	11.9	0	88.1	0
41	AP	2.1	ND	ND	ND	ND
42	AP[4]	7.6	7.6	0	74.8	8.6
43	BP(M)	2.7	19.7	2.6	67.9	9.8
44	BP(M)	0.7	14.4	0	78.4	7.1
45	BP(M)	5.1	34.3	0	65.7	0
46	BP(M)	7.6	21.9	2.2	72.6	3.3
47	BP(M)	8.0	12.5	1.1	80.6	5.7
48	BP(M)	6.2	45.5	0	54.5	0
49	BP(M)	3.3	ND	ND	ND	ND
50	BP(M)	1.9	11.9	0	79.7	8.4
51	BP(M)	0.9	53.1	8.3	38.7	0
52	BP(M)	0.2	0	0	100	0
53	BP(M)	1.3	ND	ND	ND	ND
54	BP(M)	0.2	36.7	0	43.0	20.3
55	BP(M)	1.3	49.5	0	50.5	0
56	BP(M)	10.5	15.7	1.8	76.5	6.0

Sample Number	Stage	hTERT mRNA (+α+β) (hTERT/PBDG%)	% +α+β mRNA	% -α+β mRNA	% +α-β mRNA	% -α-β mRNA
57	BP(M)	0.9	12.6	1.0	84.8	1.6
58	BP(L)	11	ND	ND	ND	ND
59	BP(L)	4.0	ND	ND	ND	ND
60	BP(L)[1]	34.4	100	0	0	0
61	BP(L)	4.6	22.2	0	77.8	0
62	BP(L)[1]	23.8	42.4	22.0	35.6	0
63	BP(L)[2]	26.3	12.1	4.7	79.1	4.1

hTERT expression profile in CML PBL from all stages of disease

This table summarises the data which is partly described in Chapter 5. ND, not done. DiagCP, diagnostic CP; AP, accelerated phase; BP, blastic phase; M, myeloid BP; L, lymphoid BP.