TELOMERASE DIRECTED GENE THERAPY

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For Anne and Peter Bilsland.

Abstract

Stabilisation of telomere length is considered to be an essential step in cellular immortalisation *in vitro* and in human cancers. The telomerase ribonucleoprotein reverse transcriptase catalyses the addition of new telomeric repeat sequence to the ends of linear eukaryotic chromosomes and counteracts the cell division associated telomeric attrition that leads to cellular senescence. Its expression has been detected in approximately 85% of all human malignancies but is not detectable in the majority of normal somatic tissues and, therefore, telomerase represents an attractive target for the development of novel molecular therapeutics. Although telomerase activity is modulated on a number of levels, a primary level of regulation is the transcription of the telomerase sub-unit genes. In the present study, I describe the development of a transcriptionally directed cytotoxic gene therapy approach targeted against telomerase positive cancer cells. Transfection experiments using fragments of the human telomerase RNA component (hTERC) and the human telomerase reverse transcriptase (hTERT) promoters revealed large differences in promoter activity between mortal cells and cancer cells. The promoter fragments were sub-cloned into plasmids containing the coding sequence of nitroreductase (NTR), a bacterial enzyme that catalyses the chemical reduction of the non-toxic pro-drug CB1954 resulting in the formation of a powerful bi-functional alkylating agent that kills both dividing and non-dividing cells. Stable cell lines harbouring hTERC-NTR and hTERT-NTR expression vectors were sensitised to CB1954 to an extent that was dependent on hTERC and hTERT promoter activity, with cell lines that had high promoter activities showing significant sensitisation, while those with low promoter activities were not significantly sensitised. The hTERC-NTR and hTERT-NTR expression constructs were cloned into adenovirus (Ad) delivery vehicles and the efficiencies of infection and expression of NTR were characterised in infected cell lines. The major RNA species that was expressed in infected cells was a splice variant that encoded a truncated NTR protein, but the function of NTR was not significantly impaired. Infection with the Ad-hTERC-NTR and Ad-hTERT-NTR gene therapy vectors resulted in a sensitisation to CB1954 that was dually dependent on promoter activity and infection efficiency. Two cancer cell lines that had high hTERC and hTERT promoter activities were significantly sensitised to CB1954, while a mortal foetal lung fibroblast cell strain and a normal adult human mammary epithelial strain, in addition to a bladder cancer cell line with low promoter activity, were not sensitised despite efficient infection with adenovirus. Therefore, the data presented herein support the further development of telomerase-nitroreductase expression vectors for anti-cancer gene therapy.

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Abbreviations

2,5-A	5'-phosphosphorylated, 2'-5'-linked oligoadenylate linkage
5-FC	5-Fluorocytosine
5-FU	5-Fluorouracil
Ad	Adenovirus
ALT	Alternative Lengthening of Telomeres
APC	Antigen Presenting Cell
BLAST	Basic Local Alignment Search Tool
b.p.	Base Pair
CAR	Coxsackie and Adenovirus Receptor
CB1954	(5-aziridin-1-yl)-2,4-dinitrobenzamide
COD	Cytosine deaminase
CR	Conserved Region
CTL	Cytotoxic T-Lymphocyte
DC	Dendritic Cells
DNA	Deoxyribonucleic acid
ER	Estrogen Receptor
ERE	Estrogen Response Element
EST	Ever Shorter Telomeres
FADD	Fas- Associated via Death Domain
GCV	Gancyclovir
HEK	Human Embryonic Kidney cells
HMEC	Human Mammary Epithelial Cells
HPV	Human Papilloma Virus
HSTK	Herpes Simplex Thymidine Kinase
HSV	Herpes Simplex Virus
hTEP1	human Telomerase Protein 1
hTERC	human Telomerase RNA Component
hTERT	human Telomerase Reverse Transcriptase
Luc	Luciferase
Max	Myc associated factor x
MHC	Major Histocompatibility Complex
mRNA	messenger ribonucleic acid
MZF2	Myeloid specific Zinc Finger protein 2
NF-y	Nuclear Factor y
NTR	nitroreductase
PCR	Polymerase Chain Reaction
PNA	Peptide Nucleic Acid
pRB	Retinoblastoma protein
PS-ODN	Phosphorothioate modified Oligodeoxynucleotide
Rap1p	Repressor/Activator Protein 1
RAR	Retinoic Acid Receptor
Rif	Rap1p Interacting Factor
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT	Reverse Transcriptase
RXR	Retinoic X Receptor
SEAP	Secreted Alkaline Phosphatase
SP1/SP3	Stimulating protein 1/ Stimulating Protein 3
T-motif	Telomerase specific motif
TRAP	Telomere Repeat Amplification Protocol
TRF	TTAGGG Repeat binding Factor

wt	wild type
WT1	Wilms Tumour suppressor 1

Units

^{o}C	degrees Celsius
bp	base pair
Bq	Bequerel
Ci	Curie
Da	Dalton
g	gram
G	gravity
h	hour
k	kilo
l	litre
m	milli
μ	micro
M	Mega
min	minute
n	nano
nt	nucleotide
p	pico
rcf	relative centrifugal force
rpm	revolutions per minute
s	second
v/v	volume for volume
w/v	weight for volume

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Declaration

I am the sole author of this thesis. All the references have been consulted by myself in the preparation of this manuscript. Unless otherwise acknowledged, all the work presented in this thesis was performed personally.

CHAPTER 1
INTRODUCTION

- Telomere and telomerase structure and function.
- Regulation of telomerase activity.
- Telomerase directed therapeutics.
- Resistance to telomerase therapeutics.
- Aims of this study.

1 INTRODUCTION

1.1 Immortality and cancer

Recent insights into tumour cell molecular biology and the tumour micro-environment have provided a wealth of possibilities for the development of novel mechanism based therapeutics. A major aim of cancer research is the identification of pathways and characteristics which differ between tumour cells and normal cells. Identification of such differences allows for the evaluation and development of mechanism based therapeutics targeted against the very features of cancer cells that are associated with malignancy.

One such tumour-associated property that may be predicted to form the basis for a new generation of tumour targeted therapeutics, is immortality. Most normal adult human somatic cells have a limited replicative lifespan *in vitro* and *in vivo*, a feature exemplified by the studies of Hayflick (Hayflick 1965). The maximum number of divisions that can be achieved is referred to as the Hayflick limit and after this maximum limit, a cell population will enter a state of growth arrest termed senescence. In contrast, cell cultures derived from malignant tumours commonly exhibit no such limit: they are able to divide indefinitely and have escaped from the normal cues to enter senescence. This characteristic is termed immortality. The underlying mechanisms of immortality are undoubtedly highly complex and almost certainly involve the co-ordinated aberration of multiple pathways for normal growth control. However, in recent years, it has become apparent that a central feature of immortalised cells is maintenance of the length and function of telomeres, the repetitive DNA sequences which cap the ends of linear chromosomes.

1.2 Telomere and telomerase structure and function

1.2.1 Telomere structure and function

1.2.1.1 Role of the telomere in cellular ageing

Telomeres are tandem repeated nucleoprotein sequences, comprising the hexa-nucleotide repeating unit $(TTAGGG)_n$ in all vertebrates tested (Moyzis et al. 1988; Meyne et al. 1989), which cap the ends of linear chromosomes and have been implicated in the maintenance of genomic stability by prevention of aberrant fusion events. Conventional

eukaryotic DNA polymerases mediate DNA replication by a semi-conservative mechanism: each strand of a parent molecule is copied such that the daughter chromosome comprises one parent strand and one newly synthesised strand. Synthesis of new strands occurs only in a 5'-3' direction from a 3'-5' template. This means that during DNA replication, only one strand (the leading strand) is completely copied. Synthesis of the complementary (lagging) strand proceeds in a discontinuous manner by extension of short stretches, termed Okazaki fragments, and is initiated from short RNA priming sequences which are subsequently removed allowing ligation of the fragments to occur (Sugino et al. 1972). The removal of the terminal RNA primer from the lagging strand means that the 3' end of the lagging strand is incompletely replicated (Watson 1972; Olovnikov 1973), leading to loss of approximately 50-200bp of telomeric sequence with each cell division. This loss of telomeric DNA leaves a 3' G-rich overhanging single stranded species (Henderson et al. 1989; Wright et al. 1997) (Figure 1.1).

This phenomenon, termed the end replication problem, is proposed to function as a "mitotic clock", counting the number of cell divisions elapsed. By this model, a cycling cell will gradually lose its telomeric DNA until one or more telomeres become critically short, signal as DNA damage and induce senescence. By this model, an increasing number of individual cells within a cycling population will progressively exit from the cell cycle until the onset of widespread growth crisis. Indeed, the observations that mean telomere lengths, analysed by Southern blot detection of terminal restriction fragments (TRF), shorten with increased population doublings in several normal human fibroblast cell strains of varying donor age (Harley et al. 1990) and that telomere length correlates strongly with remaining replicative lifespan in human skin fibroblasts (Allsopp et al. 1992; Allsopp et al. 1995) lend considerable support to the model. It has also been shown that telomere attrition in ageing fibroblasts is intrinsically associated with active cell division, but not with quiescence, both *in vitro* and *in vivo* (Allsopp et al. 1995).

The mechanisms by which telomere attrition activates senescence or apoptosis are not fully understood at this time but the studies of Vaziri et al provide some clues as to the nature of the process. Up-regulation of p53 activity leading to Cyclin Dependent Kinase inhibition by $p21^{WAF1}$ has been implicated as a major signalling event in cellular senescence (reviewed in Bringold et al. 2000; Campisi 2001). In one study, comparison of p53 DNA binding activity and p53 dependent $p21^{WAF1}$ promoter activity in 3 strains of young and aged fibroblasts revealed increased p53 DNA binding and $p21^{WAF1}$ promoter activity in the absence of increased p53 protein levels in ageing cells. Similar results were observed when

investigators examined Ataxia Telangiectasia fibroblast strains, which exhibit accelerated telomere attrition and senescence in culture, when compared to fibroblasts from normal siblings or unrelated age matched donors. Moreover, fibroblasts grown under hyperoxic conditions that accelerated the attrition of telomeres showed similar increases in p53 DNA binding and $p21^{WAF1}$ protein levels when compared with normoxic controls. Finally, the investigators demonstrated an interaction between p53 and Poly (ADP-Ribose) Polymerase (PARP) in ageing fibroblasts, leading to up-regulation of $p21^{WAF1}$ and MDM2 and showed that incubation with specific PARP inhibitors could extend cellular lifespan (Vaziri et al. 1996; Vaziri et al. 1997).

1.2.1.2 Telomere structure and regulation of telomere function

In addition to the tandem repeated nucleotide sequence $(TTAGGG)_n$, a large number of binding factors present at the telomere are implicated in its function and homeostasis. Some of the critical regulators of telomere function are discussed below, while a number of the other human telomere binding proteins are listed in table 1.1. Additionally, figure 1.2 gives a schematic representation of possible interactions of proteins at the telomere. The major functions that have been ascribed to telomeres are: (1) prevention of aberrant recombination events and genetic instability such as end-to-end fusions. (2) distinguishing chromosome ends from DNA damage. (3) telomeres are involved in modification of gene expression by gene silencing. (4) a role has been proposed for telomere function in sister chromatid separation during anaphase. The available evidence suggests that telomere length maintenance, or more specifically, the maintenance of telomere function, is a critical determinant of cellular lifespan and fate.

In cells with active telomerase, telomere seeding experiments reveal that telomere lengths are subject to homeostatic regulation: exogenously introduced telomeres are extended by the telomerase ribonucleoprotein reverse transcriptase, discussed in more detail below, which adds TTAGGG repeats to the single stranded 3' overhang of telomere termini until their length is within a range characteristic of the cell type (Barnett et al. 1993). Despite heterogeneity between telomere lengths even within the same cell (Lansdorp et al. 1996), lengths are kept within tight species specific limits (Kipling et al. 1990), suggesting the presence of a sensitive mechanism for maintenance of telomere length.

Factors implicated in this process include the proteins bound on the double and single stranded regions of the telomeric DNA, the higher order structure formed by recruitment of

other proteins by telomere binding proteins, the physical folding back of the telomere into looped structures, and the action of telomerase. By the mutual interaction of these factors, the telomere is proposed to switch between a "capped" state, in which it is protected from the action of nucleases, from the activation of damage response signals and is inaccessible to telomerase, and an "uncapped" state, in which it is unprotected and is accessible to lengthening by telomerase. A central tenet of this model is that proteins bound along the telomeric tract establish a dynamic equilibrium by which telomere length is homeostatically maintained. This model predicts that telomere shortening during cell division will reduce the number of negative regulatory proteins associated with the telomere and thereby increase the probability that a given telomere will switch to the uncapped state, allowing extension by telomerase; conversely, extension by telomerase will recruit a large number of proteins to the telomere and inhibit further elongation (reviewed in Blackburn 2000; Blackburn 2001).

A number of double stranded sequence specific telomere binding proteins that influence telomere length and function have been identified in humans and yeast (table 1.1). Among these, the protein Rap1p (Repressor/Activator Protein 1), is implicated as a central negative regulator of telomere length in yeast cells: over-expression of DNA binding mutants resulted in increased in telomere length (Conrad et al. 1990) suggesting a mode of action involving interaction with other factors which are sequestered by binding mutants. Consistent with this finding, Rap1p was subsequently demonstrated to recruit the factors Rif1p and Rif2p to the telomere (Hardy et al. 1992; Wotton et al. 1997). Both factors negatively regulate telomere length by an interaction with the carboxyl terminus of Rap1p and expression either of mutants defective in Rap1p interaction, or of carboxyl terminus mutated Rap1p defective in Rif interaction, increased telomere length. Moreover, cells deficient in both Rif1 and Rif2 showed a synergistic increase in telomere lengths relative to either single mutant. Conversely, over-expression of wild type Rif1p and Rif2p can reduce telomere length (Wotton et al. 1997). Based on these results, it has been postulated that telomere length regulation in yeast relies on a protein counting mechanism dependent on the number of Rap1p molecules bound along the telomeric tract. Consistent with this, mutation of Rap1p biding sites increases telomere length (Marcand et al. 1997).

In mammalian cells, the ubiquitous telomeric repeat binding factors TRF1 and TRF2 were identified on the basis of their ability to bind the telomere repeat sequence (Zhong et al. 1992; Chong et al. 1995; Broccoli et al. 1997) and TRF1 has been characterised as a functional homologue of yeast Rap1p, in terms of its ability to negatively regulate telomere

length in an inducible over-expression system (van Steensel et al. 1997). Recently, TRF proteins were classified as orthologues of the s.pombe Taz1 protein that binds double stranded telomere sequence and recruits factors for length maintenance (Li et al. 2000). The human TRF1 gene codes for a protein of approximately 60kDa with a highly acidic N-terminus and a C-terminal Myb-like DNA binding domain. Human and mouse TRF1s show greatest homology in the dimerisation and DNA binding domains (Bianchi et al. 1997; Broccoli et al. 1997). TRF1 binds the telomere as a homo-dimer and bends double stranded DNA (Bianchi et al. 1997), a conformation that may favour the formation of the "t-loop" structure.

When mammalian telomeres are cross-linked and purified, a proportion adopt a lasso-like conformation (the t-loop) in which conformation the single stranded overhang is proposed to be sequestered within the duplex region. The presence of a D-loop at the junction of the "lasso" suggests the involvement of a strand invasion mechanism (Griffith et al. 1999) (Figure 1.3). This might represent a mechanism whereby telomeres can maintain their function despite the presence of a single stranded region. The junction of the t-loop is bound by TRF2 (Griffith et al. 1999; Stansel et al. 2001), a TTAGGG binding factor distantly related to TRF1 that appears to be required for t-loop formation, possibly by a helicase-type action (Stansel et al. 2001). TRF2 shares most homology to TRF1 in the DNA binding domain (56% identity), homo-dimerises but does not form heterodimers with TRF1 (Broccoli et al. 1997) and may facilitate or stabilise the t-loop structure. It is proposed that TRF1 and TRF2 inhibit the action of telomerase *in cis*, possibly by generation of the t-loop.

TRFs may also contribute to telomere maintenance by generation of a higher order protein structure at the telomere. Interestingly, the human orthologue of *Saccharomyces cerevisiae* Rap1p, hRap1, was recently identified (Li et al. 2000). This protein shows homology with the yeast Rap1 proteins in the C-terminus, in addition to the central Myb domain and an N-terminal domain that also has homology with the C-terminal protein interaction domain of BRCA1. In contrast to the action of scRap1p, which is mediated by DNA binding and recruitment of Rifs to the telomere, hRap1 does not directly bind DNA but instead is recruited to the telomere by an interaction between its C-terminus and the TRF homology domain of TRF2. Over-expression of hRap1 resulted in moderately increased telomere length, but the nature of its function at the telomere remains to be clarified (Li et al. 2000). TRF2 over-expression modulates telomere length in a biphasic manner, with an initial cell division dependent decrease in length followed by extension (Smogorzewska et al. 2000).

Removing TRF2 from the telomere by expression of dominant negative analogues results in rapid ATM and p53 dependent cell death (Karlseder et al. 1999) and widespread chromosome end to end fusions (van Steensel et al. 1998). Interestingly, in another recent study (Kim et al. 2001) in which mutant template sequence not recognised by the telomere binding protein TRF1 was synthesised by the expression of mutant telomerase, cells underwent apoptosis. These data underscore the essential roles of telomere binding factors in the maintenance of normal telomere function.

Tankyrase (TANK1), a TRF1 interacting protein identified in two-hybrid screening, contains a PARP domain that ADP-Ribosylates both itself and TRF1 (Smith et al. 1998). Tankyrase associates with TRF1 via an interaction between the acidic N-terminus of TRF1 and a domain of Tankyrase containing numerous ankyrin repeats that are involved in protein-protein interaction. Modification of TRF1 by PARP activity results in inhibition of TRF1 TTAGGG binding activity *in vitro*. Modification of TRF1 by tankyrase appears to remove TRF1 from telomeres *in vivo* and promotes telomere elongation (Smith et al. 2000), although it is unclear whether TRF1 is the only telomeric protein target for modification by tankyrase or whether tankyrase is required for telomere extension by telomerase under TRF1 inhibited conditions.

It is intriguing that a number of telomere associated proteins have been identified as components of DNA damage response pathways. The involvement of PARP activity in damage signals is well characterised (reviewed in Herceg et al. 2001) and, while tankyrase activity at the telomere has been characterised to date only as a modulator of TRF1, a relative of tankyrase, TANK2, has recently been identified (Kaminker et al. 2001). TANK2 also interacts with TRF1, but in contrast to the apparent function of tankyrase, over-expression of TANK2 resulted in rapid cell death, suggesting that tankyrase family proteins may also have a role in integrating damage response signals at the telomere. Additionally, the identification of a protective role at the telomere played by the Ku proteins, (Samper et al. 2000) that are involved in the non-homologous end-joining pathway for chromosome repair, provides another interesting example of the multiple pathways required for normal telomere function.

Maintenance of telomere length and function, then, is a complex and regulated process involving the interaction of multiple components, critical to the prolonged survival of the cell, but in most adult human somatic cells telomere lengths are not maintained, but shorten with each cell division. One problem with the classical model of a critical telomere

length as the cue for cellular senescence is that the simple acquisition of shortened telomeres does not mechanistically explain what drives exit from the cell cycle. Indeed, cells with active telomerase often have short telomeres and, in experimental systems, can continue to divide despite having shorter telomeres than control cells undergoing senescence (Yang et al. 1999; Zhu et al. 1999). Another problem is that the classical model of telomere structure does not explain why functional telomeres are interpreted as natural chromosome ends rather than as double strand breaks. The capping status of a telomere may thus define how a telomere is interpreted by components of the DNA damage response path, and hence the fate of a given cell. This model of telomere length maintenance may help to explain some of these problems.

1.2.2 Telomerase function

1.2.2.1 Identification of telomerase activity

Telomerase activity, characterised as a sequence specific telomere terminal transferase activity, was first identified in *Tetrahymena thermophila* cell extracts (Greider et al. 1985). Later identification of activity in *Euplotes crassus* (Shippen-Lentz et al. 1989), *Oxytricha nova* (Zahler et al. 1988) and human cell extracts (Morin 1989) strongly suggested that a telomere terminal transferase activity was widespread amongst eukaryotes. The results of *in vitro* primer extension assays which demonstrated specificity of the activity for telomeric sequence, but not irrelevant sequences or telomere complementary sequences, suggested that this could provide a mechanism to compensate for the end replication problem in the linear DNA of eukaryotic genomes.

Experiments in *Tetrahymena* revealed that the enzyme responsible was a ribonucleoprotein reverse transcriptase, termed telomerase (Greider et al. 1987). It was subsequently recognised that the RNA subunit of *Tetrahymena* telomerase contains a sequence complementary to the telomeric sequence that was proposed to act as a template for the synthesis of new telomeric repeats (Greider et al. 1989). Support for this model came from the identification of putative template sequences in telomerase RNAs of a variety of organisms including *Euplotes* (Shippen-Lentz et al. 1990), mouse (Blasco et al. 1995) and human (Feng et al. 1995), and the mapping of the boundaries of the *Euplotes* template region by primer extension assays (Shippen-Lentz et al. 1990). In all cases, the proposed template sequence was complementary to the telomere sequence. Telomerase RNA

component (here referred to as TERC) subunits have now been identified in 35 vertebrate species (Chen et al. 2000).

Telomerase, then, is a ribonucleoprotein reverse transcriptase that catalyses the synthesis of telomere repeats from an internal RNA template (a schematic representation of the proposed activity of telomerase is presented in figure 1.4). The catalytic subunit of telomerase was first identified in *Euplotes aediculatus* (p123) (Lingner et al. 1997) and the same study identified the homologue from *Saccharomyces cerevisiae* as the EST2p gene, deletion of which had previously been demonstrated to result in telomere shortening (Lendvay et al. 1996)). Later, the human catalytic subunit was simultaneously cloned by several groups (Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997). Although initially referred to by various acronyms (hTRT, hTCS, hEST2), the telomerase reverse transcriptase is now designated hTERT. The identification of these two core components of the telomerase complex allowed the study of telomerase subunit expression and correlation with telomerase activity in human cancers, discussed in more detail below. Both subunits are required for enzyme activity and, as discussed below, both have been detected at low levels in normal tissues although they are up-regulated in malignancy (Feng et al. 1995; Avilion et al. 1996; Kolquist et al. 1998; Ramakrishnan et al. 1998).

1.2.2.2 Studies on the role of telomerase in immortality

The correlation between cellular lifespan and telomere length gave the first indications that telomere stability may impact upon development of immortal phenotypes and play a role in the progression of cancer in mammalian cells. Additionally, the definition of the yeast EST2 gene (ever shorter telomeres), deletion of which is characterised by telomere stability defects (Lendvay et al. 1996), as the telomerase catalytic subunit (Lingner et al. 1997) gave a direct indication that loss of telomerase activity could lead to telomere shortening and eventually senescence. The acquisition of telomerase activity is specifically associated with stabilisation of telomere length (Counter et al. 1992; Bodnar et al. 1998). Moreover, many cancer cells have short though stabilised telomeres (Engelhardt et al. 1997) and, in the vast majority of cases, express telomerase activity (Kim et al. 1994). This strongly suggests that telomerase activity plays a role in the acquisition of cellular immortality during cancer progression. Indeed, a study of chromosomal stability, telomere length and telomerase activity in pre- and post-immortalised human embryonic kidney cells transformed with viral oncogenes showed progressive telomere shortening and increased frequency of dicentric chromosomes with increased population doubling number

as the cells approached crisis. Immortalised cells that escaped from crisis, however, had stable telomere length, and specifically expressed telomerase activity (Counter et al. 1992). These data, together with the development of the PCR based TRAP assay (Telomere Repeat Amplification Protocol) for the sensitive detection of telomerase activity in human cell extracts led to the implication of telomerase activity as a critical determinant of sustained viability in the vast majority of human tumour cells and immortalised cell lines in tissue culture systems (Kim et al. 1994).

Direct evidence that stabilisation of telomere length by telomerase mediates the progression to an immortal phenotype came from several studies in which telomerase activity was introduced into normal primary cell strains by ectopic expression of hTERT with subsequent extension of proliferative life-span (Bodnar et al. 1998; Yang et al. 1999; Rufer et al. 2001). Conversely, transfection of dominant negative hTERT analogues, or expression of mutant hTERC molecules abrogates telomerase activity in immortal cells and, with continued passage, cells enter into senescence or apoptosis (Hahn et al. 1999; Zhang et al. 1999). Other powerful evidence of the role of telomerase in cellular immortality was presented in the study of Hahn and colleagues (Hahn et al. 1999). The investigators were able to induce tumourigenic conversion in normal human epithelial and fibroblast cells by the introduction of SV40 large T antigen, oncogenic H-ras and hTERT. Importantly, characteristics of full malignant transformation (anchorage independent growth, tumour formation in nude mice, indefinite replicative potential) were achieved only when all 3 components were present, demonstrating that expression of telomerase, although central to cell immortalisation, is not functionally equivalent to malignant transformation. This is in-keeping with the results of other studies in which acquisition of telomerase activity was shown to be insufficient for acquisition of a malignant phenotype (Jiang et al. 1999; Morales et al. 1999). Interestingly, while forced expression of telomerase can extend the lifespan of some cell types, the requirements for immortalisation may vary between cell types (O'Hare et al. 2001). A requirement for the inactivation of the Rb/p16 pathway in addition to expression of telomerase activity has been reported for immortalisation of human epithelial cells (Kiyono et al. 1998).

The model of cellular immortalisation involving telomerase as a step in tumourigenic progression in tissue culture systems is represented in figure 1.5. Telomerase activity is present during embryogenesis, but is repressed in most adult somatic cells (discussed in more detail below). Proliferation of telomerase negative cells leads to a progressive loss of telomeric sequence until one or more telomeres reach a critical length, at which time most

cells will enter senescence (also termed mortality checkpoint 1, or M1). During senescence, the majority of cells cease dividing but occasional, rare, partially transformed cells escape from the M1 checkpoint and can continue to divide, although they continue to lose telomeric DNA. Cells with critically shortened telomeres progressively exit from the cell cycle into a second growth arrested state termed mortality checkpoint 2, or M2. A minority of cells, in which expression of telomerase components is either stimulated or de-repressed, escape from this growth crisis with stabilised telomere length and function. Re-activation of telomerase activity is thus regarded as a critical late stage in cellular immortalisation.

1.2.2.3 Telomerase structure

Telomerase activity can be reconstituted *in vitro*, minimally, from its 2 core subunits, hTERC and hTERT, although other components of the holoenzyme complex play a role in the regulation of activity *in vivo*. Reconstitution of activity has been achieved in several *in vitro* systems (micrococcal nuclease digested cell extracts, rabbit reticulocyte lysate, and telomerase negative cell lines) and these studies have enhanced our understanding of the structural elements, both of hTERT and hTERC, which are involved in functional interaction.

The hTERC gene, mapped to a locus on chromosome 3q26.3 by fluorescence *in situ* hybridisation (Soder et al. 1997), encodes a mature RNA of 451nt in length. The template sequence (CUAACCCUAA) lies 46nt downstream from the transcriptional start site (Feng et al. 1995). The TERC RNA sequences which have been cloned thus far do not display a large amount of primary sequence identity in vertebrates or ciliates, but display a number of putative evolutionarily conserved functional domains, based on the predicted secondary structure derived from comparative phylogenetic analysis of 35 vertebrate TERCs (Chen et al. 2000). Evident in the vertebrate TERCs are 8 short regions of high sequence conservation (termed CR1-CR8), which are predicted to fold into 4 conserved functional domains via 10 conserved sets of distal, co-variable nucleotides putatively capable of base-pairing into helical structures designated P1-P6, P7a & P7b, P8a & P8b (Figure 1.6).

The known functions of the conserved regions and domains, shown in figure 1.6, are as follows: both the pseudoknot (CR2/CR3) and the CR4/CR5 domains are proposed to be essential for enzyme activity and hTERC/hTERT interaction based on mutational analysis using *in vitro* reconstitution systems (Autexier et al. 1996; Beattie et al. 1998; Tesmer et al.

1999; Bachand et al. 2001; Bachand et al. 2001); the box H/ACA domain is involved in protein interactions other than with hTERT and RNA stability (Mitchell et al. 1999; Dragon et al. 2000; Vulliamy et al. 2001). The template sequence (CR1, spanning nt +46 to +55) of hTERC, is predicted by phylogenetic analysis to lie in a single stranded structure accessible by the catalytic subunit and by anti-sense interference (Hamilton et al. 1997; Pitts et al. 1998). The proposed location of the template sequence is conserved in all TERC molecules (Chen et al. 2000).

The hTERT gene, mapped by fluorescence *in situ* hybridisation to a locus on chromosome 5p15.33 (Bryce et al. 2000), comprises 16 exons of sizes varying from 62nt to 1352nt and 15 introns. The coding region spans over 35kbp (Cong et al. 1999) and hTERT is subject to post-transcriptional regulation via alternative splicing (Kilian et al. 1997). The full-length transcript is predicted to be translated to the only functional protein and was simultaneously cloned by several groups (Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997). TERT translates to a protein of 1132 amino acids and expected molecular weight of 127kDa. TERT genes contain motifs characteristic of reverse transcriptases, located on individual exons whose locations vary between humans and yeast (Harrington et al. 1997; Nakamura et al. 1997). Phylogenetic analysis of the catalytic domains of reverse transcriptases places TERT genes in a sub-group of their own among RT molecules (Nakamura et al. 1997).

In addition to the conserved RT motifs 1, 2, A, B', C, D, and E, TERTs contain telomerase specific motifs N-terminal to the catalytic domain (T-motifs). Ciliate TERT molecules have additional TERT specific motifs CP and CP2. Since the action of hTERT is the hTERC-templated addition of the TTAGGG repeat to the telomere terminus, the minimal functional requirement is an interaction with the TERC component and the telomere, although a number of other proteins probably contribute to the complex. Indeed, the chaperone proteins hsp90 and p23 have recently been shown to interact directly with hTERT, mediating complex assembly *in vitro*, although they may remain associated in a stable complex after assembly (Forsythe et al. 2001).

Domains of interaction between hTERC and hTERT have recently been mapped using *in vitro* reconstitution experiments. The region spanning nucleotides 44-204 (subsequently shown to comprise the template and the entire pseudoknot domain) was identified as the minimal functional region necessary for reconstitution of telomerase activity with *in vitro* transcribed hTERC fragments following micrococcal nuclease treatment of telomerase

positive cell extracts (Autexier et al. 1996). In this study, mutational analysis identified a 30 nucleotide stretch between nt170 and nt200 as essential for catalytic activity, although a later study demonstrated that certain mutations in this region could reconstitute weak TRAP activity and that the interaction with hTERT was unaffected by the modifications in immunoprecipitation (Bachand et al. 2001) and band shift experiments (Bachand et al. 2001b). This region, interestingly, corresponds to the helix P3 which contributes to the formation of the pseudoknot domain. It has been reported that nt10-159, also comprising a large part of the pseudoknot domain, are critical and sufficient for telomerase activity (Beattie et al. 1998), but a more recent study did not reconstitute activity from the region encompassing nt1-159, although hTERC truncated to nt159 co-immunoprecipitated with hTERT confirming the importance of this region in TERC/TERT interaction (Bachand et al. 2001).

Recently, it has been proposed that hTERC contains 2 sites of interaction with hTERT (Tesmer et al. 1999; Bachand et al. 2001; Bachand et al. 2001). The region encompassing nt33-325 contains 2 inactive fragments (nt33-147, containing the template and a large portion of the pseudoknot domain, and nt164-325, containing a portion of the pseudoknot domain and the CR4/CR5 domain) that assemble with hTERT to form an activated complex (Tesmer et al. 1999). These data were recently confirmed by Bachand and Autexier, (2001), who mapped the independent hTERT binding sites to nt33-147 and 164-330.

Functional regions of TERT important for the TERC/TERT interaction and for enzyme activity have been mapped by mutational and deletional analysis using rabbit reticulocyte lysate reconstitution systems combined with co-immunoprecipitation analysis of human and yeast TERTs. Progressive truncation of the N- and C-terminals of Tetrahymena TERT resulted in peptides incapable of reconstituting telomerase activity, but defined a minimal RNA binding domain between amino acids 195 and 516 containing the ciliate TERT specific motifs CP1 and CP2 in addition to the T-motif. Additionally, C-terminal truncations of hTERT did not affect RNA binding unless the T-motif was disrupted (Lai et al. 2001). Further evidence to support the role of these motifs in TERT/TERC association comes from the observation that tetrahymena TERT molecules harbouring mutated residues within the CP1 and T-motifs, but not the reverse transcriptase motifs, greatly reduced co-immunoprecipitation of TERC (Bryan et al. 2000).

While the minimal functional requirement for active telomerase in reconstitution systems seems only to be hTERC and hTERT, it is clear that a number of other factors associate with the complex *in vivo* to regulate telomerase activity. Other components of the telomerase complex include hTEP1 (telomerase protein component 1), identified on the basis of its homology with the non-catalytic yeast telomerase sub-unit, p80 (Harrington et al. 1997). It has been postulated that hTEP1 may perform a regulatory role within the telomerase complex, but its function is not known at this time and its expression pattern does not correlate with telomerase activity in cell lines or tissues (Harrington et al. 1997; Wu et al. 1999), although hTEP1 co-purifies with active telomerase *in vitro*, interacts with p53 (Li et al. 1999), and is phosphorylated by PKCα in human breast carcinoma cells (Li et al. 1998).

Several recent reports have begun to examine more closely other components of the telomerase complex. Small nucleolar RNAs (snoRNAs) are a class of molecules involved in pre-rRNA processing and modification. They are divided into 2 groups based on the presence either of the box H/ACA motifs or of the box C/D motifs within the RNA which mediate protein interaction and assembly of small nucleolar ribonucleoproteins (snoRNPs). The telomerase RNA subunit contains boxes H and ACA in its 3' end (Mitchell et al. 1999). *In vitro* assembly of fragments of hTERC revealed binding to the H/ACA specific protein hGAR1 (Dragon et al. 2000). Additionally, dyskerin, a snoRNA interacting protein that is mutated in the X-linked form of dyskeratosis congenita and is thought to mediate the processing of rRNA, associates with hTERC and with other snoRNPs that possess a box H/ACA motif. Mutations in the dyskerin gene in X-linked dyskeratosis can reduce cellular levels of hTERC and telomerase activity (Mitchell et al. 1999).

Additional factors, identified by yeast 2-hybrid screening for proteins which interact with hTERT, are the molecular chaperone proteins hsp90 and p23. A recent study (Holt et al. 1999) illustrated that antibodies directed against p23 and hsp90, but not hsp70, could immuno-precipitate hTERT and deplete telomerase activity from a rabbit reticulocyte lysate reconstitution system. Additionally, incubation of telomerase positive fibrosarcoma cells with the hsp90 inhibitor geldanamycin could prevent telomerase activation on re-entry to the cell cycle from a quiescent state, suggesting that hsp90 is involved in assembly of active telomerase complex. Further evidence came from the lack of telomerase activity in mortal cell strains infected with hTERT expressing retrovirus in the presence of the inhibitor. Although it has been postulated that these components are involved mainly in assembly of the telomerase complex, some components of the hsp90 foldasome may

remain stably associated with the complex and may have roles in telomerase processivity (Holt et al. 1999; Forsythe et al. 2001).

These studies have contributed to the development of new concepts of telomerase activity regulation which, rather than simply being either on or off is likely to be regulated and modulated on multiple levels. A number of the known regulatory pathways that govern the expression of telomerase activity are discussed in the following sections.

1.3 Regulation of telomerase activity

1.3.1 Expression of telomerase activity in cancer and normal tissues

The advent of the TRAP assay, a highly sensitive RT-PCR assay for telomerase activity (Kim et al. 1994) has facilitated the analysis of telomerase activity across large numbers of clinical samples of normal and cancerous tissues. Telomerase activity and expression of telomerase subunits can be detected in foetal tissue from as early as the blastocyst stage during embryogenesis, but are downregulated during embryonic development (Wright et al. 1996; Yashima et al. 1998) and are not readily detectable in the majority of normal human adult somatic tissues (Kim et al. 1994; Wright et al. 1996). By contrast, the vast majority of human tumours express high levels of activity and telomerase is therefore considered to be a valid therapeutic target. It should be noted, however, that a number of studies indicate that low level telomerase activity exists, and in some cases is regulated, in some normal renewal tissues including intestinal crypts (Hiyama et al. 1996), lymphocytes (Bodnar et al. 1996), haematopoietic progenitors (Chiu et al. 1996), keratinocytes of the epidermal basal layer (Harle-Bachor et al. 1996), endothelial cells (Hsiao et al. 1997) and germ line cells (Kim et al. 1994; Wright et al. 1996).

Detection of telomerase activity in normal human tissues was first documented in germ cells (Kim et al. 1994; Wright et al. 1996). Shortly after this, several groups examined the patterns of expression of telomerase activity in cells of the blood including bone marrow haematopoietic progenitors, un-induced and activated T-cells and leukocytes. In a comparative analysis of telomerase activity in bone marrow and peripheral blood leukocytes from normal donors with activity in leukaemia samples, significant overlap between activities in normal and malignant samples was documented. In addition, low but significant levels of activity were detected in cellular fractions enriched for granulocytes,

T-cells, and monocytes and B-cells (Broccoli et al. 1995). Additionally, another study documented regulation of telomerase activity during the maturation of haematopoietic progenitor cells (Chiu et al. 1996). Cell fractions enriched on the basis of maturation associated cell surface markers were analysed for telomerase activity and showed comparatively high level expression in early $CD71^{+}$ progenitors, but not in the most primitive ($CD34^{+}$, $CD71^{lo}$) or more mature ($CD34^{-}$) populations. Stimulation with cytokines led to a small and transient increase in telomerase activity in the most primitive cells, and a rapid decrease in the early population, suggesting a role associated with maturation. Regulation of telomerase activity in the haematopoietic system has been reported by other groups: Bodnar and colleagues (Bodnar et al. 1996) described transient up-regulation of telomerase activity and hTERC expression with T-cell activation. This activation was not dependent on DNA replication or early trans-membrane signalling events and therefore it has been proposed that this represents a mechanism for transient stabilisation of telomere length to increase the lifespan of T-cells during clonal expansion.

Telomerase activity was detected in normal skin samples in a comparative study of telomerase activity in skin squamous cell carcinoma derived cell lines, *in vitro* immortalised cell lines and normal human keratinocytes. Enzyme activity was found to be located exclusively in the proliferative basal layer of epidermal tissue by enzyme mediated tissue fractionation (Harle-Bachor et al. 1996). A later study showed telomerase activity associated with various non-malignant skin conditions including sun exposure, psoriasis, and contact dermatitis induced by poison ivy, although TRAP activities of non-malignant tissue samples showed considerably lower telomerase activity than basal cell carcinoma, squamous cell carcinoma and melanoma samples (Taylor et al. 1996). This study also found telomerase enzyme activity in the epidermal layer of new-born foreskin, providing confirmation of the earlier report. It has been suggested that telomerase activity may be a marker of stem cell populations that have indefinite replicative capacity. In a more recent study, however, (Bickenbach et al. 1998) investigators used a double enrichment protocol consisting of a nuclear-label retention assay that measures indirectly the proliferation rate of sub-populations of primary cells and a collagen IV binding protocol, for enrichment of stem-cell populations. In this study, the more rapidly proliferating fraction was identified as that which had highest level telomerase activity, in-keeping with other reports which suggest that telomerase activity is not concentrated in the most primitive stem cells, but rather in cells that are more mitotically active, such as the bulb region of human hair follicles (Ramirez et al. 1997) and early haematopoietic progenitors (Chiu et al. 1996).

Interestingly, recent studies have identified the molecular defects in two forms of the hereditary disease dyskeratosis congenita, characterised by premature ageing of rapidly proliferating tissues such as hair follicles, skin and blood cells. Sufferers generally exhibit defects in wound healing and often die between the ages of 16-50 as the result of bone marrow or pulmonary failure. Dyskeratosis is linked either to X-chromosome mutation or autosomal mutations. In the case of X-linked dyskeratosis, the mutant X-locus encodes the nucleolar protein dyskerin (DKC1), a homologue of the *Saccharomyces cerevisiae* box H/ACA binding protein Cbf5p. It has been demonstrated by immuno-precipitation experiments that dyskerin interacts directly with hTERC, presumably through the H/ACA motifs and this may influence the stability of hTERC. Indeed, it was previously shown that both transcriptional and post-transcriptional mechanisms regulate cellular levels of hTERC (Yi et al. 1999), although this study did not address the nature of the post-transcriptional regulatory mechanisms. Cells expressing mutant dyskerin have reduced levels of telomerase RNA, shortened telomeres, and telomerase activity is not reconstituted by expression of hTERT (Mitchell et al. 1999). Interestingly, in a later study of several cases of autosomal dyskeratosis, an 821bp deletion was identified on chromosome 3q. In fact, further analysis revealed that the terminal 74 bases of hTERC, corresponding to the H/ACA domain, were deleted in each case (Vulliamy et al. 2001). It seems, therefore, that dyskeratosis is intrinsically linked to malfunctions in telomerase activity and possibly telomerase complex assembly. Moreover, symptoms are consistent with premature ageing of telomerase positive renewal tissues. This suggests that telomerase activity is necessary to provide the cells of a variety of normal tissues with sufficient replicative capacity to last the normal human lifespan, but more investigations are necessary to confirm this.

The function of telomerase activity in normal tissues is not clear at this time. The level of expression of telomerase activity appears to be insufficient to prevent telomere attrition in human lymphocytes (Rufer et al. 1998) and activated T-cells (Bodnar et al. 1996), yet low-level activity in normal keratinocytes maintains telomere length (Kang et al. 1998). One possibility is that detectable activity arises from low-level activity in many cells within a tissue. Alternatively, it may be that rare sub-populations of cells with high levels of activated telomerase and stable telomeres are present, but have not been identified due to the requirement for cell lysis in the TRAP assay and limitations in the protocols for telomere length detection. It has been acknowledged that infiltrating inflammatory cells or occult tumour cells in adjacent tissues may confound TRAP results in real tissue samples (Hiyama et al. 1995) and for this reason, *in situ* systems for the detection of telomerase subunits that preserve the tissue architecture may be preferable (Soder et al. 1998;

Yashima et al. 1998; Hiyama et al. 2001). However, the observation that telomerase activity is reduced in dyskeratosis patients with a concurrent failure of renewal tissue function provides some clues as to the result of malfunctioning telomerase in normal tissues.

While telomerase activity is undoubtedly present at low levels in a variety of normal human tissues, it is also clear that telomerase activity is up-regulated in the vast majority of human tumours and that the specific association of telomerase activity with human cancers represents a valid therapeutic target. In the first study utilising the TRAP assay, 90 of 101 clinical samples from tumours including colon cancer, head and neck squamous cell carcinomas, node positive breast cancer, prostate cancers, brain tumours, small cell lung cancers, and leukaemias were found to be associated with detectable TRAP activity, but most normal somatic tissues, with the exception of germ line tissues were not (Kim et al. 1994). Similarly, high-level telomerase activity has been detected in every one of the most common malignancies, with telomerase activity detectable in an average of approximately 85% of all samples (reviewed in Shay et al. 1997, Holt & Shay 1999). Notably, various studies have addressed the relative telomerase activities between paired normal and cancerous tissues in the hope that telomerase positivity may provide a useful general marker of malignancy. From this point of view, the childhood disease neuroblastoma represents an interesting case: high telomerase activity is significantly correlated with amplification of the MYCN locus, considered to be an indicator of a poor prognosis, while factors associated with a good prognosis, such as Ha-ras p21 correlate well with low activity. Additionally, telomerase activity was low or undetectable in stage IVs tumours, which often spontaneously regress. Thus, in the case of infant neuroblastoma, telomerase activity may predict clinical outcome (Hiyama et al. 1997).

Table 1.2 gives a brief summary of data derived from telomerase activity studies across a range of tumour types. Although the list is not exhaustive, its purpose is to demonstrate a trend that telomerase activity is associated with a broad range of malignancies. The examples that follow merely serve to illustrate the prevalence of expression of telomerase activity in human cancers. A study of malignant and non-malignant skin conditions identified weak telomerase activity in 44% of normal samples, but stronger activity in 83% of squamous cell carcinomas, 95% of basal cell carcinomas and 86% of melanoma samples (Taylor et al. 1996). In another study, telomerase activity was not detected in samples of normal liver, but across all histologies 85% of hepatocellular carcinoma samples were telomerase positive (Tahara et al. 1995). Detection of telomerase activity in small cell and

non-small cell lung cancers revealed high level TRAP positivity in 100% of samples from primary small cell lung cancers and their metastases, 69.2%-88.5% positivity, varying with histology, in primary non small cell samples and 50% positivity in metastases derived from non small cell lesions (Hiyama et al. 1995). A survey of neoplastic gastric lesions documented telomerase activity in 85% of primary gastric carcinomas and 95% of colorectal adenocarcinomas, but not in adjacent tissue samples (Tahara et al. 1995).

While differences in the range of positive samples within some tumour types means that more work is needed to evaluate telomerase as a potential general tumour marker before absolute conclusions regarding its usefulness for early detection can be drawn, it is clear that telomerase activity associates specifically with cancer, that a therapeutic differential exists between telomerase activity in normal and malignant tissues, and that telomerase represents an extremely attractive target for the development of novel therapies. Indeed, telomerase activity can potentially be targeted at a number of levels corresponding to levels of regulation. Telomerase regulation and opportunities for therapeutic exploitation are discussed below.

1.3.2 Regulation occurs on multiple levels

The current picture of telomerase regulation is complex: enzyme activity is likely to be controlled on many levels with multiple pathways converging to modulate the functional activity of the holoenzyme. A number of regulatory pathways have already been implicated in the normal and aberrant activity of telomerase in human cells. This section outlines briefly the major mechanisms involved that will be discussed in more detail below, with reference to relevant therapeutic targeting opportunities emerging from our current understanding.

Extensive evidence from expression studies suggests that both hTERC and hTERT are regulated on a transcriptional level and that this regulation, particularly of hTERT, is a major deterministic factor governing the activation of telomerase activity in normal and cancer cells. The cloning of the promoter regions for both the hTERC (Zhao et al. 1998) and hTERT (Cong et al. 1999; Takakura et al. 1999; Wick et al. 1999) has enabled the identification of a number of positive and negative regulators of telomerase transcriptional activity. These will be discussed in more detail below.

Other work has demonstrated that post-transcriptional mechanisms play a role in regulating telomerase activity. Kilian et al (Kilian et al. 1997) identified a number of splice variants of the hTERT transcript, which are expected to be inactive due to truncations or mutations in domains essential for catalytic activity. Interestingly, the hTERTα variant contains a deletion in the conserved reverse transcriptase motif A and has been characterised as a dominant negative inhibitor of hTERT activity (Colgin et al. 2000; Yi et al. 2000). Several studies (Ulaner et al. 1998; Ulaner et al. 2000) have begun to examine more precisely the roles of alternative splicing in the regulation of telomerase activity in various tissues.

Telomerase activity can be reconstituted *in vitro* from its 2 essential subunits, hTERC and hTERT (Autexier et al. 1996; Weinrich et al. 1997; Beattie et al. 1998; Bachand et al. 2001; Bachand et al. 2001b), although the enzyme exists in its active form at the telomere as a highly ordered multi-subunit complex. Some of the proteins that may interact with the telomere or telomerase complexes have intrinsic regulatory functions, such as the PARP domains of the TRF1 interacting proteins Tankyrase and TANK2 (Smith et al. 1998; Smith et al. 2000; Kaminker et al. 2001). PARP activity is a major mechanism for post-translational regulation of the function of nuclear proteins involved in a variety of cellular functions such as DNA damage response. The structure of the holoenzyme, therefore, represents another level on which telomerase activity is likely to be regulated, and it is now clear that other post-translational signalling events acting directly on hTERT or on other proteins involved in the complex play a role in regulation of telomerase activity. Phosphorylation status of hTERT and hTEP1 is involved in modulation of the catalytic activity of telomerase: protein phosphatase 2A (Li et al. 1997) and the c-Abl tyrosine kinase (Kharbanda et al. 2000) can both act as negative regulators of telomerase function, while PKC (Li et al. 1998; Yu et al. 2001) and Akt protein kinase (Kang et al. 1999) can act to up-regulate activity.

1.3.3 Transcriptional regulation of hTERC and hTERT expression

1.3.3.1 hTERC transcriptional regulation

hTERC up-regulation has been detected in several major types of human malignancy including tumours derived from colorectal cancer (Avilion et al. 1996; Yan et al. 2001), cervix (Soder et al. 1998), lung (Avilion et al. 1996), neuroblastoma (Reynolds et al. 1997), ovary (Park et al. 1999), and oesophagus (Hiyama et al. 1999). Feng and colleagues

(Feng et al. 1995) demonstrated up-regulation of the hTERC signal by RT-PCR in tumour cell lines derived from melanoma, leukaemia, lung, colon, and breast cancers, relative to 5 primary mortal cell strains. Additionally, a study of over 300 tissue samples from normal and tumour tissues detected hTERC signals in 43% of squamous cell carcinomas of the lung (Soder et al. 1998). *In situ* hybridisation systems for detection of hTERC (Soder et al. 1998; Park et al. 1999) clearly reveal hTERC signals concentrated mainly in tumour cells within tissues but not in adjacent normal tissue, although Hiyama and colleagues reported expression of hTERC in the basal cells of normal oesophageal mucosa and in infiltrating lymphocytes (Hiyama et al. 1999).

Indeed, low level hTERC RNA was also detected by Northern blotting in normal ovarian, testis, kidney, prostate and liver tissues (Feng et al. 1995), and up-regulation of expression of the hTERC component of telomerase has been detected in normal T-lymphocytes during activation (Bodnar et al. 1996). Thus, hTERC is expressed in sub-sets of normal tissues at lower levels than in malignant cells, suggesting an underlying mechanism for transcriptional up-regulation of hTERC during tumorigenesis. Studies of the mechanisms underlying hTERC promoter regulation should provide valuable information to enhance our understanding of telomerase regulation in normal and aberrant cells.

The cloning of the hTERC promoter region (Zhao et al. 1998) has facilitated the identification of a number of regulatory factors. The core promoter is represented in a region 272bp upstream of the transcriptional start site, containing elements typical of a pol II promoter (CCAAT box, TATA box) (Figure 1.7 (b)) that are highly conserved among the proximal promoters of 35 vertebrates (Chen et al. 2000) (supplemental data published on e-journal). The core promoter sequence contains 4 functional sites for up-regulation of activity by SP1 and down-regulation by SP3 as determined by binding studies and mutational analysis, in addition to transfection experiments (Zhao et al. 2000). The hTERC CCAAT box is bound by the trimeric transcription factor NF-y, and binding to this site is essential for basal promoter activity, as mutation of the site, or transfection of dominant negative analogues of NF-y abrogated basal activity in reporter assays. Transcriptional activity can also be modulated by an activity of pRB, the retinoblastoma gene product, by a mechanism that is unclear. Other potential sites for modulation of promoter activity include putative sites for E2F binding, GATA-1, C/EBP and c-Ets-2. The hTERC gene is contained within a 733bp CpG island (66% GC content) that extends into the promoter region. Various degrees of methylation of this sequence have been observed in some cell lines and tissues, interestingly, while mortal cell strains and normal somatic tissues showed

no methylation, three immortal cell lines that have a telomerase independent mechanism for telomere lengthening (ALT cells) and do not express hTERC showed hyper-methylation of the sequence, suggesting that transcriptional silencing by hTERC promoter methylation may play a role in selection of the ALT phenotype (Hoare et al. 2001).

1.3.3.2 hTERT transcriptional regulation

Detection of hTERT expression has commonly been achieved with the use of RT-PCR, but immunohistochemical detection was recently reported (Hiyama et al. 2001). Using these systems, high level hTERT expression has been detected in the majority of major types of human malignancy, including renal cell carcinoma (Paradis et al. 2001), oral dysplasias and cancers (Kim et al. 2001), squamous cell lung carcinoma (Shibuya et al. 2001), small cell lung cancers (Hiyama et al. 2001), colorectal cancers (Hiyama et al. 2001), pancreatic cancers (Hiyama et al. 2001) and hepatocellular carcinoma (Hiyama et al. 2001). However, several studies have also revealed weak expression in subsets of cells in a variety of normal tissues including breast, colon, lung, ovary, prostate, small intestine, spleen, stomach, testis, and uterus (Kolquist et al. 1998; Ramakrishnan et al. 1998; Hiyama et al. 2001) and in normal cell strains derived from breast colon, ovary, pancreas and prostate (Ramakrishnan et al. 1998). It is clear, then, that hTERT expression is not exclusively restricted to tumour cells and tissues, however strong relative differences exist in the expression of hTERT mRNA between normal and cancer samples and it is therefore of interest to understand the mechanisms that underlie TERT transcriptional regulation.

The simultaneous cloning of the hTERT promoter region by several groups (Cong et al. 1999; Takakura et al. 1999; Wick et al. 1999) has facilitated a number of studies into its transcriptional regulation. The transcriptional start site has variously been mapped to a region approximately 60-112bp upstream of the translational start signal. Figure 1.7 (a) gives a schematic representation of the major transcription factor binding sites known to be involved in the transcriptional regulation of hTERT. The hTERT promoter is a TATA-less sequence, containing 2 functional sites for c-Myc/Max or Mad/Max binding (E-boxes), in addition to 5 sites for modulation by SP1 within the proximal promoter. Upstream sequences include 4 binding sites recognised by Myeloid Specific Zinc Finger 2 (MZF2), an imperfect palindromic Estrogen Response Element involved in activation by ligand bound Estrogen Receptor (Kyo et al. 1999; Misiti et al. 2000) with an additional Estrogen Receptor half site adjacent to an SP1 binding motif and a site recognised by the Wilms Tumour suppressor protein, WT1. The proximal promoter region lies within a CpG island

that extends into exon2. Another small CpG island is located several hundred bp upstream (Wick et al. 1999). Investigations of the possible role of methylation in repression of hTERT did not correlate methylation status with hTERT expression, suggesting that methylation is not a general mechanism for hTERT promoter repression in telomerase negative cells, although treatment of an ALT cell line with the demethylating agent 5-azaC could induce hTERT transcription (Devereux et al. 1999; Dessain et al. 2000).

In contrast, hormone mediated modulation of hTERT transcription is likely to represent an important mechanism for regulation of telomerase activity in hormone responsive tissues, and a number of hormones have been implicated in hTERT regulation. Estrogen up-regulates hTERT transcription and telomerase activity, both by direct Estrogen receptor binding to an Estrogen Response Element in the hTERT promoter (Kyo et al. 1999; Misiti et al. 2000) and by induction of c-Myc mediated activation of transcription (Kyo et al. 1999). Other hormones that can affect hTERT transcription include retinoids that act to down-regulate telomerase activity and hTERT transcription via a pathway dependent on signalling via the Retinoic Acid Receptor (Pendino et al. 2001).

Various oncoproteins appear to regulate the expression of hTERT. Over-expression of the Human Papillomavirus type 16 E6 oncogene in early passage human keratinocytes and mammary epithelial cells results in induction of telomerase activity independently of the p53 degradation pathway (Klingelhutz et al. 1996). In a recent study, it was demonstrated that up-regulation telomerase activity by over-expression of E6 is due to activation of the hTERT promoter and that this activation required the presence of the proximal SP1 and c-Myc binding sites localised within the first 300bp of the promoter region (Oh et al. 2001). Over-expression of c-Myc in primary human mammary epithelial cells and diploid fibroblasts up-regulated hTERT mRNA and telomerase activity (Wang et al. 1998). Consistent with a view of the Myc/Max heterodimer as a critical regulator of the hTERT promoter is the observation that over-expression of c-Myc up-regulates telomerase activity and hTERT expression in EBV transformed B-cells (Wu et al. 1999) and that this was dependent on the presence of E-boxes in the promoter, although another study concluded that the activating effects of c-Myc are context specific and are disabled in some immortal cell lines (Drissi et al. 2001). Additionally, the identification of the c-Myc antagonist Mad as a repressor of hTERT transcription both by over-expression (Oh et al. 2000) and during differentiation of a leukaemia cell line (Xu et al. 2001) suggests that the balance of interactions of these factors with the promoter are probably important for hTERT promoter regulation. It is clear that c-Myc is an important regulator of hTERT transcription,

although maximal promoter activity appears to require the co-operation of c-Myc and SP1 (Kyo et al. 2000; Oh et al. 2001).

In addition to the identification of transactivators of hTERT transcription, an understanding of negative regulators binding at the hTERT promoter is likely to be critical to the understanding of TERT regulation during normal development. Negative regulatory factors influencing hTERT transcription include p53, which down-regulates hTERT mRNA independently of p53 consensus binding motifs in the hTERT promoter, instead eliciting its effects through inhibition of SP1 binding (Kanaya et al. 2000; Xu et al. 2000). MZF2 specifically binds consensus sequences within the hTERT promoter to down-regulate activity (Fujimoto et al. 2000) and the Wilms Tumour suppressor WT1 is also a negative regulator of hTERT promoter constructs (Oh et al. 1999). It was recently reported that E2F-1 represses hTERT promoter activity and mRNA expression (Crowe et al. 2001).

1.3.4 Post-transcriptional regulation of hTERT

The hTERT gene contains 16 exons, 15 introns and multiple sites for alternative splicing (Kilian et al. 1997; Wick et al. 1999). The differential expression of splice variants has been detected by RT-PCR in normal and immortal cell lines and tissues. All the splice variants other than the full-length transcript are inactive due to insertions, deletions, or truncations within the reverse transcriptase motifs (Kilian et al. 1997; Wick et al. 1999) but the most well characterised variants α, β, and α+β are expected to complex with hTERC since the N-terminal domains regarded as important for hTERT/hTERC interaction are unaffected by the mutations. In-keeping with this, expression of the full-length transcript correlates with telomerase activity (Yi et al. 2001), while the hTERTα variant is a dominant negative inhibitor of telomerase activity (Colgin et al. 2000; Yi et al. 2000). This suggests a role for splice variation in post-transcriptional regulation of hTERT expression and, therefore, telomerase activity. Studies are ongoing to assess the importance of hTERT alternative splicing in the regulation of telomerase activity. Expression of the full-length transcript correlates with telomerase activity and with maintenance of telomere length during development of foetal heart, kidney, and liver (Ulaner et al. 1998; Ulaner et al. 2001), and with telomerase activity in uterine neoplasias (Ulaner et al. 2000).

1.3.5 Post-translational regulation

Post-translational regulation of hTERT and other components of the telomerase holoenzyme undoubtedly affect telomerase activity in cells. A recent study indicated that components of the hsp90 foldasome could interact with telomerase components *in vitro* and *in vivo* to mediate assembly of an active complex (Holt et al. 1999). Interestingly, components of the hsp90 chaperone complex are often up-regulated in cancers. In a model of prostate cancer progression, it was shown that hsp90, p23, hsp70 and hsp27 were all up-regulated in malignant cells relative to parental cells and that the expression of these factors correlated with increased telomerase activity in the absence of up-regulation of hTERC or hTERT mRNA. Moreover, addition of these foldasome components to extracts of parental prostate cells in an *in vitro* reconstitution system increased telomerase activity, suggesting a novel mechanism for up-regulation of telomerase activity whereby increased levels of chaperones could increase the amount of properly folded telomerase complex to up-regulate activity during cancer progression (Akalin et al. 2001).

While hsp90 and p23 have been shown to be essential for de novo generation of functional telomerase complexes, they are not thought to modulate activity after folding, although they may remain associated with the complex and play a role in complex stability (Forsythe et al. 2001). In fact, a number of proteins may associate with telomerase *in vivo*, including the telomerase associated protein hTEP1. The function of hTEP1 is not known at this time, however p53 co-immunoprecipitates with hTEP1 and down-regulates telomerase activity (Li et al. 1999). In this study, p53 mediated regulation was inhibited by moderate concentrations of an hTEP1 derived peptide, providing evidence that the effect was genuinely derived from a post-translational mechanism involving modulation of telomerase activity via modification of hTEP1.

It has become increasingly clear in recent years that the phosphorylation state of hTERT and other components of telomerase is a major factor that influences telomerase function. Regulation of telomerase activity by phosphorylation is mediated by the action of a variety of protein kinases. Up-regulation of enzyme activity has been reported through phosphorylation by Akt tyrosine kinase (Kang et al. 1999) and by Protein Kinase C isoforms α and ξ in breast (Li et al. 1998) and nasopharyngeal cancer cells (Yu et al. 2001) respectively, while Protein phosphatase 2A (Li et al. 1997) and the c-Abl tyrosine kinase (Kharbanda et al. 2000), a major player in DNA damage response, have been reported to down-regulate the activity of telomerase in cell lines.

Although the signalling pathways that lead to activation or repression of telomerase activity by post-translation modification are not well defined at this time, it is increasingly clear that these events are important for enzyme activity. Future studies will enhance our understanding of the multiple complex pathways that converge to modulate the activity of telomerase and may provide novel targets for therapeutic intervention.

1.4 Telomerase therapeutics

1.4.1 Anti-telomerase therapeutics

The unique biology and function of telomerase and the complexity of its regulation afford a number of potential targeting opportunities directed at various levels of biological regulation. Many of the therapeutic strategies proposed to target telomerase in cancer have been tested extensively *in vitro*, and within available *in vivo* models, and it seems certain that a number of telomerase therapeutics will find their way into clinical trials before long. The sections below discuss a number of the possible targets.

1.4.1.1 Targeting transcriptional regulation of hTERC and hTERT expression

The ability to interfere with transcription of genes involved in cancer has been seen as an attractive approach for the development of novel therapies and, therefore, it is of paramount importance to understand the underlying mechanisms governing hTERC and hTERT transcription. As described above, transcriptional regulation is the major mechanism governing hTERC expression, although post-transcriptional mechanisms may play a role in stability of the RNA (Yi et al. 1999). The hTERC promoter can be positively regulated by binding of the zinc finger transcription factor SP1 at several sites in the proximal promoter, while SP3 down-regulates promoter activity in reporter assays (Zhao et al. 2000). The retinoblastoma gene product, pRb, also up-regulates promoter activity by a mechanism that is not clear. A critical finding of this study was the central importance of the CCAAT box binding factor, NF-y, as a basal transcriptional activator: transfection of dominant negative mutants of NF-yA (Mantovani et al. 1994) abrogates basal activity of hTERC proximal promoter constructs in a bladder carcinoma cell line (Zhao et al. 2000), suggesting a possible strategy for development of transcriptional inhibitors directed against the specific activity of NF-y at the hTERC CCAAT box.

Studies of mechanisms governing hTERT transcription have similarly revealed a number of positive and negative regulatory factors. The c-Myc oncoprotein has been found in several studies (Wang et al. 1998; Greenberg et al. 1999; Wu et al. 1999) to be a positive regulator of TERT transcription. The Myc-Max/Mad-Max network of transcription factors are intrinsically involved in the control of cell proliferation, cell cycle progression and apoptosis and this signalling network is often deregulated in cancer (reviewed Zhou et al. 2001). Myc and Mad heterodimerise with Max to form the Myc/Max or Mad/Max transcriptional regulators that recognise the same DNA sequence and antagonise each other's effects. Induced cellular differentiation of a human leukaemia cell line has been found to be associated with a switch from Myc/Max to Mad/Max binding at the TERT promoter (Xu et al. 2001) and other studies have also identified Mad as a repressor of hTERT transcriptional activity (Oh et al. 2000).

The tumour suppressor protein p53 (Kanaya et al. 2000; Xu et al. 2000) and the cell cycle regulator E2F-1 (Crowe et al. 2001) act to negatively regulate TERT transcription; in one recent study (Kanaya et al. 2000) introduction of wild type p53 into a cervical carcinoma cell line by means of a recombinant adenovirus was shown to down-regulate telomerase activity, highlighting one possible therapeutic approach of studies of transcriptional regulation. In this study, no obvious growth inhibition or apoptosis as a direct result of the classical tumour suppressor effects of p53 was observed, but the investigators did not analyse the growth inhibitory effects of chronic suppression of telomerase activity under long term culture conditions.

While targeting the transcriptional regulation of hTERC and hTERT at the level of the promoter, either by targeted expression or transduction of relevant regulatory molecules or by screening for promoter interactive drugs may be a feasible strategy, a more attractive approach may be the modulation of upstream signalling events leading to transcriptional activation. For instance, it is now clear that regulation of telomerase activity can be mediated by differential actions of a variety of hormones. While estrogen and androgens are thought to up-regulate telomerase activity, retinoids have been reported to down-regulate this activity and induce differentiation of leukaemia cells.

The effects of retinoids can be mediated by signalling through 2 related classes of receptor, retinoic acid receptors (RAR) or retinoic X receptors (RXR), in a pathway involving cAMP. A panel of promyelocytic leukaemia cell lines expressing variants of the RAR-α subunit was used in a study into the effects of retinoids on cellular differentiation and

telomerase activity (Pendino et al. 2001). The parental cell line differentiates in the presence of retinoids, while 2 sub-lines do not. One of the sub-lines, however, is competent to undergo maturation via RXR signalling in the presence of cAMP elevating agents. Using these cells, Pendino et al were able to dissect the various retinoid signalling pathways and to demonstrate down-regulation of hTERT mRNA and telomerase activity by RAR dependent signalling in a pathway distinct from differentiation, suggesting that retinoids may be therapeutically useful against maturation resistant cells.

Misiti et al (Misiti et al. 2000) identified binding of Estrogen Receptor-α (ER-α) at a degenerate sequence for the Estrogen Response Element (ERE) in the hTERT promoter. Further analysis demonstrated that ER-α could activate de novo hTERT transcription in the presence of estrogen and thereby up-regulate telomerase activity in telomerase negative cells derived from oestrogen responsive tissues. Tamoxifen competes with estrogen binding at the ER-α and is the chemotherapeutic drug of choice for the treatment of ER-α positive breast carcinoma. It has been shown that tamoxifen can down-regulate telomerase activity and viability of a human breast carcinoma cell line (Aldous et al. 1999); it may be, therefore, that targeting upstream signal pathways leading to promoter activation, such as hormone signalling paths, will prove an attractive approach for the treatment of specific disease types.

Another therapeutic possibility that has attracted considerable interest recently is the development of transcriptionally directed cytotoxic gene therapy using the telomerase promoter sequences to drive expression of a therapeutic transgene. A number of tumour associated promoter activities such as the prostate specific antigen promoter (PSA) or carcinoembryonic antigen promoters (CEA), and regulatable promoters, such as promoters based upon the bacterial tet operon (Gossen et al. 1992) have been described and evaluated for use in this kind of therapeutic approach, but the specificity and prevalence of hTERC and hTERT promoter activity in cancer cells suggests that these sequences may be especially well suited to this kind of approach. A number of the systems that have recently been proposed are outlined and discussed in a later section.

1.4.1.2 Targeting hTERC and hTERT RNA

The hTERC component of telomerase is an interesting target for a number of anti-sense approaches based either on blocking the function of the template region by base pairing or by actively targeting the molecule for degradation using (5'-phosphorylated 2'-5'-linked

oligoadenylate)-linked oligonucleotides (2,5-A oligonucleotides) or hammerhead ribozymes. One problem with using RNA molecules as therapeutic agents is their instability when administered as drugs. For this reason, ideal anti-sense agents are either administered encoded within an expression construct such as a plasmid vector or a virus, or as chemically stabilised analogues of the active RNA. A number of approaches have been developed to stabilise RNA and some of the stabilised molecules have been applied to anti-telomerase studies.

A retrovirus engineered by Bisoffi et al (Bisoffi et al. 1998) to express an oligomer of the template sequence (UUAGGG) was demonstrated to potently inhibit TRAP activity, decrease telomere length and viability in 2 human kidney carcinoma cell lines, while other studies using phosphorothioate stabilised antisense RNA molecules directed against the RNA component have demonstrated potent inhibitory effects on telomerase activity. The precise mechanism of action of phosphorothioate oligodeoxynucleotides (PS-ODN's), however, has been proposed to be independent of sequence; evidence was presented in one study (Matthes et al. 1999) that PS-ODN's directed against the template region of hTERC in fact elicited their effects in a sequence independent manner. Moreover, phosphorothioate modification of the backbone of a telomeric sequence primer enhanced telomerase activity in TRAP assays; it was suggested that inhibitory effects could be attributed to interaction with the primer binding site of hTERT, rather than the hTERC template region.

Another approach to targeting RNA that has been described is the use of hammerhead ribozymes. Hammerhead ribozymes are short catalytic sequences of RNA 40-50 bases in length consisting of a catalytic domain with ribonuclease activity against trinucleotide sequences, preferentially GUC, flanked on either side by specific complementary sequences that direct the ribozyme to its target RNA (for review, see Lewin et al. 2001). Several potential sequences for ribozyme cleavage exist in hTERC and, while not all specific sites have been evaluated, a notable target is the GUC trinucleotide inside the template region. Several groups have developed ribozymes directed against this sequence and the ability of these molecules to down-regulate telomerase activity has been demonstrated in 2 endometrial carcinoma cell lines (Yokoyama et al. 1998), 2 melanoma cell lines in addition to 3 surgical specimens of melanoma (Folini et al. 2000), and extracts of 2 human hepatocellular carcinoma cell lines (Kanazawa et al. 1996), although conclusions regarding the influence of hTERC inhibition on cell proliferation and telomere length regulation from these studies was more unclear: in the study of Folini et al, no

significant effects on cell proliferation and telomere length were observed after 20 population doublings in stable clones of malignant melanoma cells with reduced telomerase activity, while Yokoyama et al reported reduction in telomere length and slowed cell division in all stable endometrial cell lines tested. Kanazawa et al did not evaluate the effects of their ribozyme *in vivo*.

More recently, Yokayama et al further reported that the 5' untranslated region of hTERT mRNA also presents a suitable sequence for targeting of ribozymes (Yokoyama et al. 2000). Several ribozymes directed against sequences throughout the TERT mRNA were developed, but most failed to demonstrate anti-telomerase activity, possibly due to secondary structural elements of the hTERT message. Ribozyme directed against the 5' end, however, was able to cleave TERT mRNA and down-regulate telomerase activity. Another TERT directed ribozyme (Ludwig et al. 2001), targeted to the T motif of hTERT mRNA was able to cleave TERT mRNA, down-regulate telomerase activity and telomere length and induce apoptosis in human breast carcinoma cell lines when delivered in an adenoviral vector. Moreover, stable clones harbouring the anti-TERT ribozyme showed enhanced apoptotic response to the topoisomerase inhibitors doxorubicin and etoposide.

An alternative approach to actively target specific RNA sequences for degradation is by the use of 2,5-A oligonucleotides. The 2,5-A moiety activates RNAse L and, therefore, incorporation of this moiety to anti-sense oligonucleotides can actively target RNAse L to specific RNA sequences. This approach has been successfully used against the hTERC component to down-regulate telomerase activity and induce apoptosis of malignant glioma cells, both in culture and in subcutaneous and intra-cranial xenograft models (Kondo et al. 1998; Mukai et al. 2000). Worth noting, however, is that in these studies massive apoptosis occurred in the target cell populations within 4-5 days post-treatment, a time period which is inconsistent with effects dependent on telomere shortening but resembles more closely effects associated with telomere uncapping, discussed below.

1.4.1.3 Targeting post-translational regulation

It is now clear that post-translational modification of constituents of the telomerase complex plays an important role in modification of enzyme activity. Specific inhibition of PKC using compounds such as bisindolmaleimide or antisense against specific PKC isoforms α and ξ, respectively, down-regulated telomerase activity in breast (Li et al. 1998) and nasopharengeal (Yu et al. 2001) cancer cells. Consistent with this finding,

telomerase activity could be down-regulated by the action of protein phosphatase 2A in human breast cancer cells (Li et al. 1997). A number of other post-translational modification events also influence the activity of telomerase. Phosphorylation by Akt protein kinase up-regulated telomerase activity in a human melanoma cell line and activity was abrogated by incubation with the kinase inhibitor Wortmannin (Kang et al. 1999). While the actions of kinases such as PKC are known to regulate multiple complex signalling pathways, it is possible that the expression of specific anti-sense inhibitors of kinase action could be targeted to telomerase positive cancer cells to down-regulate telomerase activity. Alternatively, high throughput screening systems may identify drugs that can specifically abrogate the post-translational enhancement of telomerase activity.

1.4.1.4 Targeting holoenzyme function

Among the first pieces of positive evidence that telomerase inhibitors could be used to genuine therapeutic effect were two studies in which dominant negative hTERT mutants were introduced into human cancer cells (Hahn et al. 1999; Zhang et al. 1999). The investigators were able to demonstrate telomere shortening and, with continued passage, cells underwent senescence and apoptotic cell death. The advantage of dominant negative mutants over reverse transcriptase inhibitors, discussed below, clearly lies in the specificity of inhibition; however, in order to adapt such an approach to a therapeutic environment, clearly an appropriate delivery system is a necessity.

A number of investigators have examined strategies based on the use of reverse transcriptase inhibitors to down-regulate the functional activity of telomerase. One of the most well characterised reverse transcriptase inhibitors currently in use in a therapeutic setting is AZT (azidothymidine), a potent inhibitor of HIV-1 reverse transcriptase and, therefore, one of the major drugs prescribed for the management of HIV. Although AZT is not specifically targeted to telomerase and the activated analogue is a general inhibitor of polymerase activity, several studies have examined the effects of AZT on telomerase activity in cancer cell lines and have shown an inhibitory effect, although the therapeutic value is uncertain. AZT was able to inhibit cloning efficiency in 4 human breast cancer cell lines (Melana et al. 1998) and could inhibit telomerase activity in a dose dependant fashion, but another study into the effects of AZT on human lymphocytes concluded that the telomerase inhibitory effects of AZT were not cytotoxic, but merely led to a transient suppression of cellular growth which could be reversed by removal of the drug (Beltz et al. 1999).

An interesting compound, which has been identified by screening *in silico* for compounds with pharmacological properties resembling those of the weak telomerase inhibitor berberine, is FJ5002 (Naasani et al. 1999). This compound was identified as a considerably more potent inhibitor of telomerase activity and has been demonstrated to act in the fashion expected of a "classical" telomerase inhibitor: continued passage of cells in the presence of FJ5002 led to replication dependent shortening of telomeres with concurrent increase in aneuploid metaphases and apoptotic cells. Recently, an additional small molecular inhibitor of telomerase activity designated BIBR1532 was characterised. Incubation with this compound led to telomerase inhibition, telomere shortening, reversible growth inhibition, chromosome end-to-end fusions and senescence with concurrent changes in the expression of a number of genes implicated in cell cycle checkpoints and senescence, such as up-regulation of $p21^{waf1}$ and MDM2, and down-regulation of cyclins A and B. Interestingly, the telomere length regulator TRF1 was also up-regulated. The investigators did not observe apoptosis, but rather senescence was induced. Cells pre-treated with inhibitors for extended periods exhibited marked decreases in tumourigenic potential when injected into nude mice (Damm et al. 2001).

1.4.1.5 Telomere interactive agents

Another way in which telomerase positive cells could be targeted in a manner which is specific, but not dependant on telomere shortening is by targeting the telomere cap. In a recent study (Kim et al. 2001), it was demonstrated that ectopic expression of template mutated telomerase RNAs designed to have sequences not recognised by telomere binding proteins led to decreased DNA synthesis and cell proliferation without telomere shortening or inhibition of the endogenous telomerase activity in breast and prostate cancer cell lines and in a xenograft model. Strategies targeted at telomeric uncapping, then, may represent an effective way to target cancer cells more rapidly than by the "classical" effects of inhibition of telomerase activity.

A similar approach involving targeting of telomere function is the use of G-quadruplex interactive agents as telomerase inhibitors. Formation of the telomeric G-quadruplex, by planar stacking of guanine base tetrads in the (TTAGGGG)n sequence (models of G-quartet formation reviewed in (Han et al. 2000)), has been characterised *in vitro* and may be involved in several cellular processes including telomere capping and termination of telomere elongation by telomerase if it is formed *in vivo*. Compounds that interact with G-quadruplex structures such as substituted acridines, therefore, may represent a new class of

specific telomere or telomerase targeting agents. Several groups have applied molecular modelling approaches to the design of compounds that interact with these sequences and have been able to demonstrate inhibition of telomerase activity (Read et al. 2001).

1.4.2 Immunotherapy

Enhancement of the anti-tumour immune response has recently aroused considerable interest as a therapeutic approach. The complexity of the immune system, naturally, allows for a number of strategies to be employed. One way in which immune responses can be specifically targeted to particular antigens that has been demonstrated is by the *ex vivo* manipulation of autologous antigen presenting cells (APCs) such as macrophages or Dendritic Cells (DCs). Antigenic peptide sequences are processed by the proteasome of APCs and transported to the Endoplasmic Reticulum where they can interact with the products of major histocompatibility (MHC) alleles such as the MHC I type Human Leucocyte Antigen (HLA) 2. Subsequent budding of the Golgi and transport to the cell surface results in the presentation of antigen-MHC complexes for interaction with the T-Cell Receptor (TCR) of CD8+ populations of Cytotoxic T-Lymphocytes (CTLs).

It has been demonstrated repeatedly that peptides that interact with MHC molecules can be introduced into APCs, either as peptides or as DNA vaccines by the use of expression vectors, and thereby enrich specific CTL populations *in vitro* and *in vivo* (for review of cancer vaccination approaches, see (Minev et al. 1999)). Moreover, it has recently been shown that several peptide sequences of the hTERT protein match consensus sequences for interaction with HLA2 and can be naturally processed (Vonderheide et al. 1999; Minev et al. 2000), suggesting that there may be an endogenous and pre-existing anti-TERT immune response which could be enhanced by *ex vivo* manipulation of autologous APC's to generate a broadly active anti-tumour immune response. Indeed, transduction of peripheral blood mononuclear cells from a prostate cancer patient (Vonderheide et al. 1999) with HLA2 interactive sequences from hTERT resulted in an enriched population of anti-TERT specific cytolytic T-cells, as judged by dose-response of radiolabel release to increasing ratios of effector to target cells, which were able to effectively lyse HLA2+, TERT+ tumour cell lines derived from ovarian cancer, malignant melanoma and multiple myeloma, in addition to freshly isolated primary tumour cells from patients presenting with acute myeloid leukaemia and non-Hodgkins lymphoma, while normal blood cells from the same patients were unaffected.

1.4.3 Transcriptionally directed cytotoxic gene therapy approaches

A number of cytotoxic gene therapy strategies based on transcriptional targeting of the hTERC and hTERT promoters have been proposed recently; several transgenes, such as the pro-drug activating enzymes Herpes Simplex Thymidine Kinase (HSTK) and bacterial nitroreductase; apoptotic effectors; the noradrenaline transporter; and the diptheria toxin gene have been proposed as candidates for the development of gene therapy approaches. The advantages of each system will have to be evaluated thoroughly in future studies.

Majumdar et al (Majumdar et al. 2001) described an hTERT promoter-HSTK expression system which sensitised tumour cells derived from osteosarcoma, pancreatic cancer, medulloblastoma and fibrosarcoma to the effects of the pro-drug Gancyclovir. In this study, three normal human fibroblast cell lines, as well as normal retinal pigmented epithelial cells were unaffected. Moreover, *in vivo* transduction with adenoviral vectors containing the expression construct resulted in decreased tumour volumes and prolonged survival in mice bearing osteosarcoma derived xenografts with no increase in liver enzymes or histopathology associated with a cytotoxic effect on the liver.

Several studies have examined the effects of restricting the expression of apoptotic mediators using hTERT promoter sequences. Genes for Bax (Gu et al. 2000), Caspase 8 (Koga et al. 2000), a novel form of caspase 6 engineered to be constitutively active (Komata et al. 2001), and most recently FADD (Koga et al. 2001) have been variously expressed with the induction of apoptosis in tumour cell lines derived from malignant glioma, malignant melanoma, breast cancer, lung, while expression and apoptosis was not detected in normal human cell lines. Efficient cell killing has also been demonstrated using both hTERC and hTERT promoters to drive expression of the diptheria toxin A gene (Abdul-Ghani et al. 2000) and by using the hTERC promoter to drive expression of the noradrenaline transporter gene in glioma cells (Boyd et al. 2001), facilitating the uptake of [^{131}I]-MIBG, the drug of choice for treatment of thyroid cancer.

The present study describes the use of the transcriptional regulatory sequences of both hTERC and hTERT to drive expression of the bacterial nitroreductase gene, which converts the pro-drug CB1954 to a cytotoxic form (Knox et al. 1992). We previously described clear differentials in the activity of both promoters between normal and cancer cell lines, although hTERC promoter activity was always stronger than that of hTERT, and

reported efficient cell killing in tumour cell lines, derived from cervical, ovarian, lung and colon cancers, which were stably transfected with telomerase promoter-nitroreductase constructs. Moreover, sensitisation to the pro-drug CB1954 was retained *in vivo* in xenografts of the stable cell lines. In this model, a single tail vein administration of the drug, at concentrations which were not acutely toxic, could significantly reduce tumour volumes in cervical and small cell lung cancer cells. However, we observed a dependence on high promoter activity which may represent a potential limitation to this kind of approach; ALT cell lines tested in our system, as expected, were not sensitised to the effects of the drug while most telomerase positive cancer cell lines were, but a subset of telomerase positive cancer cell lines having low promoter activities were also not significantly sensitised. This suggests that only highly expressing tumour cells may be efficiently targeted by this approach (Plumb et al. 2001). These data are presented and discussed at greater length in the present study.

One of the most important characteristics of any gene targeted therapeutic strategy must be its ability to target cancer cells while leaving normal cells relatively unaffected. From this point of view, telomerase is an excellent candidate for transcriptional targeting and the initial data from telomerase promoter directed gene therapy systems that have been reported shows considerable promise. An efficacious and selective anti-tumour effect has been described in a large number of cells derived from tissues of unrelated origin and this effect has been preserved *in vivo* in xenograft models and in adenoviral (Majumdar et al. 2001; Gu et al. 2000) and cationic (Koga et al. 2000) models of delivery. Moreover, as telomerase based targeting potentially provides us with 2 promoters of differing strengths and tissue specificity, and since a number of transgene systems have already been described, there is the potential for the development of different combinations of promoter-transgene constructs for use in different situations. However, the optimisation of gene therapy approaches will require combinations of restricted transcription and delivery.

1.4.4 Resistance to telomerase therapeutics

While telomerase activity has been detected in all major human malignancies, it is not a prerequisite for tumorigenesis. While it is clear that a few tumour types, including small cell lung cancer (Hiyama et al. 1995; Sarvesvaran et al. 1999); and cervix (Soder et al. 1998) are clear candidates for anti-telomerase therapy, telomerase positivity of clinical samples derived from other cancer types such as non-small cell lung cancers and endometrial malignancies has been variable. A study of telomerase activity in small cell

versus non-small lung cancers (Hiyama et al. 1995) recorded high level TRAP positivity in 100% of samples from primary small cell lung cancers and their metastases but only 69.2%-88.5% positivity, varying with histology, in primary non small cell samples and only 50% positivity in metastases derived from non small cell lesions. The model that was proposed to explain this heterogeneity was that large solid tumours that display variable frequency of telomerase positivity could contain a fraction of partially transformed, telomerase negative mortal cells. Whether or not this would present a problem for telomerase based therapies is unclear, as the growth of mortal subpopulations within a heterogeneous solid tumour, or the metastases derived from them may be self limiting, although it is conceivable that spontaneous immortalising events may occur post-therapy.

A more worrying finding for the evolution of telomerase therapeutics as "universal" anti-tumour agents is that a number of human cancers contain cells which elongate their telomeres by an alternative mechanism, termed the ALT pathway (Bryan et al. 1997) which may be associated with telomerase transcriptional repression by methylation of the hTERC promoter (Hoare et al. 2001). The ALT phenotype, characterised by the sudden appearance of telomeres of heterogeneous length, is believed to be based on a recombinogenic mechanism of telomere extension similar to that active in telomerase mutant yeast strains which escape senescence (Lundblad et al. 1993). Acquisition of the ALT phenotype is associated with acquisition of a novel type of Promyleocytic Leukemia Body (PML body) termed the ALT associated PML body, or APB (Yeager et al. 1999). These structures have been found to include a number of the human homologues of proteins involved in the maintenance of telomere length in the absence of telomerase in mutant yeast strains that escape from senescence. Moreover, telomerase activity appears to be the dominant mechanism for telomere extension in cell fusion experiments, although it is not clear whether the 2 mechanisms can co-exist within the same cell. If the use of telomerase inhibitors in cancer therapy is actually comparable to the experimental inhibition in yeast strains and if the alternative mechanisms of telomere extension can be activated by telomerase inhibition, or are latent within telomerase positive cells, it is conceivable that the use of telomerase inhibitors may actually select for the ALT phenotype, leading to more recombinogenic forms of cancer.

In order to overcome these potential problems, several options exist: it may be necessary to evaluate the use of telomerase inhibitors in combination with other conventional treatment modalities. It has been reported (Kondo et al. 1998) that inhibition of telomerase activity by stable expression of an anti-TERC RNA increased the sensitivity of human malignant

glioma cells to cisplatin induced apoptosis *in vitro* and decreased overall viability, while Ludwig et al (Ludwig et al. 2001) showed that an anti-hTERT ribozyme could enhance the apoptotic effect of topoisomerase inhibitors. Other studies have also correlated telomerase activity and telomere length with sensitivity to conventional chemotherapeutic agents although definitive effects remain elusive.

An alternative strategy, applicable to the development of cytotoxic gene therapy and an integral part of many systems such as TK/GCV and nitroreductase/CB1954, is to "target" untransduced cells, or cells which are resistant to primary effects of a therapeutic strategy with a bystander effect. For the development of this kind of strategy, enzyme/pro-drug systems with an active metabolite which can kill both dividing and non-dividing cells, or cells which are hypoxic, would be most desirable; thus, systems such as bacterial nitroreductase/CB1954 or horseradish peroxidase/indole-3-acetic acid (Greco et al. 2000) are not limited in their efficacy by the proliferation dependence which is characteristic of some other systems.

1.5 Aims of the study

1.5.1 Development of an hTERC and hTERT directed cytotoxic gene therapy model

Several recent studies have used hTERT and hTERC transcriptional regulatory sequences to drive expression of a variety of transgenes in the development of tissue culture models of gene therapy (Abdul-Ghani et al. 2000; Gu et al. 2000; Koga et al. 2000; Boyd et al. 2001; Koga et al. 2001; Komata et al. 2001; Majumdar et al. 2001). This approach is attractive, as it will not have the problems of phenotypic lag expected from the use of telomerase inhibitors in the clinic (Keith et al. 2001, White et al. 2001 for reviews). The development and evaluation of alternative promoter-transgene systems has clear benefits: each system has its own advantages and disadvantages and it is of interest to evaluate a number of transgenes for use with these promoters. In this study, I document the development of a tissue culture model of telomerase directed cytotoxic gene therapy using promoter fragments of both hTERC and hTERT to drive expression of the bacterial nitroreductase gene in order to sensitise cancer cells to the cytotoxic effects of CB1954. The plan of experimentation is presented in figure 1.8.

1.5.2 Identification of differentials in hTERC and hTERT promoter activity in cell lines

Regulation of transcription of hTERC and hTERT genes is thought to be a major deterministic factor governing the differential expression of telomerase activity between normal and cancer cells (Feng et al. 1995; Avilion et al. 1996; Soder et al. 1998; Yi et al. 1999; Hiyama et al. 2001). Therefore, it should be possible to detect differences in hTERC and hTERT promoter activity between normal and cancer cells in tissue culture systems by the use of a luciferase reporter assay. The initial aims of the study were to quantify differences in hTERC and hTERT activity between normal and cancer cells, and thereby validate the telomerase promoters as tools for the development of a transcriptionally directed cytotoxic gene therapy strategy. In chapter 3, the results of transient transfection reporter assays using previously characterised hTERC and hTERT promoter sequences to drive expression of the firefly luciferase gene in a wide range of normal, cancer and ALT cell lines are presented. The results showed a clear differential in activities of both promoters between normal and cancer cells and gave an indication of the relative efficiency of transgene expression from these sequences between normal and cancer cells. Moreover, the hTERC sequence was a stronger promoter in all cancer cell lines tested, yet still retained a cancer cell specificity, having high level activity in cancer cells and significantly lower activity in normal and ALT cells. From these results, the hTERC and hTERT sequences were validated for further study as part of a suicide gene therapy approach for therapy of cancer cells.

1.5.3 Bacterial nitroreductase/CB1954

A number of cytotoxic gene therapy approaches based on the use of enzyme/pro-drug activation systems have been proposed. Each has its associated benefits and limitations. The efficacy of enzyme/pro-drug therapies of cancer will be limited by a number of factors: these include the mechanism of action of the activated drug and intrinsic cell specific differences in sensitivity to the type of damage induced by it, in addition to the levels and specificity of expression of the pro-drug activating enzyme within tumour cells and the efficiency and selectivity of delivery.

Several of the systems which have been pioneered are intrinsically dependant on cellular replication for maximal cytotoxic effect. It has been postulated that this may be problematic for anti-tumour therapy since large solid tumours often contain a fraction of

viable quiescent cells (Durand et al. 1998; Durand et al. 1998b) that represent a major obstacle to effective therapy (reviewed in Brown et al. 1998). Gene targeted systems that rely on cellular proliferation are, therefore, likely to be less effective systems than those which can target both dividing and non-dividing cells. A good example of such a system is bacterial nitroreductase/CB1954, which has been shown to exhibit efficacy against both dividing and non-dividing tumour cells in tissue culture assays (Bridgewater et al. 1995; Weedon et al. 2000).

Chapter 4 documents the development and characterisation of a panel of stable cell lines expressing nitroreductase under the control of hTERC, hTERT or CMV promoters. Cell lines were selected for characterisation on the basis of their hTERC and hTERT promoter activities in luciferase assays (chapter 3). Cell survival assays (MTT assay) indicate that telomerase promoters could be used to drive high-level expression of NTR in those cancer cell lines that had high promoter activities, and thereby sensitise cells to CB1954 treatment in a manner dependent on high promoter activity. The hTERC-NTR and hTERT-NTR expression constructs are therefore validated for further study using a more realistic model of delivery.

1.5.4 Adenovirus gene delivery vehicles

A major problem with genetic therapies at the present time is the inefficiency of gene transfer to specific cell populations *in vivo*. Among the most well characterised vehicles for gene delivery are adenovirus vectors. Adenoviruses are associated with infections of the upper respiratory tract and eye (reviewed in Shenk 1996, Horowitz 1996). Their replication within cells is largely dependent on the expression of the products of the E1a gene, the first viral gene to be expressed. E1a is alternatively spliced into the E1a 12s and 13s gene products, which can mediate up- and down-regulation of host cell and viral gene expression by a variety of mechanisms (reviewed in Russell 2000).

The development of first-generation adenoviral vectors, deleted in the E1 region, which can be grown in E1 trans-complementing cell lines such as the 293 embryonic kidney cell line, and the proliferation of simplified systems for cloning into adenovirus (He et al. 1998) has allowed for the development of replication defective adenovirus as a transgene delivery vehicle. The efficiency of adenovirus mediated transgene delivery to both dividing and non-dividing cells and tissues is good and therefore, adenoviruses are the vectors of choice for many gene therapy applications.

Chapter 5 documents the evaluation of adenovirus a system for efficient delivery of transgenes into cell lines used in this study. Using a reporter virus with the E.Coli LacZ gene under the control of a CMV promoter, infectivity was assessed in a panel of cell lines that have been characterised previously in this study for hTERC and hTERT promoter activity. Additionally, cloning of hTERC-NTR and hTERT-NTR expression constructs into the E1 region of an adenovirus 5 backbone is described. The subsequent vectors, Ad-hTERC-NTR and Ad-hTERT-NTR are characterised for their ability to transduce cells with the NTR expression constructs and to sensitise cell lines to CB1954.

Sequencing of NTR transcripts expressed in a cervical carcinoma cell line revealed the correct transcriptional start sites for hTERC and hTERT (Feng et al. 1995; Cong et al. 1999; Wick et al. 1999), indicating that correct transcription from the hTERC and hTERT promoters was retained in the adenoviral backbone. However, the sequences revealed alternative splicing of the transcript resulting in a 187bp deletion encoding an in frame 22 amino acid truncation of the NTR protein in addition to mutation of a further 5 residues. Characterisation of the mutation by a variety of bioinformatics approaches did not predict any adverse effect on the function of NTR. Expression of NTR was characterised by Northern and Western blotting to ensure that expression followed the expected patterns. The function of expressed NTR was tested in MTT assays to ensure that the mutant NTR retained the ability to sensitise cells to CB1954.

The data indicate that the Ad-hTERC-NTR and Ad-hTERT-NTR gene therapy vectors could efficiently transduce a variety of cell lines with NTR and that sensitisation to CB1954 was dependent in part on hTERC and hTERT promoter activities and in part on infectivity. Additionally, a cervical cancer cell line that was sensitised to the effects of CB1954 in MTT survival assays was established as a xenograft model to test the efficacy of Ad-hTERC-NTR and Ad-hTERT-NTR transduction followed by CB1954 challenge *in vivo*. The data indicate that adenovirus vectors harbouring hTERC and hTERT-NTR expression constructs could sensitise human tumour cells to the effects of CB1954 both *in vitro* and *in vivo* and, therefore, that telomerase directed gene therapy using the bacterial nitroreductase gene represents a valid therapeutic approach for the treatment of malignant disease.

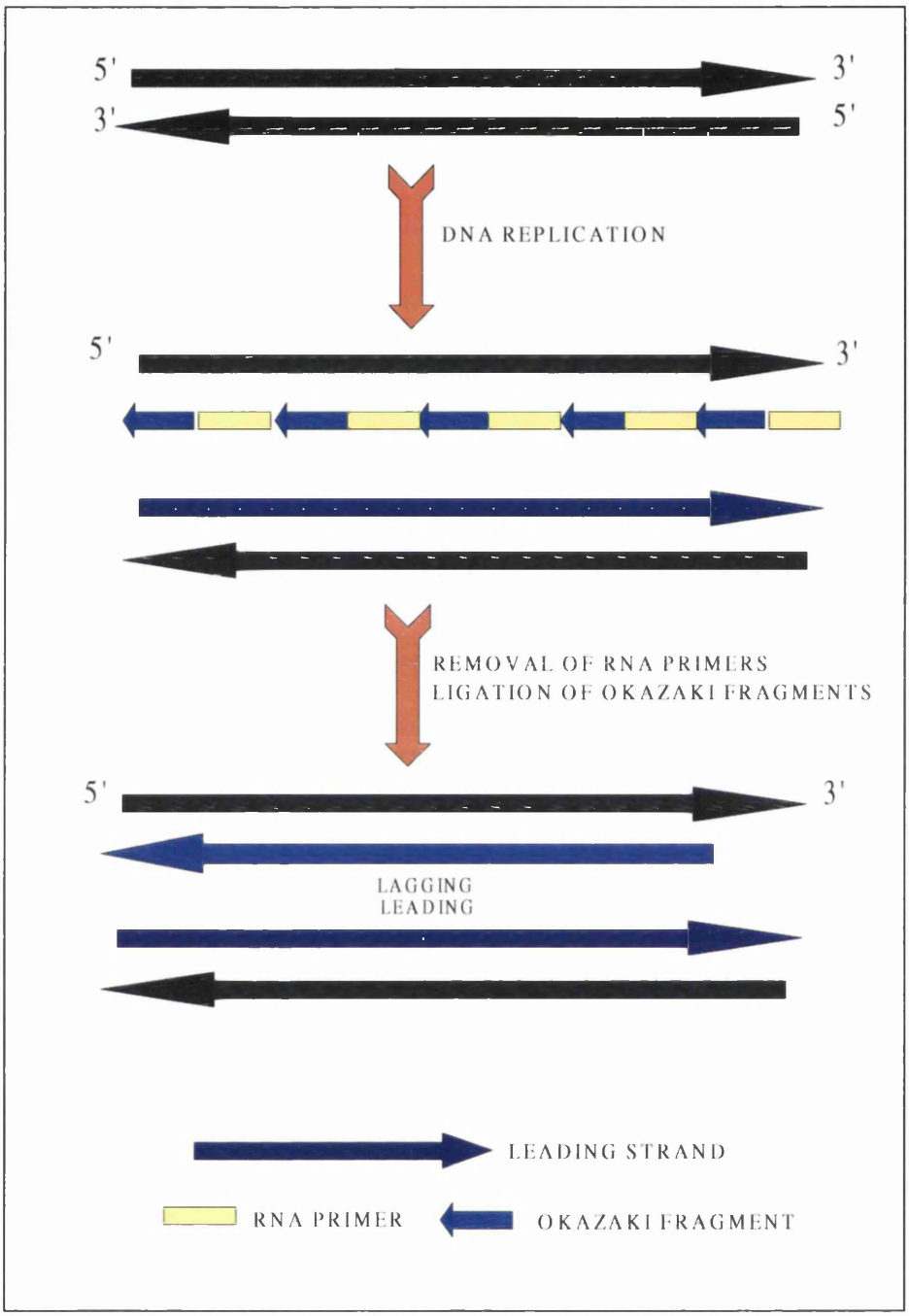


Figure 1.1: The end replication problem for linear eukaryotic chromosomes.

The figure illustrates the end replication problem. Due to the mono-directional activity of DNA polymermase, only a single strand of a parent DNA molecule (the leading strand) is completely replicated. Synthesis of the other strand (lagging strand) proceeds in a discontinuous manner in short stretches termed Okazaki fragments. Synthesis of Okazaki fragments is primed from short complementary RNA sequences that are removed, allowing ligation of the Okazaki fragments. The removal of the terminal RNA primer means that the extreme terminus of the lagging strand template is incompletely replicated.

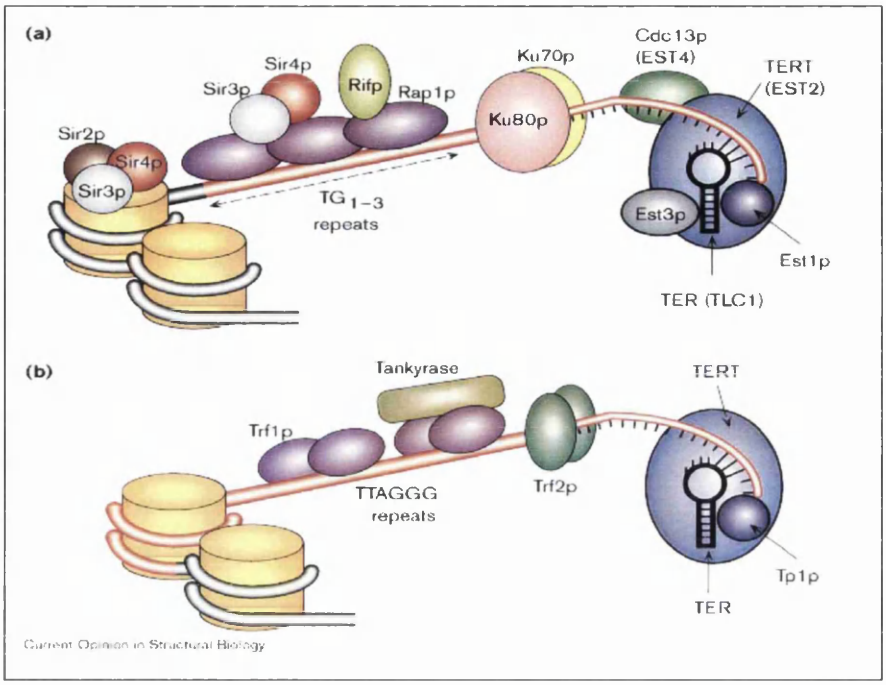
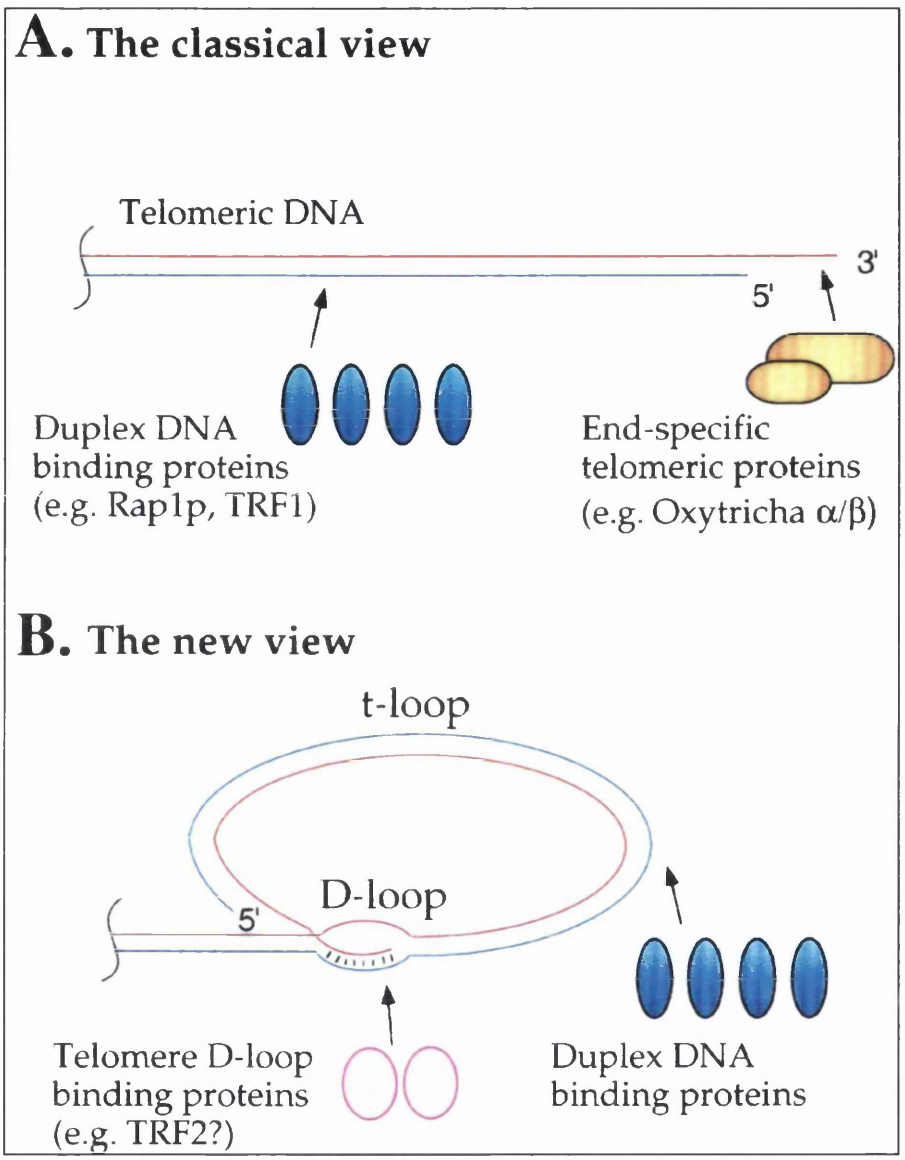


Figure 1.2: Schematic representation of possible protein interactions at the telomere.

The figure illustrates a number of the factors known to influence telomere function in (a) S. Cerevisae and (b) humans. Histone octamers are represented by orange cylinders. Telomeric and non-telomeric DNA regions are represented by red and grey tubes. Other components of the putative complexes are labelled. From O'Reilly et al 1999.



Greider, C.W. (1999) Cell 97(4): 419-22

Figure 1.3: Schematic model of the t-loop at mammalian telomeres.

The figure shows (a) the old view and (b) the new view of a possible mechanism by which the single stranded ends of mammalian telomeres are disguised from the DNA damage response machinery. Some lower eukaryotes such as Oxytricha are known to have end-binding proteins that may prevent recognition of the telomere as DNA damage. It was recently discovered, however, that when mammalian telomeres are cross-linked and purified, they adopt a looped conformation in which the single stranded region is sequestered by a strand invasion mechanism.

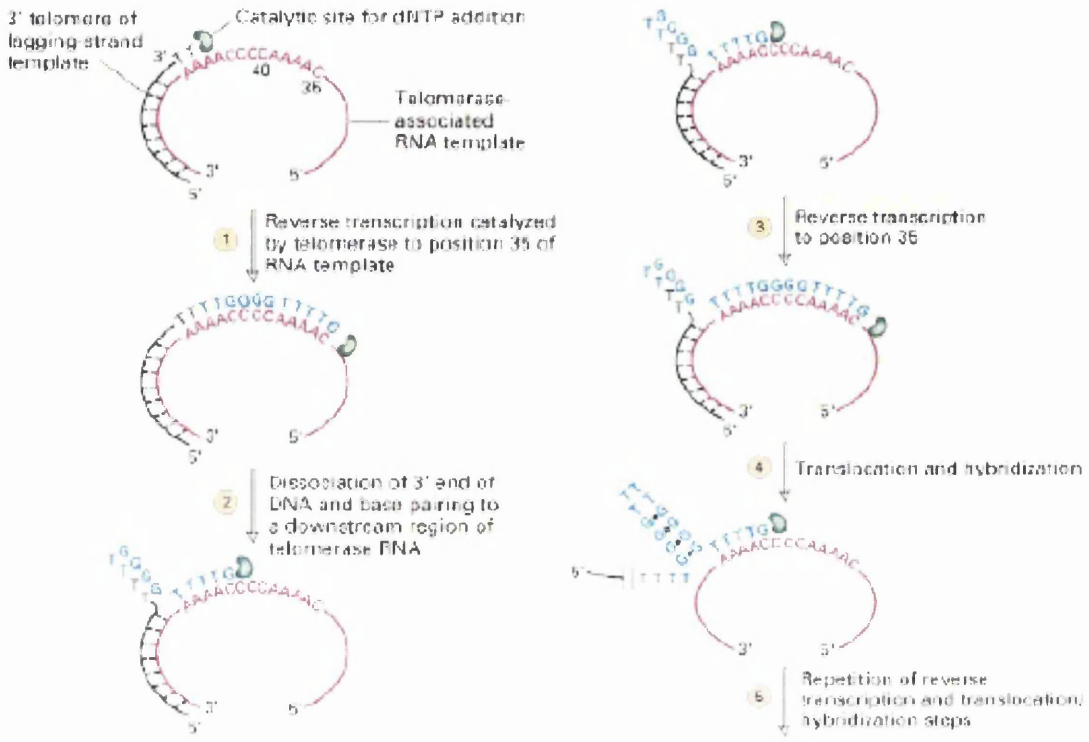


Figure 1.4: Representation of the possible mechanism of telomere extension by telomerase.

The RNA component of telomerase contains a template sequence complementary to 1.5 telomeric repeats from which new telomeric sequence is reverse transcribed in a processive manner. The model that has been proposed is outlined in the figure. The template sequence associates with overhanging single stranded telomere sequence, allowing reverse transcription of a single, new telomere repeat to proceed. The nascent repeat then dissociates and base-pairs again with a downstream region of the template, allowing reverse transcription of another repeat to proceed. Thus, by a mechanism that couples translocation and hybridisation to the catalytic function of telomerase, it is possible to synthesise large tracts of telomeric repeats.

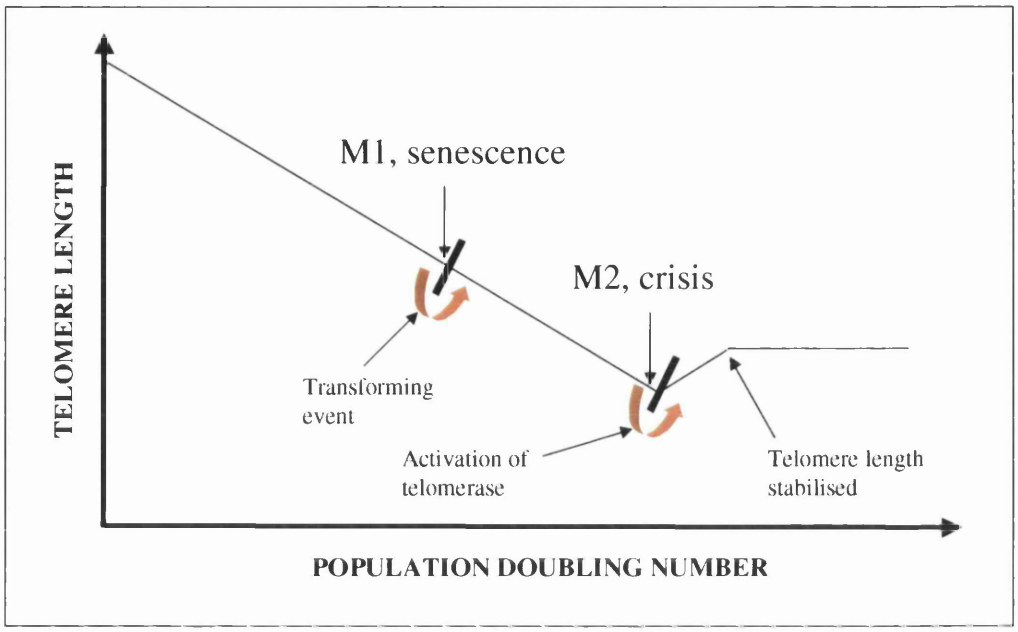
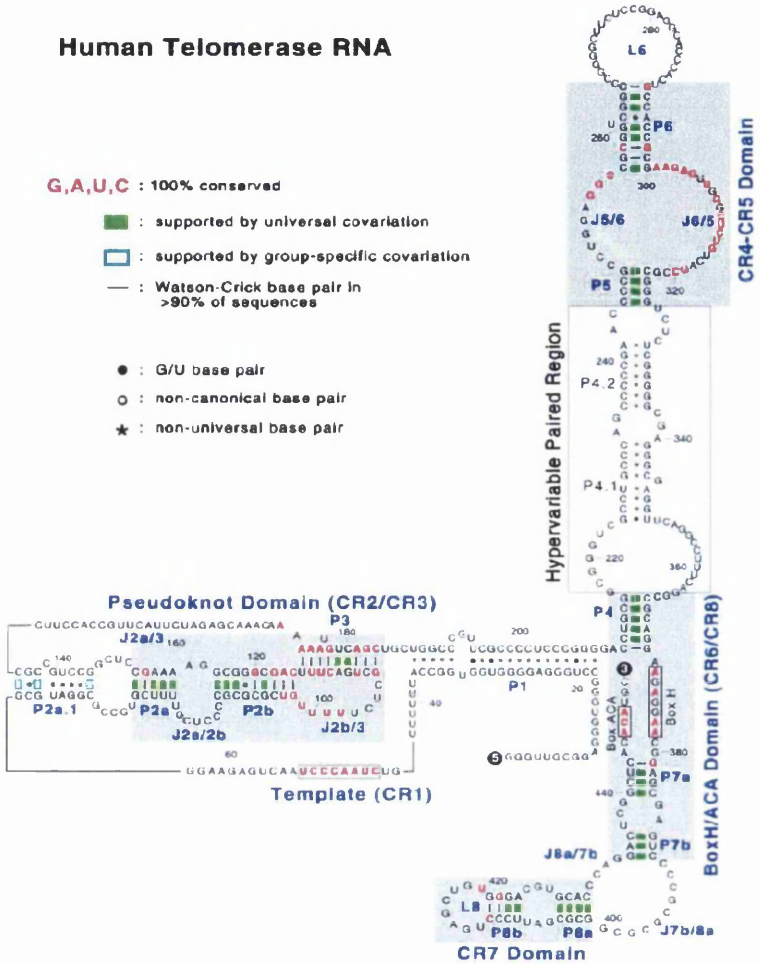


Figure 1.5: Reactivation of telomerase activity as a critical late stage in immortalisation.

By the current model, the cells of an exponentially growing mortal cell population will continue to divide, gradually losing telomeric DNA until the telomeres of individual cells are sufficiently shortened to activate the pathways that lead to senescence. Thus, an increasing number of cells will exit from the cell cycle as the age of the culture increases. A transforming event, such as inactivation of the p53 and pRb tumour suppressor proteins by SV40 large T antigen may allow a few rare cells to bypass senescence and continue to divide, while continuing to lose telomeric DNA. At a point when telomeres are critically shortened, the cell population undergoes a widespread growth crisis from which a few cells that have stabilised their telomere length by reactivation of telomerase may emerge as immortal cell lines.



Chen et al, Cell 100, 503-514 (2000)

Figure 1.6: Conserved secondary structure of mammalian telomerase RNA components (TERCs).

Phylogenetic analysis of the proposed folding patterns of 35 mammalian TERC molecules reveals the predicted conserved secondary structure represented above. Folding of the molecule via the helices designated P1-P8 allows the conserved regions of primary sequence (CR1-CR7) to adopt specific, conserved functional structures. The template sequence lies in a single stranded region that is accessible to anti-sense intervention and to the telomere. The pseudoknot domain is important for TERC/TERT interaction and for enzyme activity, as is the downstream CR4/CR5 domain. The box H/ACA domain is involved in protein interactions other than with hTERT that may be necessary for RNA stability.

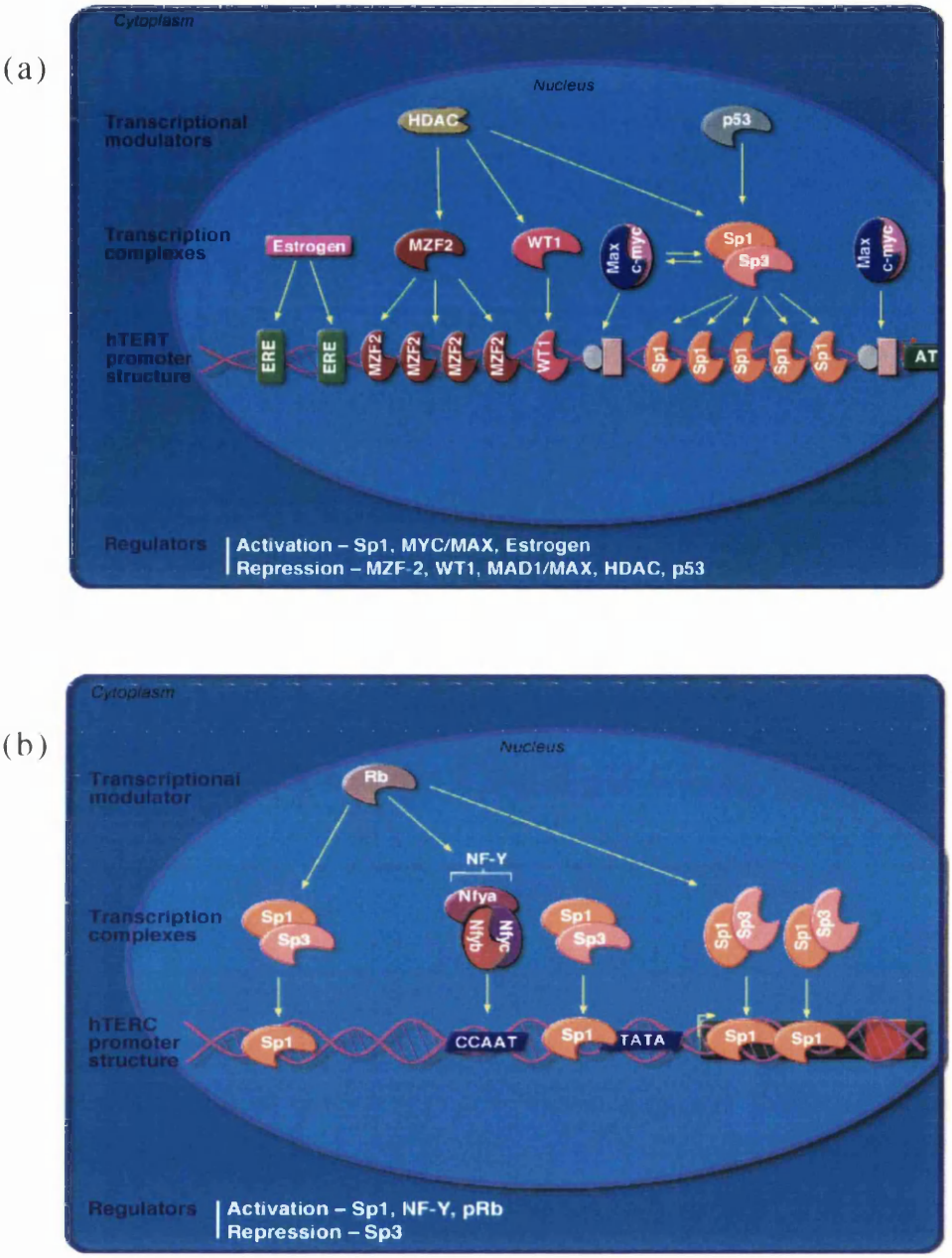


Figure 1.7: Schematic representation of the regulatory elements of the hTERC and hTERT promoter regions.

Transcription of telomerase genes appears to be a major level on which enzyme activity is regulated and a number of regulators of (a) hTERT and (b) hTERC transcription have been identified. Transcription of hTERT is up-regulated by estrogen, Myc/Max, and SP1 and is down-regulated by MZF-2, WT1, Mad/Max, P53, HDACs and E2F. The hTERC promoter region contains elements typical of a pol II promoter (TATA box, CCAAT box) that are strongly conserved across 35 mammalian species. Up-regulation of hTERC is mediated by SP1, pRb and by NF-Y, while transcription can be repressed by SP3. The pathways shown can also be viewed at http://www.biocarta.com.

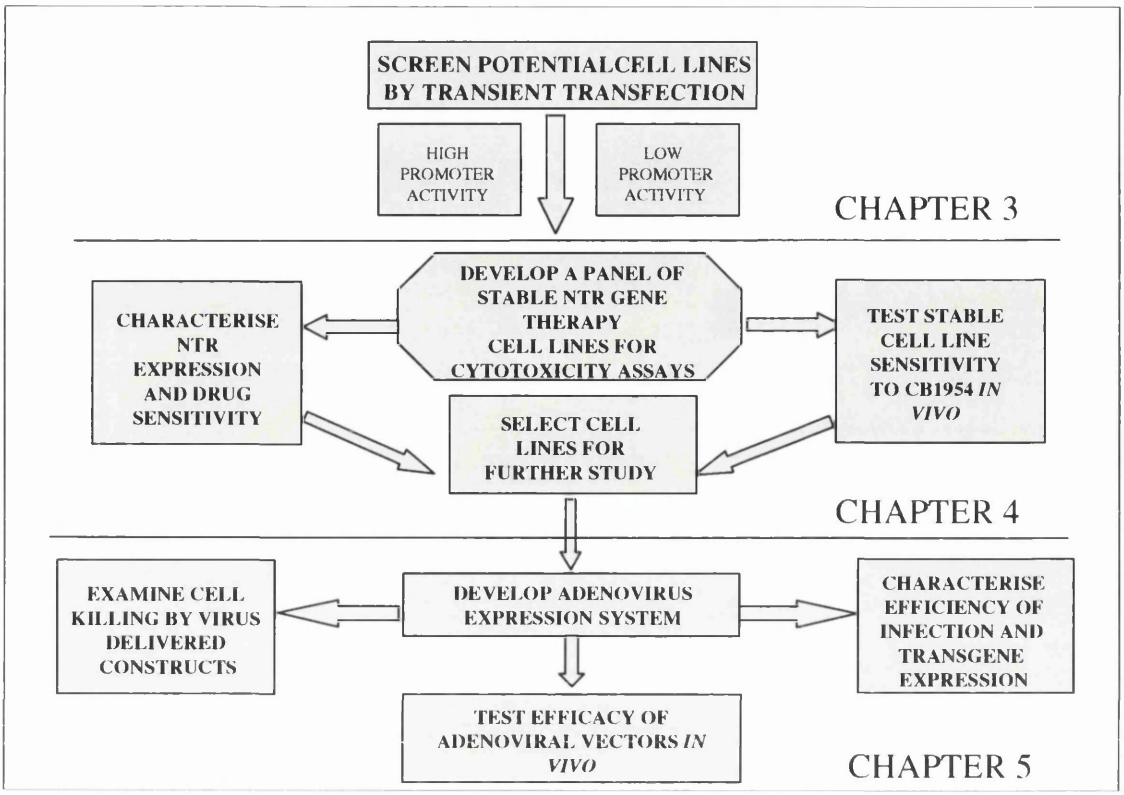


Figure 1.8: Plan of investigations undertaken in this thesis.

Chapter 3 documents the quantification of differentials in hTERC and hTERT promoter activities between cancer and mortal cells. A number of cell lines with low and high hTERC and hTERT promoter activities were selected for the generation of stable cell lines with the bacterial nitroreductase (NTR) gene under the control of hTERC and hTERT promoters. Chapter 4 details the validation of hTERC and hTERT promoters for use in a transcriptional directed anti-cancer gene therapy strategy using the bacterial nitroreductase gene to sensitise cancer cells to the effects of the pro-drug CB1954. In chapter 5, the hTERC-NTR and hTERT-NTR expression constructs are cloned into adenovirus gene therapy vectors to examine the efficiency of infection, transgene expression and sensitisation to CB1954 in a panel of cancer and mortal cells.

Telomeric factor	Telomere length regulation effect	Other effects
TRF1	Negative. Primary mode of action probably by recruitment of other factors to a higher order complex.	DNA bending action may promote configuration favourable for T-loop formation.
TRF2	Negative. Overexpression leads to transient decrease in telomere length.	Contribution to T-loop formation. Protection against end-to-end fusions. Inhibition leads to cell cycle arrest and apoptosis.
Tankyrase	Positive. ADP-ribosylation by tankyrase leads to inhibition of TRF1 binding.	May modify other nuclear factors by ADP-ribosylation. Also found in mitochondria.
Tankyrase 2		Over-expression caused apoptotic cell death.
Rap1p	Possible negative effects. Overexpression caused moderate increase in telomere length, possibly by titrating out binding factors. Recruited to telomere by TRF2.	
TIN2	Negative. Recruited by TRF1. Binding mutants increase telomere length in the presence of functional TRF1.	
POT1		Single stranded end-binding factor. Protection of telomere ends. Pot1$^{-/-}$ cells show rapid loss of telomere and subtelomere sequences.
WRN		Interaction with Ku heterodimer enhances 3', 5', and blunt end exonuclease activity. Possible role in double strand break repair.
Ku70/80		Subunit of DNA-PK. Prevents telomere-telomere fusions regardless of telomere length. Roles in non homologous end joining pathway for double strand break repair.
MRE11A		Homologous recombination and double strand break repair. Part of Mre11-RAD50-NBS1 complex.
RAD50		Homologous recombination and double strand break repair. Part of Mre11-RAD50-NBS1 complex.
NBS1		Homologous recombination and double strand break repair. Part of Mre11-RAD50-NBS1 complex.
ATM	Mutations in ATM protein in Ataxia Telangietasia cells result in accelarated telomere attrition.	Roles in signal transduction and cell cycle control.

Table 1.1: Protein factors interacting with mammalian telomeres.

The table outlines some of the components of telomere complexes that have been identified to date. Proteins identified in mammalian and yeast telomeric complexes have a variety of functions including roles in telomere length homeostasis and capping status in addition to roles in telomere repair and recombination and positional gene silencing. In addition to the factors shown, components of the telomerase ribonucleoprotein reverse transcriptase must interact with the telomere in telomerase positive cells.

TISSUE TYPE	HISTOLOGY	TRAP +/TOTAL	COMMENTS	REFERENCES
LUNG	Normal (adjacent tissue)	3/68 (4%)	May represent inflammatory infiltrate	Hiyama et al
	Small cell carcinoma	15/15 (100%)		Hiyama et al
	Non-small cell carcinoma	98/125 (78%)		Hiyama et al
SKIN	Normal epidermis	4/9 (44%)	Weaker activity than carcinoma samples	Taylor et al
	Basal cell carcinoma	73/77 (83%)		Taylor et al
	Squamous cell carcinoma	15/18 (95%)		Taylor et al
	Melanoma	6/7 (86%)		Taylor et al
LIVER	Hepatocellular carcinoma – all stages	28/33 (85%)	Strong activity in 22/28	Tahara et al 1995
	Normal	0/4 (0%)		Tahara et al 1995
	Non-malignant chronic liver dieases	25/46 (54%)	Weak activity in all positive samples	Tahara et al 1995
GASTRIC	Primary gastric carcinomas – all histologies	17/20 (85%)		Tahara et al 1995
	Adjacent tissue	0/20 (0%)		Tahara et al 1995
	Colorectal adenocarcinomas	19/20 (95%)		Tahara et al 1995
	Adjacent mucosa	0/20 (0%)		Tahara et al 1995
BREAST	Normal	0/6 (0%)		Sugino et al 1996
	Fibrocystic disease	0/17 (0%)		Sugino et al 1996
	Carcinoma - all histologies	52/71 (73%)	Strong activity in 36/52	Sugino et al 1996
	Carcinoma - invasive ductal	45/54 (83%)	Strong activity in 31/45	Sugino et al 1996
	Carcinoma – invasive lobular	5/11 (45%)	Strong activity in 4/5	Sugino et al 1996
	Lymph node metastasis – node positive	22/30 (73%)	Strong activity in 17/22	Sugino et al 1996
	Lymph node metastasis – node negative	30/41 (73%)	Strong activity in 19/30	Sugino et al 1996
	Metastatic lesion	7/8 (88%)	Strong activity in 3/7	Sugino et al 1996
BLADDER	Normal epithelium	0/7 (0%)		Lin et al 1996
	Dysplasia	1/2 (50%)	Low activity	Lin et al 1996
	Bladder cancer	39/40 (97%)	Activity related to clinical stage – 16/16 (100%) grade 3 tumours had high activity, 62% grade 2 tumours had high activity, 20% grade 1 tumours had high activity.	Lin et al 1996

Table 1.2: Summary of TRAP assay results in normal and malignant human tissues.

The specific association of telomerase activity with human cancer has been documented in almost all human malignancies. The table gives a selection of some of the telomerase activity studies that have compared normal and malignant tissues.

CHAPTER 2
MATERIALS AND METHODOLOGY

2 MATERIALS AND METHODOLOGY

2.1 Materials

2.1.1 Plasmid vectors

2.1.1.1 Luciferase reporter plasmids (Chapter 3)

pGL3-Basic; basic, promoter-less cloning vector; negative control for luciferase assay; contains the firefly luciferase gene.

pGL3-Control; positive control vector for luciferase assay; contains the firefly luciferase gene driven by the SV40 promoter and enhancer sequences.

pGL3-hTERC; hTERC reporter vector; contains the firefly luciferase gene driven by an 876bp fragment of the hTERC promoter.

pGL3-hTERT; hTERT reporter vector; contains the firefly luciferase gene driven by a 541bp fragment of the hTERT promoter.

2.1.1.2 Co-transfection control plasmids (Chapter 3)

pSEAP-Control; positive control vector for SEAP assay; contains the SEAP gene under the control of the SV40 promoter and enhancer sequences; used for normalisation of transfection efficiency within a single cell line by cotransfection with luciferase plasmids.

2.1.1.3 Nitroreductase gene therapy plasmids (Chapter 4)

pd2NTR-Basic; basic, promoter-less cloning vector; contains the E.Coli nitroreductase gene. Generated by Rania Kakani.

pd2NTR-CMV; gene therapy vector for construction of stable cell lines; contains the E.Coli nitroreductase gene driven by the CMV immediate early promoter. Generated by Rania Kakani.

pd2NTR-hTERC; gene therapy vector for construction of stable cell lines; contains the E.Coli nitroreductase gene driven by an 876 bp fragment of the hTERC promoter. Generated by Rania Kakani.

pd2NTR-hTERT; gene therapy vector for construction of stable cell lines; contains the E.Coli nitroreductase gene driven by a 541bp fragment of the hTERT promoter. Generated by Rania Kakani.

2.1.1.4 Adenovirus cloning plasmids (Chapter 5)

pShuttle; transfer vector for cloning into pAdeasy-1 by homologous recombination in bacteria; contains a multiple cloning site for transgene insertion and the adenovirus serotype 5 left and right arms and terminal repeats.

pShuNT; intermediate transfer vector for cloning into pAdeasy-1; the E.Coli nitroreductase Sal I fragment from pd2NTR-hTR is ligated into the multiple cloning site of pShuttle.

pShuNT-hTERC; transfer vector for cloning into pAdeasy-1; the Hind III fragment of the hTERC promoter from pd2NTR-hTR has been ligated upstream of the nitroreductase gene in pShuNT.

pShuNT-hTERT; transfer vector for cloning into pAdeasy-1; the XhoI fragment of the hTERT promoter from pd2NTR-hTERT has been ligated upstream of the nitroreductase gene in pShuNT.

pAdeasy-1; cloning vector for insertion of transgenes into the E1 region of adenovirus serotype 5; contains an E1/E3 deleted adenovirus genome and sequences homologous with those in pShuttle.

pAd-NTR-hTERC; gene therapy vector for construction of recombinant adenovirus serotype 5 with the bacterial nitroreductase gene under the control of the hTERC promoter inserted into the E1 region of the adenovirus genome.

pAd-NTR-hTERT; gene therapy vector for construction of recombinant adenovirus serotype 5 with the bacterial nitroreductase gene under the control of the hTERT promoter inserted into the E1 region of the adenovirus genome.

2.1.2 Adenovirus vectors

Ad-hTERC-NTR; gene therapy vector; contains the E.Coli nitroreductase coding sequence under the control of a 876bp fragment of the hTERC promoter inserted by homologous recombination into the E1 region of an E1/E3 deleted serotype 5 adenovirus.

Ad-hTERT-NTR; gene therapy vector; contains the E.Coli nitroreductase coding sequence under the control of a 541bp fragment of the hTERT promoter inserted by homologous recombination into the E1 region of an E1/E3 deleted serotype 5 adenovirus.

Ad-CMV-LacZ; commercial reporter virus; contains the E.Coli LacZ gene under the control of the CMV immediate early promoter cloned into the E1 region of an E1/E3 deleted serotype 5 adenovirus.

2.1.3 Human cell lines used in this study

5637 bladder carcinoma cells

C33-A cervical carcinoma cells

A2780 ovarian adenocarcinoma cells

A549 lung adenocarcinoma cells

GLC4 small cell lung carcinoma cells

Colo320 double minute, colorectal adenocarcinoma cells

HT-29 colon carcinoma cells

SK-LU-1 lung adenocarcinoma cells

SUSM-1 in Vitro immortalised fibroblast cells

WI38 foetal lung fibroblast cells

IMR 90 foetal lung fibroblast cells

HMEC normal human mammary epithelial cells

NHEK normal human adult epidermal keratinocytes

HEK-293 Adenovirus E1a transformed human embryonic kidney cells

BE colon carcinoma cells

Cell Line	Cell Type	Telomerase Status (TRAP)	Comments
5637	Bladder carcinoma	Positive	Immortal cell. p53/pRb negative.
C-33 A	Cervical carcinoma	Positive	Immortal cell
A2780	Ovarian adenocarcinoma	Positive	Immortal cell
GLC4	Small cell lung carcinoma	Positive	Immortal cell
A549	Lung carcinoma	Positive	Immortal cell, gives rise to telomerase negative mortal subpopulations.
Colo320DM	Colorectal adenocarcinoma	Positive	Immortal cell
HT29	Colorectal adenocarcinoma	Positive	Immortal cell
BE	Colorectal adenocarcinoma	Unconfirmed	Immortal cell
SK-LU-1	Lung adenocarcinoma	Negative (ALT)	Immortal cell
SUSM-1	In Vitro immortalised fibroblast	Negative (ALT)	Immortal cell
WI-38	Foetal lung fibroblast	Negative	Mortal cell
IMR-90	Foetal lung fibroblast	Negative	Mortal cell
NHEK	Normal adult epidermal keratinocyte	Weakly positive	Mortal cell
HMEC	Mammary epithelium	Negative	Mortal cell
HEK-293	Embryonic kidney	Positive	Immortal cell

Table 2.1: Human cell lines used in this study.

2.1.4 Tissue Culture Reagents and Glassware

RPMI 1640 growth medium	Life Technologies
Minimum Essential Medium (MEM)	Life Technologies
Dulbeccos modified Eagles MEM	Life Technologies
Penicillin/streptomycin	Life Technologies
L-glutamine (200mM)	Life Technologies
Trypsin (2.5%)	Life Technologies
Foetal calf serum	Autogen Bioclear
Mammary epithelial cell growth medium	Clonetics
Epidermal keratinocyte cell growth medium	Clonetics
10cm^2 Falcon plates	Becton Dickinson
6-well plates	Iwaki
96-well plates	Iwaki
75 cm^2 flasks	Iwaki

2.1.5 Kits and Enzymes

Kit/reagent:	Supplier:
Superfect transfection reagent	Qiagen
Luciferase assay system	Promega
Protein assay reagent	BioRad
Great escape SEAP assay system	Clontech
Lyse-n-Go PCR reagent	Pierce
Taq core PCR kit	Qiagen
Advantage GC genomic PCR kit	Clontech
Advantage2 Taq polymerase mix	Clontech
Qiaex II gel extraction kit	Qiagen
Nucleospin II RNA extraction kit	Machery Nagel
NorthernMax blotting system	Ambion
Redi-prime II random prime labelling kit	Amersham
NE-PER protein extraction reagent	Pierce
ECL detection reagents for western blotting	Amersham
Adeasy adenovirus cloning system	Qbiogene
Restriction endonucleases and buffers	Life Technologies

	New England Biolabs
Qiaquick gel extraction kit	Qiagen
Rapid ligation kit	Roche
E.Coli DH5α competent cells	Life Technologies
S.O.C. medium	Life Technologies
Plasmid and cosmid miniprep kit	Hybaid
SMART-RACE II cDNA synthesis and PCR kit	Clontech
Advantage2 PCR kit	Clontech
TOPO-TA cloning kit	Invitrogen
RNAse A	Life Technologies
DNA molecular markers	Life Technologies
0.24-9.5kb RNA molecular marker	Ambion
Benchmark protein molecular weight marker	Life Technologies

2.1.6 *Chemicals*

Tris-hydrochloride

EDTA (Ethylenediamine tetra-acetic acid)

EGTA (ethylene glycol-bis(β-aminoethyl ether)n,n,n',n'-tetraacetic acid)

Absolute ethanol

Propan-2-ol

Sodium hydroxide

10 % SDS solution (sodium dodecyl sulphate)

Sodium chloride

Poly Ethylene Glycol 6000

Agarose

10x TBE Buffer

MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide)

Glacial Acetic acid

Tween 20 (polyoxyethylenesorbitan monolaurate)

Bromophenol blue

Xylene cyanole

Marvel (low fat powdered milk)

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase)

100 % methanol

Potassium acetate

DMSO (Dimethyl sulfoxide)

Ethidium bromide

Glycerol

α-^{32}P-deoxyctosine triphosphate

CB1954 (5-aziridin-1-yl-2,4-dinitrobenzamide)

2-mercapto ethanol

Geneticin sulphate

30% acrylamide (37.5:1 acrylamide:bis-acrylamide)

Ammonium persulfate

TEMED (n,n,n',n'-tetramethylethylenediamine)

Bicinchoninic acid solution

Copper (II) sulphate

Saturated phenol (25:24:1 phenol:chloroform:iso-amyl alcohol)

Magnesium chloride

25% glutaraldehyde

Potassium ferricyanide

Potassium ferrocyanide

2.1.7 Antisera and hybridomas

R36, rabbit polyclonal antiserum. IgG reactive against E.Coli nitroreductase. A kind gift from Dr. Steve Hobbs (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Surrey).

Anti-rabbit-IgG. HRP conjugated secondary. Reactive against rabbit IgG (Cell Signalling Technology Cat no. 7071-1).

2.1.8 Oligonucleotides for PCR and sequencing

Oligonucleotide sequences are given from 5' to 3' end:

Luc1f	CTACCGTGGTGTTCGTTTC
Luc1r	TTTGAATCTTGTAATCCTGAA
Shunt1f	GGCGTAACCGAGTAAGATTTGG
Shunt1r	TGCTGGATGGGCTGTATTGC
AdNTseq5a	CATTCCACTAAGGCATTTGATG
Adtranscript1	CAGAGTGGATGGCAAAACAG
Adtranscript2	AAAGAATATATAAGGTGGGG
AdDELr	ATCAAACGAGTTGGTGCTCATG
M13 primer set	Cat no. 46-0691/46-0690 (Invitrogen).
β-actin (exon2-3) set	Cat no. BAC 1004/BAC 1008 (Maxim biotechnology).

2.1.9 General laboratory supplies and miscellaneous

Provided by Beatson Institute Central Services:

LB-Medium (Luria-Bertani Medium)

Sterile distilled water

Sterile phosphate buffered saline (PBS)

Sterile PBS + EDTA (PE)

Sterile glassware and measuring pipettes

General:	**Supplier**
Ampicillin	Sigma
Kanamycin	Sigma
Falcon tubes 50ml and 15ml	Becton Dickinson
Universal containers 5ml, 20ml, 100ml	Bibby Sterilin
Micro-centrifuge tubes 1.5ml and 0.5ml	Elkay
Cell scrapers (rubber policeman)	Corning
Pipette tips	Elkay
X-ray film	Fujifilm
Positively charged nylon membrane	Boehringer-Mannheim
Nitrocellulose membrane	Millipore

2.1.10 Equipment

Medical Air Technologies Bio-MAT class II microbiological safety cabinet

Scharfe Systems Casy-1 cell counter

Forma Scientific CO_2 H_2O jacketed incubator

Olympus CK2 phase contrast microscope

Sigma 4K15/ Beckman GS-6R bench top centrifuges

Bio-Rad sub-cell GT electrophoresis gel tank/model 200 power supply

Pharmacia Biotech GeneQuant DNA/RNA calculator

M.J. Research PTC-200 Peltier thermal cycler

Beckman J6-MC centrifuge

Beckman Microfuge-R refrigerated micro-centrifuge

Turner Designs TD 20/20 luminometer

Alpha Laboratories Molecular Devices/ Dynex technologies MRX II microplate readers

Lab Systems Multidrop microplate filler

Kodak X-Omat 480 RA film processor

Bio-Rad Gel Doc 1000 UV transilluminator

Beckman DU650 spectrophotometer

Brunswick New Scientific G24/G25 orbital incubators

Atto AE 6450 polyacrylamide gel electrophoresis tank

Atto AE 6675 semi-dry blotting apparatus

2.2 DNA Recombination Techniques

2.2.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) mixtures were made using reagents from the Qiagen Taq-core PCR kit. Reactions typically contained 1µl template DNA along with final concentrations of master-mix components as follows: 1x PCR buffer; 0.5µM each primer; 0.2mM each of dATP, dTTP, dCTP, dGTP; 1 unit Taq polymerase; reaction volumes were made up to 25µL with sterile distilled H_20.

Cycling conditions were typically as follows: 1 initial denaturation step at 95°C for 2 minutes, followed by 25-35 cycles of 95°C 30 seconds (strand denaturation), 60°C-65°C (dependent on primer sequence) 30 seconds (primer annealing), 72°C 1 minute (chain extension). A final incubation at 72°C for 2minutes was included in all reactions. To analyse PCR products, typically 5µl was run out on a 2% agarose gel containing ethidium bromide for UV visualisation of DNA.

2.2.2 Restriction Digests

Restriction endonuclease digestion of plasmid DNA was performed as follows: 20µl reactions containing a maximum of 2µg plasmid DNA, 1x restriction endonuclease buffer, 1µl restriction endonuclease (typically 10U/µl). Reactions were made up to a volume of 25µl with sterile dH_20. Reactions were allowed to progress overnight at 37°C.

2.2.3 Gel Extraction

Extraction of restriction endonuclease digested DNA fragments from agarose gels was performed using the Qiaquick Gel Extraction Kit (Qiagen), according to the manufacturers instructions. Gels were viewed over a UV transilluminator and gel slices containing bands of interest were excised with a scalpel and 3x gel volumes of Buffer QG were added and

incubated at 50°C for approximately 10 minutes until the gel had dissolved. 1 gel volume of isopropanol was added and the sample was loaded into a spin column with collecting tube and centrifuged at 13000rpm for 1 minute. Flow-through was discarded and 750µl Buffer PE (wash buffer) was added to the spin column. The column was spun for 1 minute at 13000rpm, flow-through was discarded and the column was spun for a further minute to completely remove the wash buffer. DNA was then eluted in 50µl distilled water and the eluate was collected in a clean 1.5ml microfuge tube.

2.2.4 Ligation and Transformation

Ligation of restriction endonuclease digested DNA fragments was performed using the Roche rapid ligation kit. Typically, restriction endonuclease digested plasmid backbones and inserts were mixed at a molar ration of approximately 1:1. Next, 2µl of 5x DNA dilution buffer was added and the volume was made up to 10µl with dH_2O. 10µl 2x T4 DNA ligase reaction buffer and, finally, 1µl T4 ligase was added and the reaction was mixed and incubated at room temperature for 5 minutes. 1µl reaction was used for transformation of 50µl E.Coli DH5α.

Direct ligation of PCR products into pCR-II was performed using the Invitrogen TOPO-TA cloning kit. 1µl pCR-II was mixed with 4µl PCR product and incubated at room temperature for 5 minutes. 1µl stop solution was added and 1µl reaction was used for transformation of 50µl E.Coli DH5α.

1µl ligation reactions were mixed with 50µl competent E.Coli DH5α for 30 minutes on ice. The cells were then placed in a 42°C waterbath for 45 seconds and then returned to ice for 2 minutes. At this stage, 150µl S.O.C. medium was added to the transformation reaction and the cells were incubated for 1 hour at 37°C in a shake incubator. 100µl cells were spread onto agar plates containing an appropriate antibiotic and incubated overnight to allow the development of discrete colonies.

2.2.5 Glycerol Stocks

750µl aliquots of fresh overnight bacterial cultures were mixed with 750µl sterile 50% glycerol in Nunc cryotubes and were stored at -70°C. Maxipreps started from glycerol stocks used 50µl aliquots in 5ml Luria broth supplemented with appropriate antibiotics.

2.3 Generation of gene therapy vectors

2.3.1 Plasmid vectors

Cloning of the plasmid vectors pd2NTR-hTERC, -hTERT, -CMV, and –basic was performed by Rania Kakani (Plumb et al).

The promoter-less cloning vector pd2EGFP-1 was digested with NotI and EcoRI and the 3379bp fragment containing the SV40 poly-A signal and the Kanamycin/Neomycin resistance gene was isolated. This fragment formed the backbone of the gene therapy vectors. A 733bp NotI/EcoRI fragment containing the bacterial nitroreductase coding sequence was isolated from the vector pTargetntr1 and ligated into the pd2EGFP backbone to generate the basic cloning vector pd2NTR-basic.

The 876bp and 541bp proximal promoter sequences of hTERC and hTERT genes were isolated in a 914bp BamHI/BglII fragment and a 606bp XhoI fragment from plasmids containing these sequences which had previously been developed within the laboratory. These sequences were ligated upstream of the NTR coding sequence in pd2NTR-basic which had been digested with appropriate enzymes to form the plasmids pd2NTR-hTERC and pd2NTR-hTERT. An 896bp CMV promoter fragment was isolated by BamHI/BglII digestion of the plasmid vector pRc/CMV. This fragment was inserted upstream of the NTR coding sequence to generate the plasmid pd2NTR-CMV. The sequence and orientation of all inserts in the final panel of gene therapy vectors was checked by restriction digests and sequencing.

2.3.2 Adenovirus vectors

2.3.2.1 Initial cloning of recombinant Ad-hTERC-NTR and Ad-hTERT-NTR plasmids.

Cloning of hTERC-NTR and hTERT-NTR expression constructs into the E1 region of Ad5 genome was performed using the Adeasy system, as described in He et al (He et al). The transfer vector, pShuttle was first linearised with SalI and gel extracted. Next, the 740bp SalI fragment of pd2NTR-hTERC containing the coding sequence of bacterial nitroreductase, was digested and gel extracted. This fragment was ligated into linear pShuttle to generate the vector pShuNT. Insertion of the hTERT and hTERC promoter

sequences upstream of the NTR coding sequence was achieved by digestion of the plasmids pd2NTR-hTERT and pd2NTR-hTERC with restriction enzymes XhoI and HindIII, respectively, and the 606bp (hTERT promoter) and 889bp (hTERC promoter) fragments were isolated and ligated upstream of the NTR gene in XhoI or HindIII digested pShuNT to generate the transfer vectors pShuNT-hTERC and pShuNT-hTERT.

Transfer of expression constructs to the E1 region of the adenovirus backbone was accomplished by electro-co-transformation of the plasmid pAdeasy1 and the PmeI linearised intermediate cloning vectors pShuNT-hTERC and pShuNT-hTERT. Mixtures of plasmids and bacteria were pulsed with 2.8kV in a Hybaid Cell Shock electroporator. Homologous recombination occurred in the E.Coli strain BJ5183. Colonies containing Kanamycin resistant BJ5183 were selected and minipreps performed. PacI digestion of extracted DNA revealed several transformants with the correct banding pattern indicating that homologous recombination had taken place. Miniprep DNA from recombinant BJ5183 was then transformed into DH5a cells for maxipreparation of and long term storage of glycerol cryopreserved bacterial cultures. Sequence and orientation of inserts was confirmed by PCR using the primers Shunt1f and Shunt1r in 25µl PCR reactions containing final concentrations of 1x PCR buffer; 0.5µM each of the primers; 0.2mM each of dATP, dTTP, dCTP, dGTP; 1 unit Taq polymerase; volumes were made up to 25µL with sterile distilled H_20. Sequencing reactions were performed using the Applied Biosystems Big Dye Terminator system and reagents (Big dye terminator cycle sequencing reaction mixture) according to the manufacturers instructions using the primers shunt1f or shunt 1r in individual sequencing reactions.

2.3.2.2 Large scale preparation of infectious Ad-hTERC-NTR and Ad-hTERT-NTR

These manipulations were carried out by Q-Biogene in fulfilment of a contract with them.

Generation of infectious adenovirus vectors was performed by transfection of HEK293 E1a trans-complementing cells and harvesting of infectious supernatants. HEK293 cells, containing the E1a gene were plated out to give 70% confluence on the day of transfection

and were transfected with 5μg of each of the PacI linearised plasmids Ad-hTERC-NTR and Ad-hTERT-NTR using a calcium phosphate transfection.

50μl DNA (0.1μg/μl) was mixed with 169μl dH_2O and 5μl 2M $CaCl_2$. An Additional 26μl $CaCl_2$ was added after mixing and a second tube containing 250μl HBS (Hepes Buffered Saline) was prepared. Air was bubbled through the HBS with a pipette and during this time, the DNA/$CaCl_2$ mixture was added. Cells were removed from incubation and the transfection mixture was added drop-wise to the medium. Cells were incubated overnight, then the transfection solution was removed and the cells were rinsed in PBS. Cells were then incubated for approximately 14 days to allow for the formation of a cytopathic effect (CPE). After this stage, small scale amplification was performed using an initial volume of 0.1ml of crude virus from cell lysate supernatant to infect 10^5 cells. Virus was released from cells by 3 cycles of freeze/thawing between -20°C and 37°C. After several rounds of amplification using aliquots of up to 45ml crude virus released in tissue culture supernatant to infect up to $3x10^8$ 293 cells, the large-scale adenovirus preparation was performed by freeze/thawing to release virus and purification by $CsCl_2$ banding. Quantification of the viral titrations in Viral Particles (V.P.) was performed by measurements of optical density ($O.D._{260}$) and measurements of Plaque Forming Units (P.F.U.) were performed by plaque assay.

2.4 DNA Analysis

2.4.1 Minipreparation of plasmid DNA

Minipreparations of plasmid DNA were performed using the Hybaid Plasmid and Cosmid Miniprep kit according to the manufacturers instructions. Briefly, 1.5ml fresh bacterial culture was pelleted in a 1.5ml micro-centrifuge tube by centrifugation at 13000rpm for 1 minute in a Beckman refrigerated micro-centrifuge and the supernatant discarded. The pellet was resuspended in 50μl Pre-Lysis Buffer and vortexed to mix. 100μl Alkaline Lysis Buffer was added to the suspension and mixed by pipetting repeatedly until the solution became clear and viscous. Next, 75μl Neutralising Solution was added and mixed by vortexing briefly. The tube was spun for 2 minutes at 13000rpm to pellet cellular debris and the supernatant was removed to a spin filter.

Binding Buffer, containing a silica gel matrix was shaken to suspend the silica and 250µl was added to the spin filter, pipetting up and down to mix with the sample. The spin filter was then centrifuged at 13000rpm for 1 minute and excess liquid gathered in the collection tube was discarded and the filter placed back into the tube. 350µl Wash solution, containing added ethanol, was added to the filter and the tube spun for 1 minute at 13000rpm. Excess liquid was decanted and the filter spun again to dry the pellet. The filter was then placed in a fresh collection tube and plasmid DNA eluted in 50µl dH_2O by centrifugation for 30 seconds at 13000rpm.

2.4.2 Maxipreparation of plasmid DNA

50µL glycerol stock or 0.5mL fresh culture were added into 5ml L-broth containing an appropriate antibiotic and were incubated overnight in an orbital shaker at 37°C. The following day, cultures were expanded to 100ml and again incubated overnight. Cultures were divided into two 50ml falcon tubes and centrifuged at 4000rpm, 4°C for 10 minutes in a Beckman GS-6R centrifuge.

Supernatant was then discarded and the cell pellet resuspended in 5ml Solution I (50mM Tris-HCL pH8.0, 10mM EDTA pH8.0) by vortexing. With the tubes on ice, 10ml Solution II (0.2M NaOH, 1% SDS) was added and the contents gently mixed by inversion. The tubes were then incubated on ice for 10 minutes prior to the addition of 15ml Solution III (5M Potassium Acetate, 11.5% v/v glacial acetic acid). The contents were mixed thoroughly and centrifuged for 10 minutes at 4°C in a Beckman GS-6R centrifuge. Supernatant was filtered through a gauze and at least 0.6 volumes of isopropanol added to precipitate the DNA.

The pellet was collected by centrifugation at 4°C in a Beckman GS-6R centrifuge, the supernatant was removed and the pellet was resuspended in 1ml TE buffer (pH8) containing 10µg/ml RNase A, then incubated at 55°C for 30 minutes. After RNAse A treatment, the plasmid DNA was precipitated by adding 1ml Solution IV (13.5% polyethylene glycol 6000, 1.6M NaCl) and centrifugation at 13000rpm for 5minutes in a Beckman micro-centrifuge.

The DNA pellet was next resuspended in 500µl TE buffer and an equal volume of saturated phenol added. The mixture was vortexed and spun at 13,000 rpm in a refrigerated micro-centrifuge and the aqueous layer was removed to a fresh tube. Phenol extraction was

repeated and the DNA was precipitated from the final solution by addition of 1ml 100% ethanol and centrifugation for 10 minutes at 13,000rpm, 4°C. Finally, the plasmid DNA was washed with 1ml of 70% and 100% ethanol and resuspended in 50-100µl TE buffer.

2.4.3 Nucleic acid sequencing

Dideoxy chain termination sequencing reactions were performed by the in house sequencing service using the Applied Biosystems Big Dye Terminator system and reagents (Big dye terminator cycle sequencing reaction mixture) according to the manufacturers instructions. Sequence analyses were performed on ABI-PRISM 373A or 377.

2.4.4 Quantitation of DNA/RNA

Concentrations of nucleic acids in a solution were determined spectrophotometrically using a Pharmacia Biotech GeneQuant nucleic acid calculator. The instrument was first calibrated using TE only as a blank and samples were transferred to quartz capillary tubes for measurement. Optical Density measurements were taken at 260nm. An O.D. measurement of 1 at 260 (A_{260}=1) corresponds to a concentration of approximately 50µg/ml of double stranded DNA or to a concentration of 40µg/ml for RNA. The ratio between the readings at 260nm and 280nm (OD_{260}:OD_{280}) provides a measure of the sample purity. For DNA samples, an OD_{260}:OD_{280} of between 1.8 and 2.0, and for RNA samples, an OD_{260}:OD_{280} of between 2.0 and 2.2, were taken to be sufficiently pure for all techniques used in this study.

2.5 Analysis of Gene Expression

2.5.1 Northern Blotting

Before commencing work, all work surfaces and equipment were thoroughly cleaned with RNaseZAP (Ambion).

2.5.1.1 RNA Purification

Extraction of total RNA from human cell lines was performed using the Machery-Nagel Nucleospin II total RNA isolation kit according to the manufacturers instructions. Briefly, cells grown in culture were trypsinised to release them from the tissue culture surface and

were resuspended in 10ml ice cold PBS. The suspension was then spun down at 1200rpm for 6 minutes and the supernatant aspirated. The cell pellet was then rinsed further by resuspension in a further 1ml ice cold PBS and the cell suspension was stored on ice.

Cell suspensions were transferred to DEPC-treated micro-centrifuge tubes and pellets were recovered by centrifugation at 13,000 rpm for 1 minute. The supernatant was removed and cells were lysed by vortexing in 400µl cell lysis buffer (buffer RA1) containing 4µl 2-mercaptoethanol. 300µl 100% ethanol was added and mixed by vortexing to precipitate nucleic acids and the entire contents of the tube were transferred to a spin filter and centrifuged at 10,000rpm for 1 minute. Next, 10µl DNase I was added to 90µl Dnase reaction buffer and 95µl of the reaction mix was added directly to the central membrane of the spin filter for 15 minutes at room temperature. The DNase reaction was stopped by adding 500µl buffer RA2 (DNase stop solution) and centrifuged at 13,000rpm for 1minute. The spin filters were next washed twice by addition of 600µl, then 350µl of wash buffer (RA3) containing added ethanol, followed by centrifugation at 13,000rpm for 1minute and 2minutes, respectively. RNA was eluted in 100µl RNase free dH_2O and quantified by spectrophotometry.

2.5.1.2 Sample and gel preparation

The day before electrophoresis, 25µg or 30µg equivalent RNA samples were mixed with 0.1 volumes of 5M Ammonium Acetate and 2.5 volumes of 100% ethanol, then precipitated overnight at –20°C. On the day of electrophoresis, samples were centrifuged at 13000rpm at 4°C for 15 minutes to pellet the RNA and the supernatant was decanted. After this, the tubes were spun again briefly and residual fluid removed. The pellets were then air dried and resuspended in 5µl RNase free water. 15µl formaldehyde loading dye was added to each sample and the RNA was denatured for 15 minutes at 65°C in a thermal cycler. The tubes were briefly spun in a micro-centrifuge and stored on ice until ready to load.

For a 150mL denaturing gel, 135ml RNase free H_2O was added to 1.5g agarose and melted in a microwave oven for 2 minutes. The molten gel was equilibrated to 55°C in a waterbath then, in a fume hood, 15mL 10x denaturing gel buffer (Ambion) containing formaldehyde was added. The gel was poured into a casting tray to a thickness of approximately 0.6cm and allowed to set.

2.5.1.3 Electrophoresis and blotting

Specific RNA's were separated by electrophoresis at 5V/cm for approximately 2.5 hours in 1x MOPS gel running buffer (Ambion) then transferred to a positively charged nylon membrane using a downward transfer apparatus. Construction of the transfer apparatus was as follows: a 4cm stack of dry paper towels was laid out on a flat area of bench. On top of these, 2 pieces of dry 3mm Whatman blotting paper, cut to a size slightly larger than the gel were laid. On top of the dry blotting paper, 3 pieces of blotting paper, pre-wet in transfer buffer (Ambion) were laid out and rolled out flat with a sterile pipette to remove any air bubbles. Next, the positively charged nylon filter, pre-soaked in transfer buffer, was laid on top and rolled out flat. On top of the filter, the gel was laid out and rolled flat and on top of the gel, 3 more pieces of pre-wet blotting paper were rolled out. Finally, 3 long pieces of blotting paper were rolled out and placed with one end in a reservoir of transfer buffer, to act as a bridge for the downward flow of buffer, and the entire apparatus was weighted with the casting tray and a 100ml bottle.

Transfer was allowed to continue for 1.5 hours before disassembly of the apparatus. Immediately after disassembly, the filter was washed in 1x MOPS running buffer then the RNA was UV cross-linked to the membrane in a Stratagene UV Stratalinker 2400.

2.5.1.4 Pre-hybridisation and Hybridisation

Ultrahyb pre-hybridisation/hybridisation buffer (Ambion) was first warmed to 68°C in a water bath, then 20ml was added to a large roller bottle. The membrane was added to the bottle, unrolled, and allowed to pre-hybridise for 1 hour at 42°C. After pre-hybridisation, ^{32}P-dCTP labelled probe was added to the bottle, as described below, and allowed to hybridise to the target RNA overnight at 42°C.

2.5.1.5 ^{32}P Labelling of DNA probes

Labelling of DNA probes was performed using the Redi-Prime II kit according to the manufacturers instructions. The nitroreductase DNA probe was generated by digesting the plasmid pd2NTR-hTERC with SalI (Gibco) and gel extraction of the 740bp fragment which spans the entire coding sequence of the nitroreductase gene. The control DNA probe, ribosomal 18s, was supplied by Ambion. The probe was diluted to a concentration of 25ng/µl (10ng/µl for the 18s) and 1µl was added to 44µl TE buffer. The probe was

denatured at 95°C for 5 minutes in a thermal cycler then allowed to cool on ice. The entire tube contents were spun down, transferred to a Redi-prime tube and flick-mixed. In the radioactive suite, 5µl ^{32}P-dCTP (1.85MBq total activity) was added to the tube and the contents mixed and transferred to a 37°C heated block for 10 minutes. After this incubation, 2µl 0.5M EDTA (pH 8.0) was added to the tube to stop the labelling reaction and the contents were denatured by incubation for 5 minutes at 100°C in a boiling waterbath. After this step, the tube was incubated on ice for 5 minutes, then the contents were briefly spun down. The entire contents of the tube were added to a roller bottle containing prehybridised positively charged nylon filters with cross linked RNA.

2.5.1.6 Washing filters and autoradiography

After overnight incubation at 42°C, the hybridisation mixture was decanted and the filter was subjected to 1 wash for 15 minutes at 65°C with 0.1% SDS, 2x SSC buffer (diluted from stock solution of 20x SSC (3M sodium chloride, 0.3M sodium citrate, pH 7.0), followed by a further 2-3 washes in 0.1% SDS, 0.1x SSC at 65°C. After each wash, and prior to removal from the bottle, the counts from the bottle were monitored with a geiger counter. When the counts reached approximately 30cps, the filter was removed and allowed a flat wash in 0.1% SDS, 0.1x SSC at room temperature in a plastic basin on an orbital shaker. When specific bands were detectable and the counts from the bands were approximately 5-10cps, excess fluid was blotted from the filter and it was wrapped in 2 pieces of Saran wrap.

To perform auto-radiography, in the dark room, 1 sheet of Fujifilm X-ray film was placed in contact with the filter inside an auto-radiography cassette and the cassette was left for 1-2 days at -70°C. Films were developed in a Kodak X-Omat 480 RA processor.

2.5.2 Rapid Amplification of cDNA Ends (RACE)

2.5.2.1 First strand cDNA synthesis

RNA samples were extracted from virus infected C33-A cells, as described in section 2.4.1.1, and cDNA libraries were constructed using the SMART RACE cDNA amplification kit (Clontech). The reactions produced 2 cDNA libraries for each sample,

5'RACE ready cDNA and 3'RACE ready cDNA (the incorporation of an extended 3' primer (SMART oligo) which anneals to extra nucleotides added by the terminal transferase activity of Superscript reverse transcriptase allows for the generation of complete 5' ends). For the first strand synthesis of 5' RACE ready cDNA, 1µl RNA was mixed with 1µl 5'cDNA synthesis primer (CDS), 1µl SMART II oligo and 2µl dH_20. For preparation of the 3' RACE ready cDNA, 1µl RNA was mixed with 1µl 3'CDS and 3µl dH_20 in 0.5ml micro-centrifuge tubes. Both tubes were incubated at 70°C for 2 minutes then cooled for 2 minutes on ice. The tubes were briefly spun to collect liquid at the bottom of the tubes and the following was added to each tube: 2µl 5x first-strand buffer, 1µl DTT (20mM), 1µl dNTP mix (10mM) 1µl superscript reverse transcriptase. The tubes were then incubated for 15 minutes at 42°C. The reactions were then diluted with 100µl Tricene-EDTA buffer, heated for 7 minutes at 72°C and stored at -20°C.

2.5.2.2 5' and 3' RACE

All amplifications were carried out using components of the SMART-RACE and Advantage2 Polymerase mix kits (Clontech). For amplification of the 5' and 3' ends of NTR cDNA, reverse transcribed from mRNAs of Ad-hTERC-NTR and Ad-hTERT-NTR infected cells, 2.5µl cDNA was mixed with final concentrations of the following in a 50µl reaction. For the 5' RACE reactions, 2.5µl 5'RACE ready cDNA with 1x Universal Primer Mix, 0.2µM Shunt1r, 1x Advantage 2 PCR buffer, 0.2mM each dNTP, 1µl Polymerase mix and dH_2O to 50µl.

For amplification of the 3' ends, reactions contained the following: 2.5µl 3'RACE-ready DNA, 1x Universal Primer Mix, 0.2µM AdNTseq5a, 1x Advantage2 PCR buffer, 0.2µM each dNTP, 1µl polymerase mix and dH_2O to 50µl. The amplifications were allowed to progress for 35 cycles of 94°C 30 seconds, 68°C 30 seconds, 72°C 3 minutes. After amplification, size and specificity of the amplification products was checked by electrophoresis of 5µl samples on a 2% ethidium bromide-agarose gel.

PCR reactions were directly cloned into the plasmid pCRII (TOPO-TA cloning kit (Invitrogen) for transformation in DH5a, maxipreparation of plasmid DNA and sequencing of the 5' and 3' ends of the Ad-hTERC-NTR and Ad-hTERT NTR transcripts. Sequencing reactions were performed by the Beatson sequencing service using the Applied Biosystems Big Dye Terminator system and reagents (Big dye terminator cycle sequencing reaction mixture) according to the manufacturers instructions. Individual sequencing reactions of

the 5' end contained one of the primers M13f, or M13r, while sequencing of the 3' end used the primers M13f, M13r, Adtrans1, or Adtrans2.

2.5.3 Western blotting

2.5.3.1 Protein Purification and Quantitation for Western Blotting

Purification of the cytoplasmic protein fraction for Western blotting was achieved using the NE-PER differential nuclear and cytoplasmic protein extraction kit provided by the Pierce chemical company according to the manufacturers instructions. Briefly, medium was aspirated from a 75cm^2 flask of cultured cells and the cell layer rinsed once in PBS. Cells were scraped off in 1.5ml PBS using a rubber policeman and transferred to a 1.5ml micro-centrifuge tube. The cell pellet was collected by centrifugation at 500rcf, 4°C, for 3 minutes and the supernatant was removed with a pipette.

200µl ice cold CER I (Cytoplasmic Extraction Reagent) was added and the pellet was vortexed for 15 seconds at the maximum setting to mix. The tube was incubated for 10 minutes on ice. Next, 11µl ice cold CER II was added and the sample was vortexed for 5 seconds then incubated for 1 minute on ice. Next, the sample was vortexed again for 5 seconds then spun down at 16000rcf for 5 minutes to pellet nuclei. The supernatant (cytoplasmic fraction) was removed to a clean, pre-chilled tube and stored at –70°C until quantitation.

Quantitation of cytoplasmic protein extracts was accomplished by BCA/Cu (II)SO_4 assay using a 6 point BSA standard with concentrations 80µg/ml, 100µg/ml, 200µg/ml, 400µg/ml, 1000µg/ml, 2000µg/ml. Undiluted samples and samples diluted 1:10 were incubated for 30 minutes at 37°C alongside BSA standards in a solution of BCA/Cu (II)SO_4, after which time colorimetric changes were quantified using a Dynex MRX II microplate reader.

2.5.3.2 Western Blotting

20µg protein equivalents were made up to 31µl with dH_2O and 2.5µl 2-mercapto ethanol + 16.5µl 3x Loading Buffer (6% SDS, 30% glycerol, 100mM tris pH 6.8, 0.01%

bromophenol blue) was added to each. Samples were denatured at 100°C for 10 minutes in a thermal cycler then placed briefly on ice, spun down briefly and left on ice until ready to load. Cellular proteins were electrophoresed in a 12% SDS-polyacrylamide gel with 5% stacking gel for approximately 2.5 hours at 200V in 1L 1x Running Buffer (25mM tris, 0.2M glycine, 0.1% SDS, pH8.3). After SDS-PAGE, the top, bottom and sides of the gel were trimmed with a scalpel and the proteins were blotted onto Millipore nitrocellulose membrane previously prepared by soaking for 1 minute in 100% methanol, rinsing for 1 minute in distilled water and soaking in 1x transfer buffer (48mM tris, 40mM glycine, 0.037% SDS) diluted in 20% methanol.

6 pieces of 3mm Whatman blotting paper were soaked in Transfer Buffer and placed onto to the bottom conductive plate of a semi-dry blotting apparatus and rolled out to remove bubbles. Next, the Millipore filter with gel on top was placed on top of the blotting paper and on top of that, a further 6 pieces of 3mm Whatman blotting paper soaked in Transfer Buffer were rolled out. The proteins were transferred for 1 hour at 20V. After transfer, the blotting apparatus was dismantled and the filter was blocked overnight at 4°C in TBS-T (0.7% tween 20) containing 5% non-fat dried milk.

The following day, filters were probed for 2 hours at room temperature with a 15ml of 1:50 dilution of primary antibody in TBS-T containing 5% milk. After probing with the primary antibody, filters were washed 3 times for approximately 10 minutes each with TBS-T, then probed for 1 hour with 20 ml of a 1:3000 dilution of Horse Radish Peroxidase (HRP) conjugated secondary anti-rabbit IgG antibody. Following probing with the secondary antibody, filters were washed 3 times for approximately 10 minutes each with TBS-T then bound HRP was detected using ECL western blotting HRP detection reagents (Amersham) according to the manufacturers instructions.

For each filter, 2ml ECL reagent 1 was mixed with 2ml reagent 2 and the entire 4ml volume was transferred drop wise to the filter ensuring an even distribution of the detection reagents across the filter and the filter was incubated at room temperature for 1 minute. After 1 minute, excess detection reagent was shaken off the filter and the filter was wrapped in 1 thickness of Saran wrap. The HRP signal was detected by exposing photographic film to the filter for approximately 1 minute before developing. Transferred proteins were stained with amido black for loading controls. Filters were soaked in amido black for 5 minutes at room temperature, the rinsed several times with dH_2O.

2.6 Cell Culture and reporter gene assays

2.6.1 Maintenance and storage of mammalian cell lines

All cell lines were routinely cultured in 75cm^2 flasks containing 20mL appropriate growth medium, typically supplemented with 2mM L-glutamine, 10% FCS and 2.5mL penicillin/streptomycin solution. WI-38 and IMR-90 cells were supplemented with 20% FCS. HMEC and NHEK normal adult cell lines were maintained in complete mammary epithelial and keratinocyte growth media, respectively, obtained from Clonetics. Cells were trypsinised for subculturing with 3mL trypsin (0.25%) diluted in PE (PBS + EDTA) then resuspended to an appropriate concentration in growth medium. All cell lines were regularly tested for mycoplasma contamination.

For long term storage, cells were typically resuspended to a concentration of 1x10^6 cells/ml in growth medium supplemented with 10% FCS and 10% DMSO. 1mL aliquots of cell suspension were cooled to -70°C overnight, then transferred to liquid nitrogen. To recover cells from liquid nitrogen, cryovials were warmed to 37°C then the contents were transferred to 19mL warm growth medium in a 75cm^2 flask and incubated overnight. The next day, medium containing DMSO cryopreservant was removed and normal, complete medium was added back.

2.6.2 Transient transfection

Cells were trypsinised and seeded into 6-well plates the day prior to transfection at a concentration sufficient to give 60-80% confluence on the day of transfection. For a single well, on the day of transfection, 3μg of each plasmid DNA, pGL3-Basic; pGL3-Control; pGL3-hTERC; or pGL3-hTERT (see section 2.1.1) was mixed with 97μL serum free growth medium and 7.5μL superfect transfection reagent (Qiagen) in separate tubes and incubated for 15 minutes at room temperature to allow formation of transfection complexes. Next, 600μL complete growth medium (10% FCS) was added to the transfection complexes and the growth medium was aspirated from each cell culture well. The cells were incubated for 2 hours in the presence of the transfection reaction mixtures. After transfection, cells were rinsed twice in PBS then incubated for 48 hours in appropriate growth medium. To ensure reproducibility of transient transfections, all transient transfections were carried out in duplicate and were repeated at least 3 times. As described below, transfection efficiencies between cell lines were controlled using a semi-

quantitative PCR assay, and transfection efficiencies within a single cell line were normalised both by co-transfection with a second SEAP reporter and by measurement of cellular protein equivalents.

2.6.3 Generation of stable cell lines

Human cell lines were seeded the day prior to transfection into 10cm Falcon dishes at a concentration sufficient to allow 60-80% confluence on the day of transfection. 10μg of each plasmid DNA, pd2NTR-Basic; pd2NTR-CMV; pd2NTR-hTERC; or pd2NTR-hTERT (see section 2.1.1) was mixed with 300μL serum free growth medium and 50μL superfect transfection reagent (Qiagen) in separate tubes and incubated for 15 minutes at room temperature to allow formation of transfection complexes. Next, 3mL complete growth medium (10% FCS) was added to the transfection complexes and the growth medium was aspirated from each cell culture vessel. The cells were incubated for 2 hours in the presence of the transfection reaction mixtures. After transfection, cells were rinsed twice in PBS then incubated for 48 hours in appropriate growth medium.

48 hours after transfection, the cells were trypsinised and reseeded at $1x10^6$ cells per plate in appropriate growth medium containing 1mg/ml G418 (Geneticin-sulphate). Selection medium was refreshed every 3-4 days and the cells were grown for approximately 2-3 weeks until the appearance of stable clones. At this stage, the cells were trypsinised, the clones were pooled and the cultures were expanded under selection until sufficient cells were present to store in liquid nitrogen. Stable cell lines were routinely cultured under selection except during experiments, for which the selection was removed

2.6.4 Luciferase reporter assay

48 hours post-transfection, cells were harvested by scraping from the wells of 6-well plates in 1ml PBS. Cells were spun down for 1 minute at 13000rpm and the supernatant was decanted. Cells were lysed by vortexing in 100μl 1x Cell Culture Lysis Buffer (Promega) and incubated on ice for 10 minutes. The lysates were then spun down for 1 minute at 13000rpm and kept on ice. Protein concentrations were determined by Bio-Rad assay using Bio-Rad protein assay reagent and 2.5μg protein equivalents were used for luciferase assay according to the manufacturers instructions. Protein samples were mixed with 25μl Luciferase Assay Reagent (Promega) at room temperature and the reactions were placed

into a Turner Designs TD 20/20 luminometer to measure cumulative luminosity over a 15 second period.

To ensure reproducibility, all transfections were carried out in duplicate wells and repeated at least 3 times. DNA for transfection was carefully quantitated both by spectrophotometry, using the Pharmacia Biotech GeneQuant spectrophotometer, and by direct visualisation by gel electrophoresis. In each experiment, all constructs were analysed together with the basic, promoter-less cloning vector, pGL3-Basic, and with the positive control, pGL3-Control, which contains the luciferase gene driven by the SV40 promoter and enhancer sequences.

2.6.5 SEAP reporter assay

Normalisation of luciferase reporter assays was performed by several means. Cotransfection with a second reporter (in this case, Secreted Alkaline Phosphatase, SEAP) is a useful way to control for variation in transfection efficiency within a single cell line. The SEAP assay is especially useful, as quantification of SEAP activity can be determined from cell culture supernatant and the integrity of the transfected cells is not compromised. SEAP assays were performed using the "great escape SEAP assay kit" (Clontech) according to the manufacturers instructions.

Cells transfected with luciferase reporters were cotransfected with 1.5μg SV40-SEAP reporter per well. 48 hours post-transfection, 110μL cell culture medium was removed from each well to a micro-centrifuge tube and centrifuged at 13,000 rpm for 1 minute to pellet any cells. 100μL was removed to a fresh tube. For each sample, 75μL 1x dilution buffer was added to 25μL cell culture supernatant and mixed in a 0.5μL micro-centrifuge tube. The samples were incubated at 65°C for 30 minutes then cooled on ice for 2 minutes before equilibrating to room temperature. 100μL assay buffer was added to each sample and incubated for 5 minutes at room temperature. 100μL 1.25mM CSPD substrate (diluted 1:20 in chemiluminescent enhancer) was added to each tube and incubated at room temperature for 40 minutes. Cumulative light units were measured over 15 seconds on a Turner Designs TD 20/20 luminometer and luciferase activities were adjusted by the formula: $Luc_{normalised} = Luc_{well} \times (SEAP_{mean} / SEAP_{well})$.

2.6.6 Semi quantitative PCR detection of luciferase gene

In order to control for transfection efficiency across cell lines, where the application of a second reporter would not be appropriate due to cell specific differences in SV40 promoter activity, a semi-quantitative PCR assay for the presence of the luciferase gene in genomic DNA extracted from post-transfected nuclei was developed. After luciferase assay (described above), the nuclear pellet was collected by centrifugation for 1 minute at 13000rpm, 4°C and the cell lysate supernatant was removed with an 18 gauge hypodermic needle. 50μL Lyse-N-Go PCR compatible DNA extraction reagent (Pierce) was added to each nuclear pellet and the nuclei were loosened by vigorously vortexing at maximum speed for 15 seconds. The tubes were then placed in a thermal cycler for 3 cycles of 65°C 30 seconds, 8°C 30 seconds, 65°C 90 seconds, 97°C 180 seconds, 8°C 60seconds, 65°C 180 seconds, 97°C 60 seconds, 65°C 60 seconds, 80°C 10 minutes. Cycling conditions were according to the manufacturers instructions with slight modifications (cycle number increased from 1 to 3, duration of final 80°C incubation modified from a final hold step to 10 minutes).

Standard amplification reactions typically contained 1μL nuclear lysate as template in a total volume of 25μL, together with final concentrations of 1x PCR buffer; 0.5μM each of the primers Luc1f and Luc1r; 0.2mM each of dATP, dTTP, dCTP, dGTP; 1 unit Taq polymerase; volumes were made up to 25μL with sterile distilled H_20.

Reactions for the amplification of luciferase were allowed to progress for 25 cycles of: strand denaturation at 95°C for 30 seconds; primer annealing at 60°C for 50 seconds; primer extension at 72°C for 40 seconds. Reactions for the amplification of β-actin were allowed to progress for 25 cycles of 94°C 1 minute, 60°C 1minute, 72°C 1minute. The primers (Luc1f, Luc1r) used in the amplification of luciferase are given in section 2.1.8, the forward and reverse genomic control primers for the amplification of β-actin were supplied by Maxim Biotechnologies (catalogue numbers BAC 1004/1008).

2.6.7 Adenovirus infection of mammalian cell lines

Human cell lines were seeded into 6-well plates the day prior to infection at a density sufficient to give approximately 80% confluence the following day (typically 4-5x10^5 cells

per well in 2ml appropriate growth medium). The cells were incubated at 37°C overnight in humidified incubators at an appropriate percentage CO_2.

On the day of infection, growth medium was removed from one of the wells and the cells were trypsinised and counted. Adenovirus infection suspensions were prepared by adjusting the concentration of infectious units in PBS such that 100µl contained an appropriate multiplicity of infection (M.O.I.) (either 1, 10, 50, or 100 plaque forming units (P.F.U.) per cell in 100µl in all experiments). Cells were incubated in the presence of 100µl of virus suspensions for 1 hour at 37°C with rocking of the culture vessel every 15 minutes.

After the 1 hour, the virus suspension was aspirated, fresh growth medium containing 2% FCS for infectivity assays, or 10% FCS for cytotoxicity assays was added back to the cells and the cells were incubated for an appropriate period prior to downstream assays.

2.6.8 Lac Z reporter assay for adenovirus infectivity

Cells were infected with a CMV-LacZ adenovirus, as described above, for 1 hour at 37°C at a multiplicity of infection of either 1, 10, 50, or 100 P.F.U. per cell. Following infection, cells were incubated overnight in fresh growth medium containing 2% Foetal Calf Serum. 24 hours post-infection, the medium was aspirated from the cells and the cell layer was rinsed 3 times with 4°C PBS. The cells were then fixed by incubation on ice for 20 minutes in 4ml of fixative solution per well containing 0.2% glutaraldehyde, 5mM EGTA, 2mM $MgCl_2$ in ice cold PBS.

Next, the cells were rinsed a further 3 times in 1ml PBS and after the last rinse, staining solution was added (500µg X-Gal, 2.5mM $K_3Fe(CN)_6$, 2.5mM $K_4Fe(CN)_6$ in 25mL PBS). The cells were incubated in staining solution for 24 hours in the dark. In order to assess the efficiency of virus mediated transgene transduction to cell layers, the following day the staining solution was removed and the cells were rinsed with PBS, then the proportion of blue cells was assessed. For each multiplicity of infection, 5 random fields were counted at 20x objective of a Zeiss phase contrast microscope using a 21mm diameter counting graticule. Typically, 500-1000 cells per well were counted and all experiments were repeated at least twice.

2.7 MTT assay

Cell lines or cells treated with individual viruses or at different multiplicities of infection were trypsinised and seeded in triplicate into the central 10 columns of flat bottomed 96 well plates (80 wells per plate) at a density of 800-1000 cells per well. For assays with more than one cell line, cells were seeded in the first 4 rows only (40 wells per cell line). The outer columns on either side were left as blanks, containing growth medium only. Each independent cell line or treatment was therefore seeded into one half of a 96 well plate and was set up in triplicate. Cells were incubated at 37°C in an appropriate concentration of CO_2 and allowed to divide for 2 days prior to drug administration. On the day of the drug challenge, 4-fold serial dilutions of CB1954 were prepared in cell growth medium to give 8 concentrations with an initial concentration of 400μM. Thus, the concentrations of CB1954 in the titration were as follows: 400μM, 100μM, 25μM, 6.25μM, 1.56μM, 0.39μM, 0.098μM, 0.024μM. The medium was aspirated from wells using an 18 gauge hypodermic needle attached to an aspirator and the drug was titrated across the central 8 columns of each 96 well plate by adding 100μL volumes of a single concentration to each of the wells in a single column. The column on either side of the plate which was not exposed to drug served as an untreated control against which the cytotoxic effects of CB1954 could be estimated. Cells were incubated at 37°C for 24 hours in the presence of the drug, after which the drug was aspirated and the cells were fed with 200μL of fresh growth medium and allowed to recover for a further 3 days, replenishing the medium daily.

At the end of the recovery period, medium was aspirated and 200μL fresh medium was added, in addition to 50μl of 0.5% MTT in PBS (Sigma). Cells were incubated for 4 hours in the dark, then the medium and MTT was aspirated. The purple MTT-formazan product in the bottom of each well was dissolved in 200μL DMSO and 25μL Soreensons Glycine Buffer (0.1M glycine, 0.1M NaCl, pH 10.5) using a Labsystems Multidrop plate filler and OD measurements were made at 570nm using a Molecular Devices microplate reader. For an individual experiment, each data point on kill curves were plotted as the mean percentage of the untreated control, calculated across triplicate plates (each consisting of 4 individual values) for each independent drug concentration. In order to determine the IC50 values for various treatments, the 50% y-intercept value for each individual plate was calculated using the Softmax 2.32 analysis package and the mean of these triplicate measurements was taken to be the IC50. Sensitisation values for individual treatments are

taken to be the fold difference between the IC50 values for stable cell lines harbouring a basic promoter-less vector and those harbouring telomerase or CMV promoter gene therapy vectors (or, in the case of virus infected cells, between the IC50 values of the mock infected cells and those which were infected by a gene therapy adenovirus). All experiments were repeated at least 3 times and final sensitisation values presented are the means and standard errors derived from all 3 independent experiments.

2.8 Software analysis packages

2.8.1 Sequence analysis, restriction fragment analysis and primer design

All sequence analysis, development of cloning strategies, restriction fragment analysis and primer design were performed using Vector NTI 6.0 (Informax).

2.8.2 Optical density analysis for micro-titre assays

The Softmax 2.32 microplate analysis package was used for analysis of raw and analysed data from microtitre assays and to derive IC50 values for data sets.

2.8.3 UV gel documentation

Analysis and photography of ethidium bromide agarose gels visualised under UV was accomplished using the Bio-Rad UV gel doc 1000 UV transilluminator with Molecular Analyst software.

CHAPTER 3

DIFFERENTIAL ACTIVITIES OF THE TELOMERASE hTERC AND hTERT PROMOTERS IN NORMAL AND CANCER CELLS

- Semi-quantitative detection of luciferase gene in transfected nuclei.
- hTERC and hTERT promoters are more active in cancer cells than mortal cells.
- hTERC and hTERT promoters are not universally strong in cancer cells.
- hTERC is a stronger promoter than hTERT.

3 Differential activities of the telomerase hTERC and hTERT promoters in normal and cancer cells.

3.1 Abstract

The hTERC and hTERT sub-units of telomerase are differentially regulated at a transcriptional level between normal and cancer cells. Therefore hTERC and hTERT promoter reporter constructs should restrict transgene expression to cancer cells. Luciferase assays using a 541bp hTERT promoter construct and an 876bp hTERC promoter construct indicated that tumour derived cell lines had higher activities for both promoters than two mortal foetal lung fibroblast cell strains or two telomerase negative ALT cell lines. Analysis of a larger panel of cell lines indicated that the promoters are not universally strong in cancer cell lines and that promoter activities of some human cancer cell lines more closely resemble those of ALT or mortal cells. The hTERC promoter was a stronger promoter than hTERT in all cell lines. These data validate the use of hTERC and hTERT promoter sequences in a transcriptionally directed cancer gene therapy strategy, but also suggest that not all cancer cells will necessarily be effectively targeted by cytotoxic genes under the control of these promoters. However, it might be possible to overcome possible problems arising from low level transgene expression in therapeutic models using hTERT promoter constructs with the use of the hTERC promoter.

3.2 Introduction

The hTERC component of telomerase is present at low levels in a number of tissues, including embryonic kidney cells and primary B-cells (Avilion et al. 1996), foetal lung and skin tissue, sinovial cells and adult prostate tissue, testis, ovary, brain, spleen, liver and kidney (Feng et al. 1995). However, strong up-regulation of hTERC expression in cancer has been documented in a number of studies. The levels of hTERC are elevated relative to normal tissue during colorectal carcinogenesis (Avilion et al. 1996; Yan et al. 2001) and during neuroblastoma progression (Reynolds et al. 1997). Additionally, up-regulation of hTERC has been described in cancer cell lines derived from breast, lung and colon cancers in addition to leukemia and melanoma cells (Feng et al. 1995; Avilion et al. 1996). *In situ*

hybridisation analysis reveals strong hTERC signals concentrated over tumour cells in tissue sections, but not over adjacent normal tissue (Soder et al. 1998; Park et al. 1999).

Similarly, the hTERT component of telomerase, which is expressed at lower levels than hTERC, is up-regulated in cancer. Most studies of hTERT expression have utilised RT-PCR for the detection of hTERT mRNA. Up-regulation of hTERT relative to normal tissue has been described in every major human malignancy, including renal cell carcinoma (Paradis et al. 2001), oral squamous cell carcinomas (Kim et al. 2001) and squamous cell carcinomas of the lung (Shibuya et al. 2001). Immunohistochemical detection of hTERT was recently reported in a subset of cells in normal tissues, including keratinocytes, lymphocytes, mammary epithelial cells, basal cells of intestinal crypts and villi, and basal cells of the oesophageal mucosa. However, strong activity was detected specifically in cancer cells from tissue sections of a number of tumour types, including small cell lung cancer, non-small cell lung cancer, hepatocellular carcinoma, pancreatic duct cell carcinoma, and Wilms tumour (Hiyama et al. 2001).

Thus, both components of telomerase are expressed at low levels in some normal tissues, but are up-regulated in cancer. There is considerable evidence that the expression of hTERC and hTERT are regulated at a transcriptional level. Therefore, the promoter sequences of these genes are potentially useful for the development of transcriptionally directed cancer gene therapy strategies. The efficacy of such an approach will depend on a number of factors, including the capacity of promoter sequences to drive high level transgene expression in specific target cell populations. From this point of view the telomerase regulatory sequences are excellent candidates for novel transcriptional targeting approaches due to the prevalence and specificity of expression of the hTERC and hTERT components in cancer.

The ability of an 876 bp fragment of the hTERC promoter to drive high level expression of the luciferase gene in reporter constructs transfected into bladder carcinoma cells was previously described (Zhao et al. 1998). An hTERT promoter reporter containing a 541 bp fragment of the proximal hTERT promoter, characterised as a region of high activity containing a single c-Myc binding site and 5 SP1 binding sites (Cong et al. 1999; Takakura et al. 1999; Wick et al. 1999) has been subsequently generated. These plasmid vectors, together with a commercial SV40 promoter driven positive control plasmid, pGL3-control, and the promoter-less luciferase cloning vector pGL3-basic, were transiently transfected into a number of normal and cancer cell lines.

A number of problems are associated with transient transfection systems for analysis of promoter regulation: firstly, within transfected cells, plasmids exist in an artificial configuration and copy number that can lead to aberrant function of control elements. Hundreds or thousands of plasmid molecules may enter a transfected cell while transcription factors may be present only in limited quantities. Thus, relatively few plasmids may actually associate with the full complement of factors necessary for normal promoter regulation. Moreover, the episomal and non-replicating nature of plasmids may lead to a loss of function resulting from the fact that the plasmids are not in an appropriate chromatin configuration.

The use of appropriate internal controls is considered to minimise some of the problems associated with transient transfection. Common methods used to normalise the results of transfection experiments include the use of protein equivalents or co-transfection with, and normalisation to, a second reporter such as the secreted form of human alkaline phosphatase (SEAP). A drawback to the use of this kind of internal control is that sequences in control viral promoter regions included in the internal control plasmid may compete for limiting transcription factors. Therefore, while a second reporter can be a useful control, it does not diminish the need for experimental repetition. Moreover, the activities of commonly used control promoters will vary across cell lines and, therefore, these are not good control assays across a range of cell lines. In this study, all experiments have been conducted at least three times, all transfections included positive (SV40 promoter) and negative (no promoter) control luciferase reporter constructs, and assays both of protein equivalents and a second SV40-SEAP reporter activity have been evaluated as systems to minimise transfection artefacts. No significant differences were observed between patterns of activity of the promoter constructs within single cell lines whether protein equivalents or SEAP assay was used. Therefore, all data presented herein are the results obtained with 2.5μg protein equivalents. Additionally, a semi-quantitative PCR assay that compares the relative quantities of reporter gene in post-transfected nuclear extracts has been developed to allow for analysis of transfection efficiency across cell lines.

The data presented in this chapter show clear differences in the transcriptional activity of both hTERC and hTERT promoters between normal and cancer cell lines which may be of therapeutic value and, additionally, the hTERC promoter is demonstrated to be the stronger of the two, supporting the use of both sequences in further studies of telomerase directed gene therapy.

3.3 Control experiments minimise issues of transfection efficiency

3.3.1 Development of a semi-quantitative PCR assay for transfection efficiency.

While co-transfection with a second reporter is a useful tool for normalisation of the activities of different transfected constructs within individual cell lines, the activities of commonly used control promoters, such as CMV or SV40, can vary between cell lines and, therefore, it is not an appropriate control for transfection efficiency between cell lines. During the exponential phase of PCR amplification, the amount of product formed is directly related to the amount of input DNA; this has been exploited recently for the development of quantitative real-time PCR systems based on measurements of fluorescence intensity. For this reason, it was decided that a semi-quantitative PCR assay for detection of the luciferase gene in genomic DNA extracts from post-transfected nuclei would give a reasonable indication of transfection efficiencies between cell lines. The use of this approach assumes the fulfilment of several key criteria: genomic DNA must be released with a similar efficiency from nuclear pellets of all samples; the assay must be genuinely semi-quantitative and therefore able to detect transfection of different amounts of input DNA; finally, detection must be within the exponential phase of the PCR amplification. Figures 3.1, 3.2 and 3.3 show the results of experiments designed to evaluate the validity of this assay based on these criteria.

Figure 3.1 shows an experiment designed to determine a cycle number at which detection of the luciferase gene in genomic DNA extracts from post-transfected nuclei would still be within the exponential phase of amplification: genomic DNA was released from post-transfected nuclear pellets in 50μl of the PCR compatible cell lysis reagent "Lyse-n-go", as described in materials and methods. 1μl aliquots were amplified alongside high (10ng) and low (10pg) concentrations of pGL3 control plasmid for 20-35 rounds of amplification. While the band intensities of the products of the high input concentrations of plasmid DNA appeared to plateau between 25 and 35 cycles, the low concentrations had not reached the plateau phase even after 35 cycles and the band intensities of the standards did not overlap at any cycle number. Samples of genomic DNA extracted from the nuclei of cells

transfected with 3μg of luciferase positive control plasmid were amplified in parallel for 25 cycles. The band intensities of these products were considerably less than those of the high concentration of standard at all cycle numbers and greater than those of the low concentration standard, except at the highest cycle number. Based on these results, an optimal cycle number of 25 cycles was selected for future experiments.

The next requirement that had to be satisfied was to ensure that genomic DNA containing tranfected luciferase plasmids could be extracted from the nuclear pellets of post-transfected cells with a similar efficiency across all samples assuming standard transfection conditions. To this end, 11 samples were transfected with 3μg pGL3 plasmid and 48 hours later, samples were lysed according to the luciferase assay protocol. Nuclear pellets were spun down and DNA released into 50μl Lyse-n-go. Reactions containing 1μl nuclear extract were amplified for 25 cycles and the products were run out on an agarose gel. Figure 3.2 shows the similarity in band intensities of products of the reactions containing post-transfection nuclear extract, providing evidence that the DNA extraction protocol used in this assay can efficiently and consistently release DNA from the nuclei of multiple samples.

In figure 3.3, the semi-quantitative characteristics of the assay are evaluated: 5637 cells were transfected in duplicate with a titration of pGL3 control plasmid (either DNA with no transfection reagent, lanes 3 and 4; 100ng, lanes 5 and 7; 500ng, lanes 9 and 11; or 2500ng, lanes 13 and 15). 48 hours later, genomic DNA was extracted from nuclear pellets and amplified. The increase in band intensity with increasing amounts of input DNA (lanes 3, 4, 5, 7, 9, 11, 13, 15) indicated that the assay could detect relatively large differences in transfection efficiency. Loading controls are shown in the lower panel. As a control to prevent artefacts arising from the presence of DNA in transfection mixtures that may not have entered the cell, 2 wells were incubated in the presence of DNA but with no added transfection reagent (lanes 3 and 4). The absence of a band in these products indicated that the assay detected exclusively DNA that had been internalised. As an additional control to ensure that products were amplified from DNA extracted from the nuclear pellet, rather than contaminating DNA from the cytosolic supernatant, DNA was extracted from the cytosolic fractions of the lysate using the Qiaex II gel extraction kit and these extracts were subjected to amplification. The banding pattern in lanes 5-16 indicated that the majority of DNA in the samples was found in the nuclear DNA extracts, but small amounts could be extracted from the supernatant at higher transfected plasmid concentrations. That the clean-up removed PCR inhibitors is supported by the presence of a weak band in the

supernatant sample in lane 18; no DNA was transfected in these cells, but the lysate was spiked with 50ng pGL3 control plasmid prior to the clean-up. These results indicated that PCR detection of luciferase could distinguish large differences in transfection efficiency and, together with the results in figure 3.1and 3.2, suggested that the assay had a useful application as a part of a larger overall approach to minimising problems associated with transfection efficiency.

Figure 3.4 shows an example of the application of this assay to transfection experiments. Mean promoter activities of duplicate wells in this representative experiment in 5637 bladder carcinoma, C33-A cervical carcinoma and A549 lung adenocarcinoma cells are shown in figure 3.4(a): in this experiment, 5637 cells had an hTERC promoter activity some 79 times greater than that of hTERT, while this value was 35 and 4 for A549 and C33-A cells, respectively. C33-A cells had the greatest hTERC and hTERT activity of all 3 cell lines, approximately 30-fold greater than either 5637 or A549 for the hTERT promoter and approximately 1.8- and 4-fold greater than 5637 and A549, respectively, for the hTERC promoter. The figure also illustrates the variation which was observed for the SV40 promoter between cell lines, lending strength to the argument that co-transfection with a second reporter driven by a strong promoter such as SV40 is not an appropriate control for transfection efficiency between cell lines. In 5637 cells, the SV40 promoter was stronger than both hTERC and hTERT, while in A549 cells SV40 activity was greater than hTERT but of a similar level to hTERC. Lastly, in C33-A, both hTERC and hTERT promoters were stronger than the constitutively active SV40 promoter.

Figure 3.4 parts (b) and (c) illustrate the use of semi-quantitative detection of the luciferase gene in DNA extracted from post-transfected nuclei as a control for transfection efficiency: (b) shows that all luciferase band intensities, both within cell lines and between cell lines, were similar, while (c) shows the β-Actin loading control. The similarity of band intensities provides strong evidence that observed differences in promoter activities between cell lines were due to genuine, cell-specific regulation of promoter activity and not due to major differences in transfection efficiency. This approach was applied to all transfection experiments described in this chapter and all samples from all cell lines exhibited similar band intensities both within and between cell lines suggesting that transfection efficiencies were broadly similar between cell lines, with the exception of Colo320dm and BE cells which demonstrated average band intensities across samples of approximately 2-fold higher and lower, respectively, than other cell lines. The luciferase

assay data for these 2 cell lines have not been modified, since the assay is not a direct measure of transfection efficiency.

3.4 Differential activities of the telomerase hTERC and hTERT promoters in normal and cancer cells.

3.4.1 The hTERC and hTERT promoters are more active in cancer cell lines than mortal and ALT cells

Since transcriptional regulation of telomerase genes has been proposed to be a major mechanism involved in the transition to an immortal phenotype in cancer progression, it is of considerable interest to begin to understand how hTERC and hTERT promoters function in normal and cancer cells. In order to directly compare differences in promoter activity between normal and cancer cell lines, a transient transfection approach was adopted: 3μg luciferase reporter plasmids containing fragments of either the hTERC or hTERT promoter were transfected into a panel of mortal and cancer cell lines. 3μg each of hTERC-luciferase, hTERT-luciferase, SV40-luciferase and a basic luciferase cloning vector lacking a promoter were transfected into each cell line used in each experiment. 5637 cells were included in all experiments alongside other cell lines in order to ensure that relative differences in promoter activities observed between cell lines were repeatable. To control for possible differences in transfection efficiency of the different constructs within a single cell line a second reporter was employed, as described above, and to ensure observed differences between the luciferase activities of cancer and mortal cell lines tested were not due to differences in transfection efficiency across cell lines, a semi-quantitative PCR assay described above, was employed. Figures 3.5 and 3.6 show the summary of experimental data for the (a) hTERC and (b) hTERT promoters in mortal and cancer cell lines.

There is a clear differential in the activities of both promoters between normal and cancer cell lines, with hTERC promoter activities for the telomerase positive cancer cells illustrated in figure 3.5(a) lying in the range between 249.7 light units (5637 bladder carcinoma) and 2002.7 light units (A2780 ovarian adenocarcinoma). 2 other cell lines, Colo320dm (colorectal carcinoma) and GLC 4 (small cell lung cancer) had comparatively high promoter activities of 1957 and 1479 light units respectively, although it should be noted that luciferase specific semi-quantitative PCR detected a higher transfection

efficiency in Colo320dm than in other cell lines. The mortal foetal lung fibroblast cell strains IMR90 (60.1 light units) and WI38 (6.1 light units) had low promoter activities within the ranges of 4.2-fold (smallest difference) to 33.4-fold (largest difference) lower than the cancer cell lines for IMR-90 and 40.8-fold (lowest) to 328-fold (highest) for WI-38. This gave a clear indication that the hTERC promoter is a valid target for therapeutic strategies.

The hTERT promoter (figure 3.5(b) was in all cases less active than hTERC, but the differential in activity could be considered to be more pronounced, in that the mortal fibroblast strains showed no luciferase activity above background light levels (0.7 light units for IMR-90 and 0.09 light units for WI-38). In contrast, the cancer cell lines had luciferase activities ranging from 29.3 light units (GLC4) to the extremely high activity of Colo320dm (833.6 light units). Again, it should be noted that Colo320dm had a higher transfection efficiency than other cell lines which had lower hTERT activities (41.2 light units for A2780 and 79.4 light units for C33a). The fold differences between mortal and cancer cell hTERT promoter activities could not be precisely quantified, as light units detected in the mortal cells were not above background and were not integers.

Although these experiments demonstrated a valid therapeutic window for the use of both telomerase promoters, the analysis of a larger panel of cancer cell lines, as shown in figure 3.6, revealed a large variation in the capacity of these promoters to drive transgene expression between cancer cell lines. The hTERC promoter (figure 3.6(a)) was highly active in the 5 cell lines discussed above (5637; C33-A; A2780; Colo320dm; GLC4), but a subset of immortal cell lines displayed much lower activities more comparable with mortal cells. BE colon carcinoma cells and the ALT cell line SK-LU-1 had luciferase activities of 0.2 (background levels) and 3.4 (approximately half that of the mortal strain WI-38), respectively, while 2 other telomerase positive cancer cell lines, A549 and HT-29, also had low activities of 69.6 light units and 83.5 light units respectively. Surprisingly, the ALT cell line SUSM-1, which has been shown to shut off hTERC promoter activity by methylation (Hoare et al. 2001), showed a low to moderate activity of 143 light units, suggesting that transient transgene expression from the exogenously introduced hTERC promoter is possible in this cell line.

As shown in figure 3.6(b), 5637 cells, which had a moderate activity for the hTERC promoter had very low hTERT activity (4 light units), as did A549 (2.9 light units), HT-29 (2.1 light units), SUSM-1 (2.7 light units) and BE (background levels). These levels of

activity were within the range of 7- to 397-fold lower than the highly active cancer cell lines and, therefore, were more similar to the activities of mortal cell strains. This suggests that strategies for cytotoxic transgene expression based exclusively around the hTERT promoter may be limited in the range of cells that can be efficaciously targeted and suggests a role for use of the hTERC promoter in circumstances where hTERT cannot effectively target expression.

3.4.2 The hTERC promoter is more active than hTERT in cancer cell lines

Figure 3.7 shows a summary of the activities of the hTERT promoter relative to hTERC derived from 3 independent experiments across the cell lines in this model. Although a relative figure could not be quantified in those 4 cell lines (WI-38, IMR-90, BE, SK-LU-1) which had only background levels of hTERT promoter activity, the remaining 9 immortal cell lines all had high hTERC activity which ranged from 2.9-fold (colo320dm) to 66.8-fold (5637) greater than hTERT. Most cell lines had an hTERC:hTERT ratio in the range of approximately 20- to 40-fold difference (HT-29, 24.9-fold; A549, 28-fold; A2780, 32.6-fold; GLC4, 34.8-fold; SUSM-1, 43-fold, in ascending order) but colo320, C33-A and SuSa cells all had hTERT activities comparable to hTERC (2.9-fold, 4.1-fold and 5.7-fold respectively). The range of activities between these two promoters suggests that the hTERC promoter drives higher level expression of transgenes than the hTERT promoter and may therefore ultimately prove more useful than hTERT for particular therapeutic settings.

Thus, not only is the hTERC promoter validated for use in transcriptionally based therapeutics, but the probability arises that exclusive use of the hTERT promoter in telomerase gene therapy may limit the target range to exceptionally highly TERT expressing tumour cells. Therefore, the development of a double-edged system using both hTERC and hTERT promoters allows an element of choice and may be preferable to a strategy based upon a single expression construct.

3.5 Discussion

The data presented here show large differences in the activity of both the hTERC and hTERT promoters between mortal and cancer cells. These data are consistent with the idea that the components of telomerase are differentially regulated at a transcriptional level

between mortal and cancer cells and lend weight to the argument that these promoters may prove useful for transcriptionally directed anticancer gene therapy strategies. Both hTERT and hTERC were stronger promoters in cancer cells than in normal cells, with fold differences in activity between mortal and cancer cells of up to 328-fold for the hTERC promoter while effectively no hTERT activity was detected in mortal cell strains. These differences were not the result of large differences in transfection efficiency (as assayed by semi-quantitative PCR) but, rather, reflected cell specific regulation of the activity of the hTERC and hTERT promoters.

The hTERC and hTERT promoters are not universally strong promoters in cancer cell lines. Analysis of 13 cancer, ALT and mortal cell strains revealed a range of activity within cancer cell lines which are likely to be the result of regulation by undefined cell specific factors. Interestingly, A549 lung adenocarcinoma cells have previously been shown to give rise to mortal subpopulations with no detectable telomerase activity when plated out as single cells (Katakura et al. 1997), suggesting that a fraction of these cells may have no promoter activity, thereby masking the activity of positive cells in transfection experiments.

The relationship between telomerase holoenzyme activity and transcriptional regulation of telomerase components is not absolute: numerous other post-transcriptional and post-translational regulatory mechanisms such as alternative splicing (Kilian et al. 1997; Ulaner et al. 2000) phosphorylation (Li et al. 1997; Li et al. 1998; Yu et al. 2001), the action of hsp90 and p23 chaperone proteins (Holt et al. 1999; Akalin et al. 2001), hTERT multimerisation (Beattie et al. 2001), and telomere capping status (reviewed in Blackburn 2001) may modulate enzyme activity. Therefore, low telomerase activity detected by TRAP assay does not necessarily preclude the use of transcriptionally directed gene therapy against telomerase positive cancer cell lines. An essential component of efficacious gene therapy systems, however, is the ability to drive high level transgene expression in a target cell population. An interesting outcome of this study, therefore is the observation that the hTERC promoter can consistently drive higher level luciferase expression than hTERT.

Figure 3.1: Determination of cycle number for semi-quantitative PCR amplification of the luciferase gene from post-transfected nuclear extracts.

PCR reactions containing either water (lane 1), 10ng or 10pg (lanes 2-9) of pGL3control plasmid were allowed to proceed for 20 cycles (lanes 2 and 3), 25 cycles (lanes 4 and 5), 30 cycles (lanes 6 and 7), or 35 cycles (lanes 8 and 9). In parallel, reactions containing 1μl nuclear DNA extract from 5637 cells transfected with 3μg pGL3control, as described in materials and methods, were amplified for 25 cycles. Relative band intensities of the 2 control standards and the transfected samples at 25 cycles confirmed that the detection was still within the exponential phase of amplification. Experiments were repeated 3 times. The figure shows a representative gel.

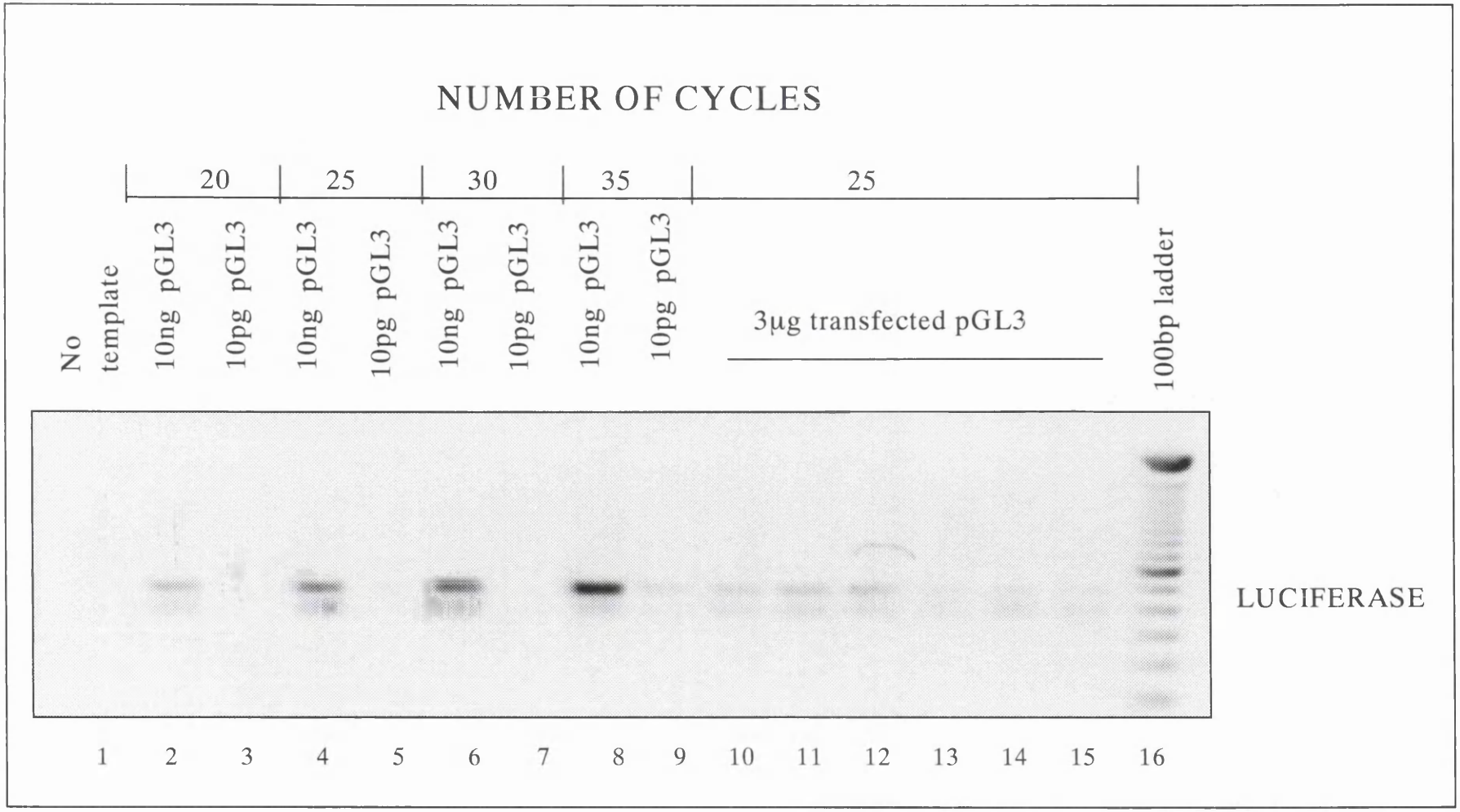

NUMBER OF CYCLES
20
25
30
35
25
No template
10ng pGL3
10pg pGL3
10ng pGL3
10pg pGL3
10ng pGL3
10pg pGL3
10ng pGL3
10pg pGL3
3µg transfected pGL3
100bp ladder
LUCIFERASE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 3.2: Validation of the equal efficiency of the DNA extraction protocol between samples for semi-quantitative PCR.

5637 cells were transfected with 3μg pGL3 plasmid DNA and 48 hours later, cells were lysed according to the luciferase assay protocol. Nuclear pellets were collected and DNA was extracted in 50μl "lyse-n-go". 1 μl aliquots were amplified for 25 cycles. The similarity in band intensities in lanes 3-13 indicated that the DNA procedure was of similar efficiency across multiple samples.

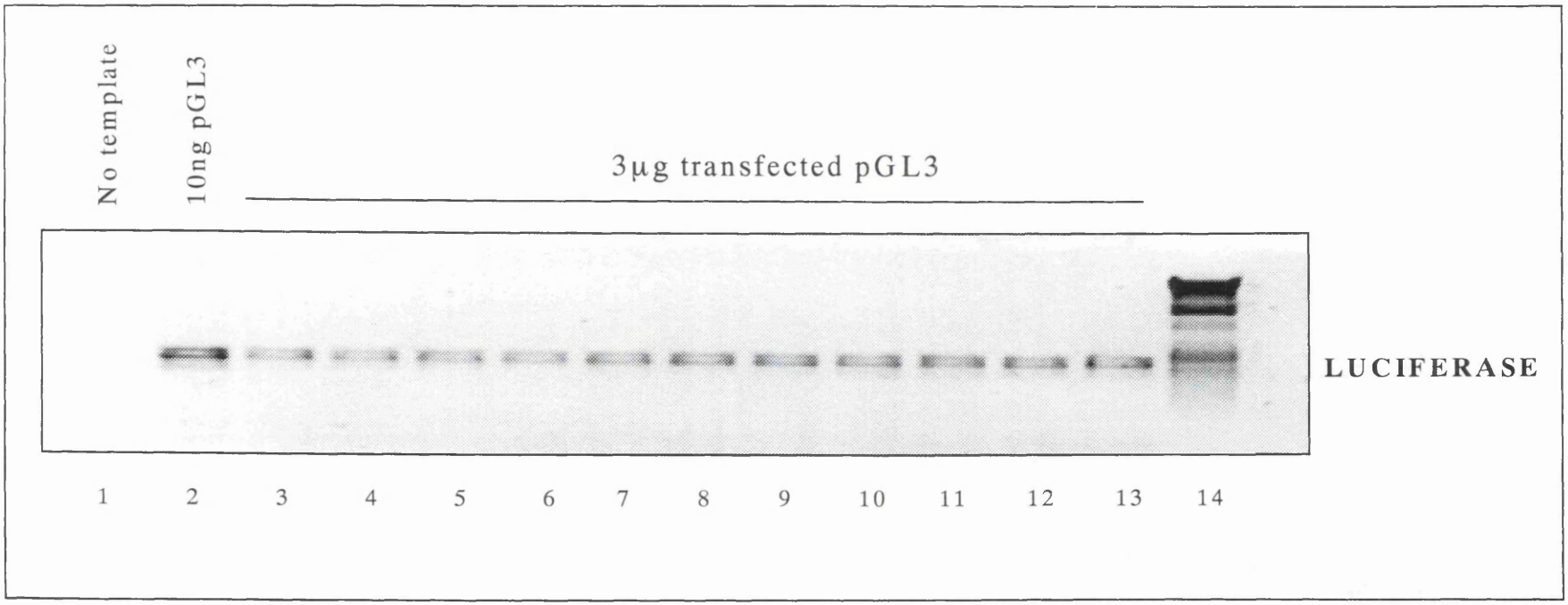

No template
10ng pGL3
3µg transfected pGL3
LUCIFERASE
1
2
3
4
5
6
7
8
9
10
11
12
13
14

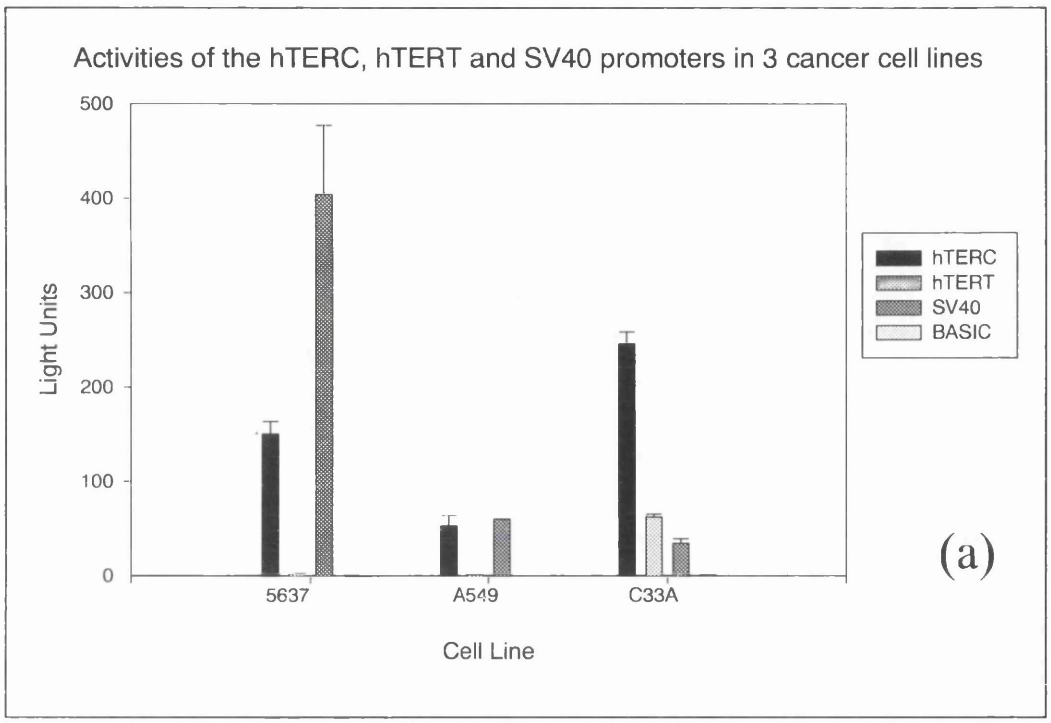
Figure 3.3: Semi-quantitative PCR detection of luciferase gene in post-transfected nuclear extracts detects a titration of transfected DNA.

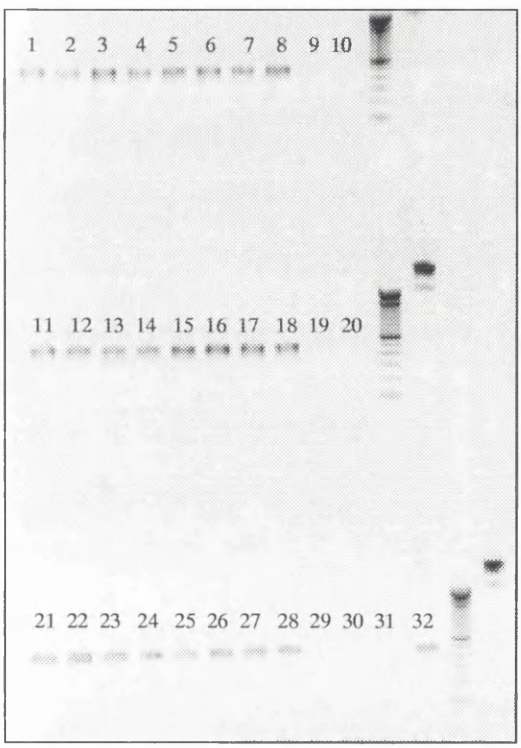
5637 cells were transfected with a titration of pGL3control plasmid (transfection reagent with either no DNA, 100ng, 500ng, or 2500ng) and 48 hours later cells were lysed according to the luciferase assay protocol, genomic DNA was extracted from nuclear pellets and 1μl extract was amplified for 25 cycles (lanes 3, 4, 5, 7, 9, 11, 13, 15). Increasing band intensities with increasing input DNA confirmed the semi-quantitative nature of the assay. To ensure that the reaction primarily detected DNA extracted from nuclei, the cytosolic (protein) supernatant fraction was cleaned with the QiaEx II gel extraction kit and the cleaned samples were subjected to amplification (lanes 6, 8, 10, 12, 14, 16). Spiking the cytosolic fraction of one of the untransfected samples with 50 ng plasmid DNA prior to clean-up (lane 18) revealed a weak band, confirming that the cleaning process removed any PCR inhibitors. Detection of the β-Actin gene revealed similar quantities of genomic DNA in all samples. Experiments were repeated 3 times. The figure shows a representative gel.

LUCIFERASE
No template
20ng pGL3control
No transfected DNA
No transfected DNA
100ng pGL3
100ngcytosolcleanup
100ng pGL3
100ngcytosolcleanup
500ng pGL3
500ngcytosolcleanup
500ng pGL3
500ngcytosolcleanup
2500ng pGL3
2500ngcytosolcleanup
2500ng pGL3
2500ngcytosolcleanup
No DNAcytosol
DNA spikedcytosol
100bp ladder
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
B-ACTIN
No template
100ng genomic DNA
No transfected DNA
No transfected DNA
100ng pGL3
100ng pGL3
500ng pGL3
500ng pGL3
2500ng pGL3
2500ng pGL3
λ/Hind III
100bp ladder
1 2 3 4 5 6 7 8 9 10 11 12

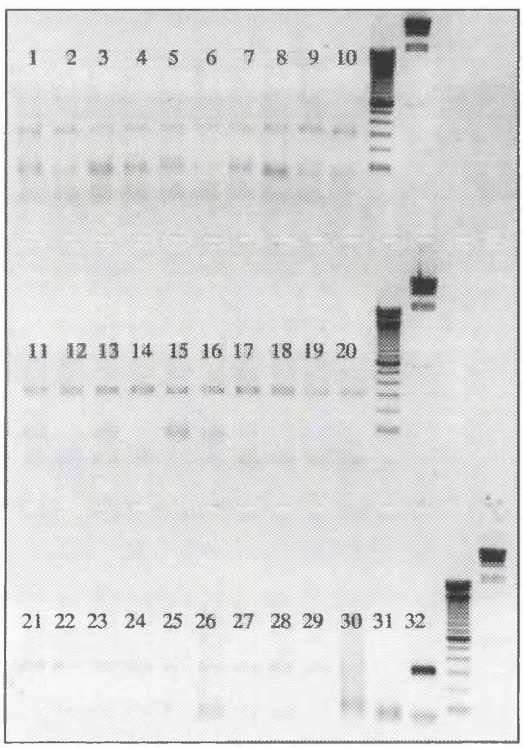
Figure 3.4: Semi-quantitative detection of luciferase gene in post-transfected nuclear extracts reveals similar transfection efficiency between cell lines.

(a) shows the results of a single luciferase assay in 5637 bladder carcinoma, C33-A cervical carcinoma, and A549 lung adenocarcinoma cells. Cells were transfected in duplicate with each of hTERC-and hTERT-luciferase in addition to SV40-luciferase and a basic, promoter-less vector. Bars represent the mean and standard error of the duplicates. (b) shows the detection of luciferase gene from post-transfected nuclear extracts. Lanes 1-10 are 5637 cells, lanes 11-20 are C-33A cells and lanes 21-30 are A549 cells. Lanes 31 and 32 are no template and 10ng plasmid DNA controls. Lanes 1,2,11,12,21,22 are extracts from cells transfected with hTERC-luciferase; extracts from lanes 3,4,13,14,23,24 were transfected with hTERT-luciferase, while the cell extracts in lanes 5,6,15,16,25,26 and 7,8,17,18,27,28, respectively, were transfected with SV40-luciferase and the basic vector. Cells whose extracts are shown in the empty lanes 9,10,19,20,29,30 were incubated with DNA but no transfection reagent. The similarities in band intensity, both for different constructs within a single cell line and for constructs between cell lines, suggests that the observed differences in light units in the luciferase assay reflect genuine cell-specific differences in promoter activity and not large differences in transfection efficiency. (c) shows the control detection of β-actin. Lane numbers are identical to those in (b).


Activities of the hTERC, hTERT and SV40 promoters in 3 cancer cell lines
Light Units
500
400
300
200
100
0
5637
A549
C33A
Cell Line
hTERC
hTERT
SV40
BASIC
(a)


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
21 22 23 24 25 26 27 28 29 30 31 32

(b)


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
21 22 23 24 25 26 27 28 29 30 31 32

(c)

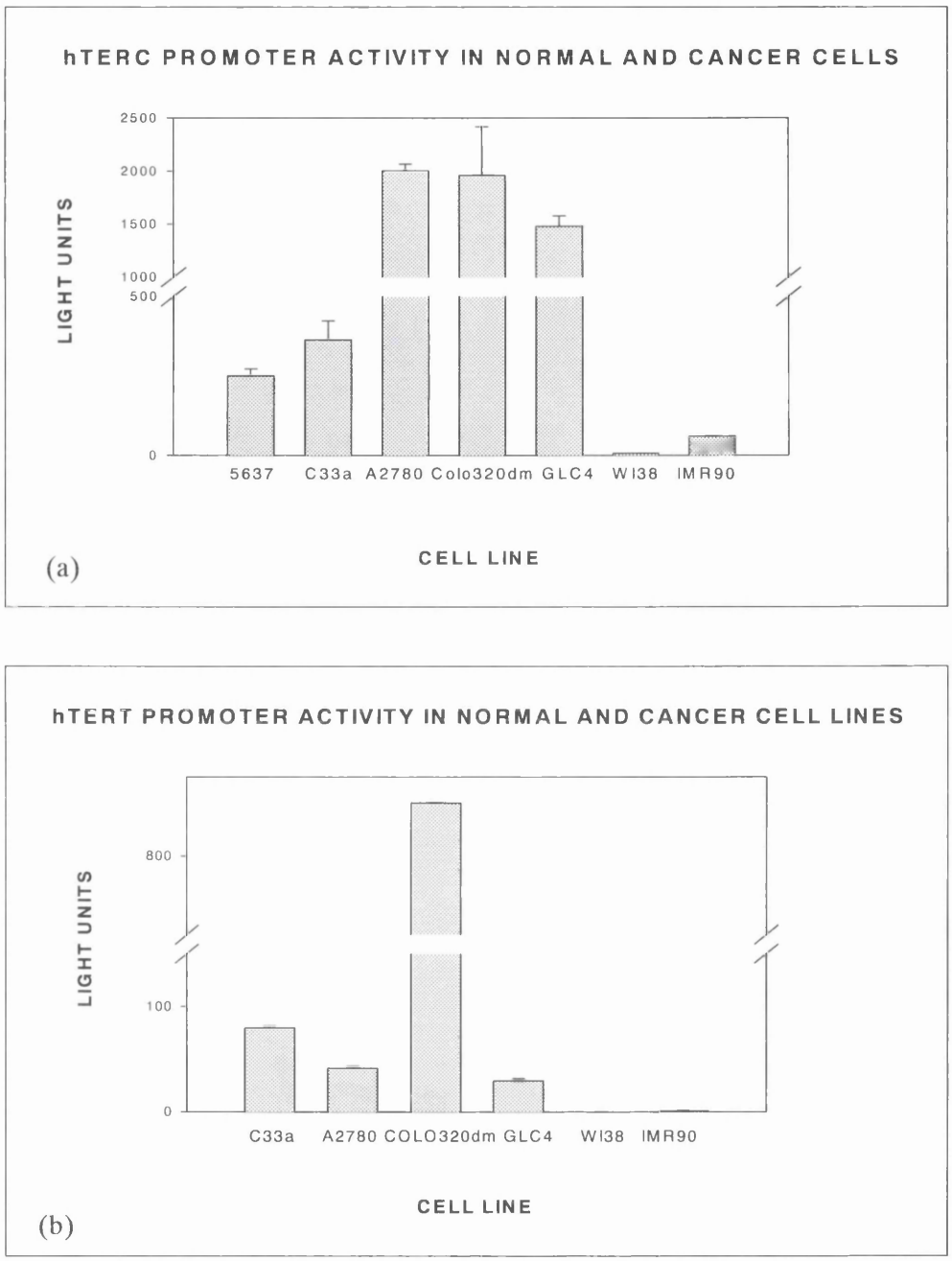


Figure 3.5: Differential activities of the hTERC and hTERT promoters between mortal and cancer cell lines.

Cells were transfected in duplicate with 3μg plasmid DNA containing the luciferase gene under the control of (a) hTERC or (b) hTERT promoters and incubated for 48h. Cells were lysed and 2.5μg protein equivalents were calculated using Bio-Rad protein assay reagent. Relative light units in 2.5μg equivalents were quantified in a luminometer (Turner Designs TX20/20) for each of 5637 bladder carcinoma, C33-A cervical carcinoma, A2780 ovarian adenocarcinoma, Colo 320dm colorectal adenocarcinoma, GLC4 small cell lung cancer, WI-38 and IMR-90 foetal lung fibroblast cells. All experiments were repeated at least 3 times and included positive (SV40) and negative (promoterless) control luciferase plasmids. Results shown are from a representative experiment for each cell line. 5637 cells were included in each experiment as a control for relative promoter activities between cell lines. Transfection efficiencies were checked by semi-quantitative PCR.

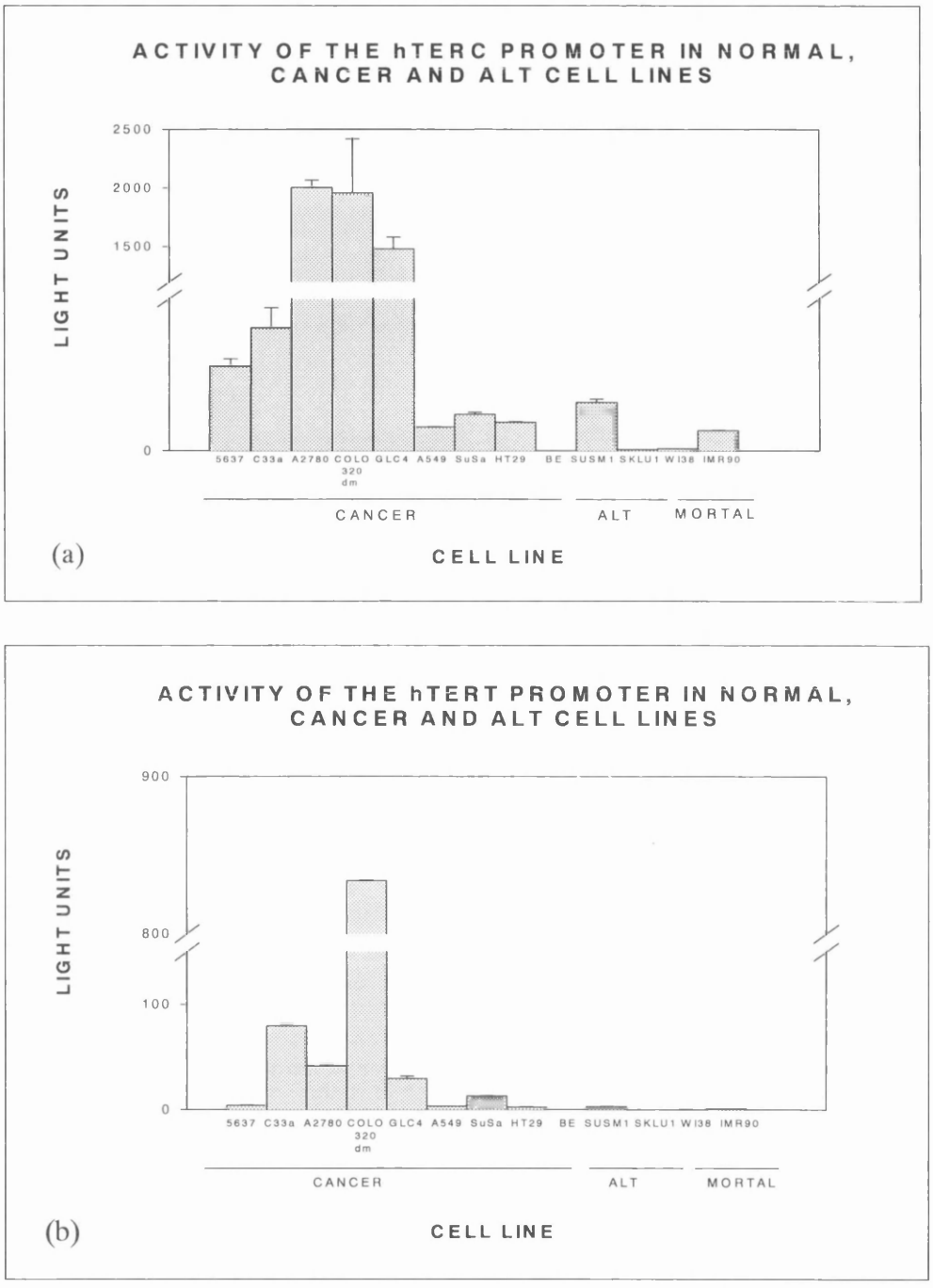


Figure 3.6: hTERC and hTERT promoters are not universally strong in cancer cell lines.

Cells were transfected in duplicate with 3μg plasmid DNA containing the luciferase gene under the control of (a) hTERC or (b) hTERT promoters and incubated for 48h. Cells were lysed and 2.5μg protein equivalents were calculated using Bio-Rad protein assay reagent. Relative light units of 2.5μg equivalents were quantified in a luminometer (Turner Designs TX20/20) for each of the cancer cell lines 5637 bladder carcinoma, C33-A cervical carcinoma, A2780 ovarian adenocarcinoma, Colo 320dm colorectal adenocarcinoma, GLC4 small cell lung cancer, A549 lung adenocarcinoma, SuSa testicular teratoma, HT-29 and BE colon carcinoma. Additionally, the mortal cell strains WI-38 and IMR-90 foetal lung fibroblast cells and the immortal, telomerase negative ALT cell lines SK-LU-1 and SUSM-1 were assayed. All experiments were repeated at least 3 times and included positive (SV40) and negative (promoter-less) control luciferase plasmids. Results shown are from a representative experiment for each cell line. 5637 cells were included in each experiment as a control for relative promoter activities between cell lines. Transfection efficiencies were checked by semi-quantitative PCR.

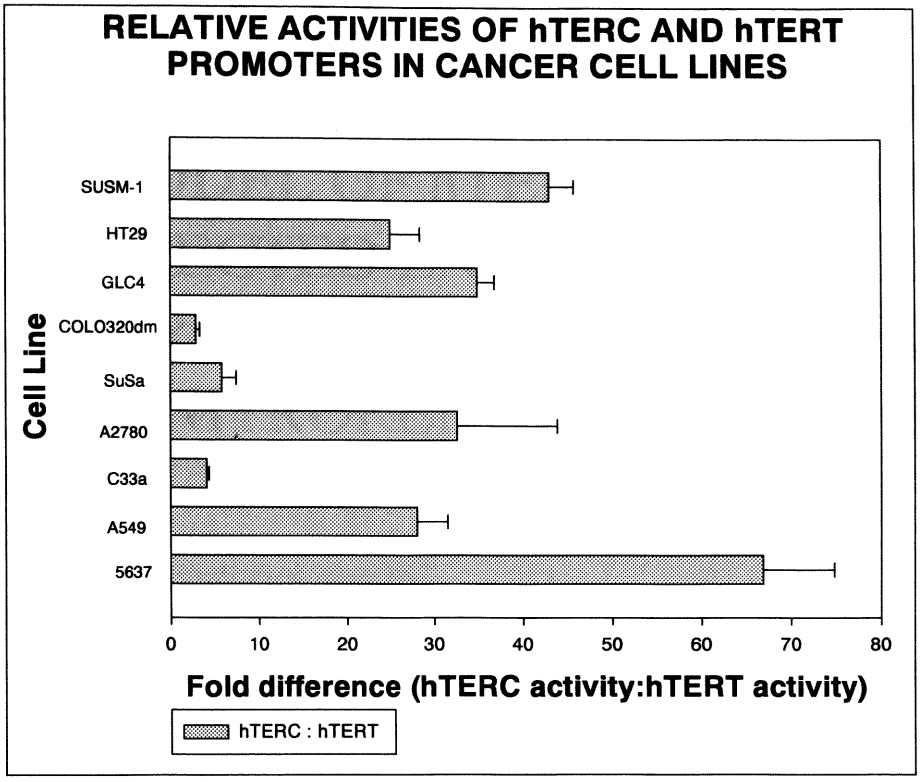


Figure 3.7: The hTERC promoter is stronger than hTERT.

Cells were transfected in duplicate with 3μg plasmid DNA containing the luciferase gene under the control of hTERC or hTERT promoters and incubated for 48h. Cells were lysed and 2.5μg protein equivalents were calculated using Bio-Rad protein assay reagent. Relative light units of 2.5μg equivalents were quantified in a luminometer (Turner Designs TX20/20) for each of the cancer cell lines 5637 bladder carcinoma, C33-A cervical carcinoma, A2780 ovarian adenocarcinoma, Colo 320dm colorectal adenocarcinoma, GLC4 small cell lung cancer, A549 lung adenocarcinoma, SuSa testicular teratoma, HT-29 colon carcinoma and the telomerase negative ALT cell line SUSM-1 were assayed. All experiments were repeated at least 3 times and included positive (SV40) and negative (promoterless) control luciferase plasmids. Relative activities of the promoters were taken to be the fold difference in light units between the promoters in an individual experiment. Results shown are the mean values and standard errors calculated across 3 independent experiments for each cell line. Relative values could not be calculated for the cells with only background hTERT activity (WI-38, IMR-90, SK-LU-1, and BE). 5637 cells were included in each experiment as a control for relative promoter activities between cell lines. Transfection efficiencies were checked by semi-quantitative PCR.

CHAPTER 4

DEVELOPMENT OF A TISSUE CULTURE MODEL OF hTERC AND hTERT DIRECTED ENZYME/PRO-DRUG THERAPY USING THE BACTERIAL NITROREDUCTASE GENE

- Development of hTERC-NTR and hTERT-NTR gene therapy plasmids.
- Generation of stable cell lines harbouring gene therapy plasmids.
- Promoter dependent sensitisation to CB1954.
- hTERC-NTR and hTERT-NTR sensitise human cancer cells to CB1954 *in vivo*.

4 Development of a tissue culture model of hTERT and hTERC directed enzyme pro-drug therapy using the bacterial nitroreductase gene.

4.1 Abstract

In order to directly address the question whether hTERC and hTERT promoter sequences can drive sufficient expression of a therapeutic transgene within cancer cells to validate their use in a pre-clinical model of telomerase gene targeted therapy, nine sets of stable cell lines harbouring hTERC-NTR and hTERT-NTR plasmids were generated. Cell lines were selected on the basis of promoter activities measured by luciferase assay. Expression of bacterial nitroreductase was analysed by northern and western blotting in promoter-less, CMV-NTR, hTERC-NTR and hTERT-NTR stable cell lines, and the relative efficiency of sensitisation to the pro-drug CB1954 by hTERC and hTERT promoter mediated expression of bacterial nitroreductase was analysed by MTT assay. Results were compared both within and between sets of stable cell lines. Analysis of the fold sensitisation to CB1954 by hTERC-NTR and hTERT-NTR expression, across all sets of stables, revealed that the cell lines fell into two main groups: those cell lines that were significantly sensitised to pro-drug and those that were not. The four cancer cell lines selected for their high hTERC and hTERT promoter activities were all significantly sensitised to CB1954, as expected, while the other cell lines assayed did not exhibit more than 2-fold sensitisation which was not considered significant. Thus, hTERC and hTERT promoter activities are predictive of the efficiency of cell killing by hTERC-NTR and hTERT-NTR expression combined with CB1954 treatment, and only cell lines with highly active hTERC and hTERT promoters are effectively targeted by the approach. These data validate the use of hTERC and hTERT promoter sequences for use in combination with the NTR/CB1954 suicide gene therapy system.

4.2 Introduction

Cancer therapies are often limited in their efficacy due to a variety of factors. Poor circulation and hypoxic non-dividing cell populations within large solid tumours are major obstacles to effective chemo- and radiotherapy (reviewed in Brown et al. 1998).

Additionally, the mechanisms of action of many of the classical chemotherapeutic drugs such as alkylating agents, platinating agents, and anti-metabolites are targeted to cellular events such as DNA replication that are characteristic of dividing cells, not of tumour cells. This often can result in an inability to distinguish tumour cells and rapidly proliferating normal cells leading to dose limiting toxicity. Strategies to overcome this have included combinatorial treatment regimes in which several cytotoxic agents with different modes of action are administered concurrently to increase the overall concentrations of cytotoxic drug. However, dose-limiting toxicity still represents a major problem which gene therapists hope to overcome by the use of tumour restricted cytotoxic gene therapy. In genetically directed enzyme/pro-drug therapies (GDEPT), researchers seek to limit to target cell populations the expression of an enzyme whose action is the conversion of a relatively non-toxic pro-drug to an active, toxic derivative. These systems were first pioneered by the use of the Herpes Simplex Thymidine Kinase gene (HSTK) which catalyses the mono-phosphorylation of the guanylate analogue Gancyclovir (GCV). GCV-MP can be further phosphorylated to di- and tri-phosphate forms by cellular kinases and can then be incorporated into newly synthesised DNA strands. GCV-TP lacks the 3'OH on the deoxyribose and 2'C-3'C bond necessary for DNA chain elongation and thereby acts to terminate chain extension.

Although HSTK/GCV is in many ways the paradigm for GDEPT, a number of other enzyme pro-drug activation systems have been proposed. An attractive system is bacterial nitroreductase/CB1954. CB1954 (5-Aziridin-1-yl 2,4-dinitrobenzamide (fig 4.1)) is a weak mono-functional alkylating agent that first aroused interest as a potential anti-cancer agent on the basis of its effectiveness against the rat Walker tumour (Cobb et al. 1969). Subsequent investigations failed to detect such an effect in human tumours and cell lines and CB1954 was not considered an appropriate anti-cancer agent until the elucidation of the mechanism of its efficacy against the Walker tumour (Knox et al. 1988). These cells express an enzyme, DT-Diaphorase which can catalyse the reduction of the 4-nitro function of CB1954 to a hydroxlamino derivative which is subsequently acylated in an interaction with cellular thioesters to form a powerful bifunctional alkylating agent which introduces a high frequency of poorly repaired inter- and intra-strand cross-links into DNA (Knox et al. 1991; Friedlos et al. 1992; Knox et al. 1992). The aziridine function of CB1954 interacts with the O6 position of deoxyguanine, allowing the activated hydroxylamino moiety to interact with a C8 position of deoxyguanine on the opposite strand. The human and mouse homologues of DT-Diaphorase possess an inactivating Tyrosine to Glutamine mutation at amino acid 104 (Chen et al. 1995). Tissue culture

assays using human cells with enzyme activity comparable to Walker cells required approximately 500-5000-fold higher concentrations of CB1954 to elicit similar cytotoxic effects. Moreover, the K_{cat} value for reduction of the 4-amino group by the rat enzyme was shown to be around 6-fold higher than for the human, indicating that the human enzyme is less efficient than the rat at the bioactivation of CB1954 (Boland et al. 1991). The identification of bacterial nitroreductase as a functional homologue of the rat enzyme has renewed interest in the use of CB1954 as part of a GDEPT strategy utilising bacterial nitroreductase as the activating enzyme.

In vivo, nitroreductase functions as a homodimer that is complexed with an internal molecule of flavin mononucleotide (FMN) which functions as an electron donor in the 2-step bio-activation of CB1954 (Parkinson et al. 2000). The system offers a number of advantages over the more common systems HSTK/GCV and COD/5-FC. Firstly, bio-activated CB1954 induces a p53 independent apoptotic response that leads to widespread toxicity in both dividing and non-dividing cells (Bridgewater et al. 1995; Weedon et al. 2000). Thus, the system is not limited in its effect to cells that are actively cycling, as is the case with HSTK/GCV. Second, the formation of the ultimate cytotoxic species does not appear to require the involvement of other cellular enzymes, and hence, the kinetic bottleneck that has been proposed to limit the efficacy of HSTK/GCV therapy does not occur (Knox et al. 1991; Akyurek et al. 2001). Thirdly, the active species is membrane permeable and induced a strong bystander effect in cells that were not transduced with enzyme (Bridgewater et al. 1997). The importance of a bystander effect to target untransduced neighbouring cells is paramount to the development of these systems as genuine therapeutic tools and from this point of view, NTR/CB1954 is an excellent candidate for development. Indeed, a recent study which compared directly the cytotoxicity and bystander effects of 4 enzyme/pro-drug systems in proliferating thyroid carcinoma cells concluded that the bystander effect induced by CB1954 was superior to that of GCV (Nishihara et al. 1998). Additionally, the pro-drug CB1954 and its derivatives are radio-sensitising agents (Walling et al. 1987), which may enable the development of combinatorial therapies. Lastly, CB1954 is one of many drugs which can be activated by NTR, suggesting that different drugs may be employed in order to overcome any inherent cellular resistance to activated CB1954 derivatives, thereby enhancing the versatility of nitroreductase expression systems for GDEPT (Bailey et al. 1996).

NTR has been delivered to human tumour cell lines by stable and transient transfection, in retrovirus vectors and, most recently, in an adenoviral vector (Bridgewater et al. 1995;

Drabek et al. 1997; Green et al. 1997; McNeish et al. 1998; Weedon et al. 2000). An efficacious effect has been documented against tumour cells derived from human ovarian, pancreatic, colorectal, cervical and small cell lung cancers both *in vitro* and *in vivo* (Bridgewater et al. 1995; Drabek et al. 1997; Green et al. 1997; McNeish et al. 1998; Weedon et al. 2000). Moreover, CB1954 has already been evaluated in clinical trials as a potential anti-cancer agent and the drug is well tolerated at comparatively high doses in humans. In a recent phase I study of CB1954 as a single agent, investigators were able to administer intravenous doses of up to 24 mg/m^2 before any significant toxicity was observed. Peak serum levels of CB1954 were judged to be sufficient to allow conversion to the active species to occur (Chung-Faye et al. 2001).

Transcriptional restriction of expression of NTR has been accomplished using hTERC and hTERT promoters in a panel of cell lines having high and low promoter activities (Plumb et al). In this chapter, NTR/CB1954 is evaluated as a candidate enzyme/pro-drug therapy system for transcriptional restriction by the hTERC and hTERT promoters in stable cell line models. Nitroreductase expression constructs with the NTR gene under the control of hTERC, hTERT, or CMV promoters, or in basic promoter-less vectors, are stably introduced into a panel of 9 cell lines whose promoter activities have been characterised by luciferase assay in chapter 3. Cancer cell-specific, promoter dependent regulation of expression of NTR limits the cytotoxic effects of CB1954 to those cell lines that had a high promoter activity, leaving the cells with low promoter activities broadly unaffected. Transcriptional restriction of NTR expression with hTERC and hTERT promoters is thereby validated as a potentially useful targeted approach to the cytotoxic gene therapy of cancer cells.

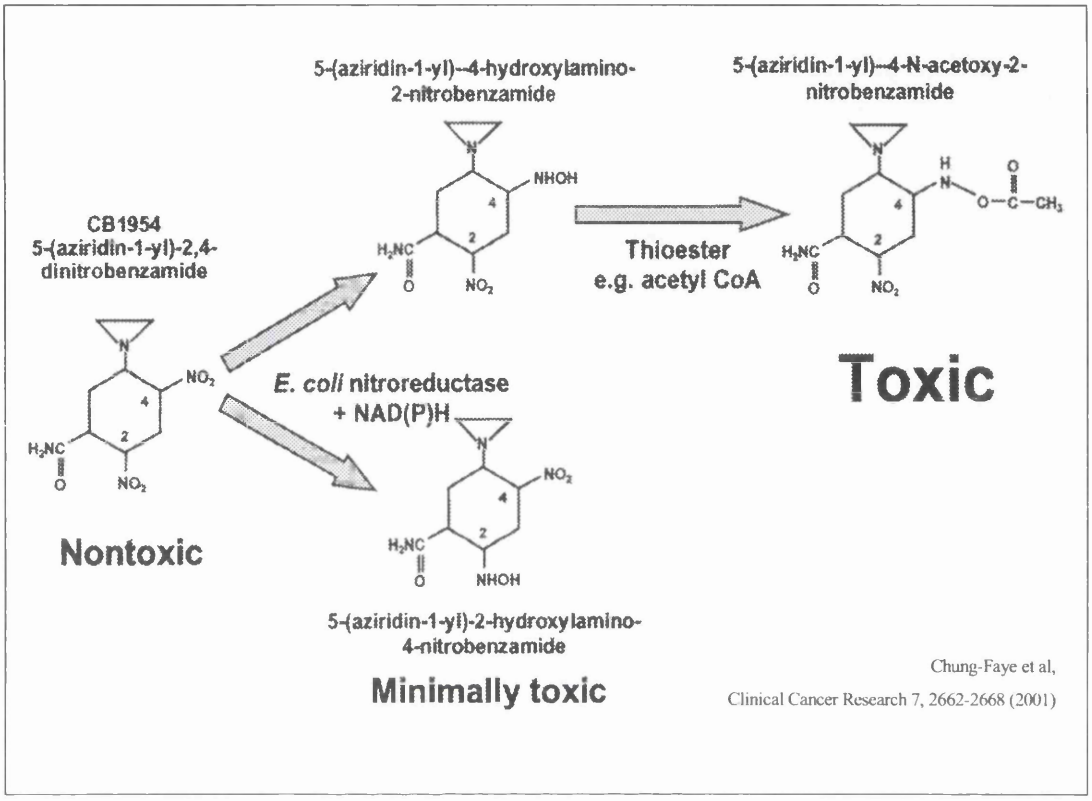


Figure 4.1: Mechanism of bioactivation of CB1954 by bacterial nitroreductase.

The reduction of the 4-amino group to a reactive 4-hydroxylamino intermediate precedes acylation by endogenous thioesters. The acylated species is a powerful bifunctional alkylating agent able to induce a high frequency of poorly repaired inter-strand crosslinks between deoxyguanine nucleotides. Both 4-hydroxylamino and 2-hydroxylamino derivatives are generated in the first reaction, but only the 4-hydroxylamino goes on to form the ultimate cytotoxic species (Knox, 1991).

4.3 Development of telomerase-nitroreductase plasmid vectors.

A family of plasmid vectors containing the NTR coding sequence under the control of either CMV, hTERC, hTERT promoters, or in a basic promoter-less construct were developed within the laboratory by Rania Kakani (Plumb et al). Vector feature maps are given in figure 4.2. Each vector contained the Kanamycin/ Neomycin resistance gene to enable the development of stable cell lines. The purpose of vector construction was to enable nitroreductase to be expressed by the telomerase promoters or by the CMV promoter in stable cell lines, thereby allowing direct comparisons between the capacity of each promoter to drive enzyme expression and sensitise cells to the toxic effects of activated CB1954.

PLASMID NAME	PROMOTER	DESCRIPTION
Pd2NTR-Basic	None	Basic, cloning vector for generation of CMV, hTERC and hTERT gene therapy vectors. Contains nitroreductase gene. Negative control for stable transfection experiments.
Pd2NTR-CMV	CMV	Contains nitroreductase gene driven by CMV promoter and enhancer sequences. Positive control for stable transfection experiments.
Pd2NTR-hTERC	hTERC	Contains nitroreductase gene driven by 876bp fragment of the hTERC promoter. Telomerase gene therapy vector.
Pd2NTR-hTERT	hTERT	Contains nitroreductase gene driven by 541bp fragment of the hTERT promoter. Telomerase gene therapy vector.

Table 4.4.1: Characteristics of nitroreductase gene therapy vectors.

A description of the construction of the vectors is given in materials and methods. Vectors were used for the development of stable cell lines to enable direct comparisons of the expression of NTR from each promoter in a number of cell lines and its effects on sensitisation of cells to the toxic effects of activated CB1954.

4.4 Selection and characterisation of stable cell lines harbouring nitroreductase gene therapy vectors.

In order to assess the validity of a cytotoxic gene therapy approach using the telomerase promoters to drive expression of bacterial nitroreductase, the plasmid vectors described in table 4.1 were stably transfected into a range of cell lines. Using superfect transfection reagent, 10^6 cells were transfected in 10cm dishes with 10μg plasmid DNA for each plasmid. 48 hours after transfection, cells were selected in 1mg/ml geneticin-sulphate for approximately 2 weeks until the appearance of stable foci. At this point, stable clones were pooled and each culture was expanded under selection to give a final total of 4 sets of stable pools of clones (basic, CMV, hTERC and hTERT nitroreductase clones) for each of 9 parental cell lines (5637, C33-A, A2780, GLC4, Colo320dm, A549, HT29, SUSM-1, SK-LU-1) (5637, C33-A and A2780 clones generated by Rania Kakani (Plumb et al. 2001)). Due to the limited proliferative lifespan of WI-38 and IMR-90 cells, these could not be selected as stables. However, since the results of transfection assays (Chapter 3) suggest that the two telomerase negative ALT cells lines (SK-LU-1 and SUSM-1) have promoter activities comparable with those of the normal diploid fibroblasts, these have been selected for use as surrogate negative control cell lines.

To ensure that nitroreductase was expressed in stable pools and that expression followed the expected promoter dependent pattern, each pool was analysed by Northern and Western blotting for nitroreductase. The results of Northern blots using 25μg total RNA from each stable pool to detect the nitroreductase transcript are given in figure 4.3. Signals consistent with the expected transcript size of approximately 1kb were detected in lanes corresponding to hTERC-NTR, hTERT-NTR, and CMV-NTR transfected cells. As expected, none of the lanes containing RNA extracted from the Basic pools in any cell line showed a signal for NTR mRNA. Figure 4.4 shows the detection of a protein species consistent with the expected molecular weight of NTR of 24kDa using Western blotting. Relative band intensities for individual lanes were comparable in both Northern and Western blots.

In the majority of cell lines, the strongest signal was detected in the lane corresponding to NTR expressed from the CMV promoter with the hTERC and hTERT promoters giving signals of a similar and lower intensity, although hTERC was usually stronger than hTERT (5637, C33a, SK-LU-1, A2780). Notable exceptions were A549 cells, which had the strongest signal in the lane corresponding to the hTERC promoter, with barely detectable

signal for the CMV and undetectable signal for hTERT. A2780 stable cell lines, despite showing high activity of both hTERC and hTERT promoters in transient transfection analysis had a low proportion of NTR mRNA relative to total steady state mRNA compared with other cell lines. Surprisingly, the NTR signal for the Colo320dm hTERC stable pool was of a low intensity compared with hTERT; additionally, hTERT was more intense than the CMV signal in this cell line.

4.5 Cancer cell specific, promoter dependent cell killing after treatment with CB1954.

The use of stable pools of clones allows for direct comparisons to be made regarding the effects of expression of a pro-drug converting enzyme on sensitisation to the pro-drug between a number of cell lines. Analysis of cytotoxic cell responses to the pro-drug CB1954 was performed by MTT assay in triplicate for each promoter. Cytotoxicity data were analysed using Softmax 2.32 analysis software and IC50 values for independent experiments were calculated from the mean value of triplicate measurements of the 50% y-axis intercept. Table 4.2 gives mean IC50 values for CB1954 sensitivity for cells transfected with various promoter-nitroreductase combinations derived from 3 independent experiments and Figures 4.5 and 4.6 show representative cytotoxicity curves for sets of basic, CMV, hTERC and hTERT clones in each of 5637, HT-29, SUSM-1, SK-LU-1, A549, C33a, A2780, GLC4, and Colo320dm. All cell lines were assayed at least 3 times. Figures presented are representative curves.

Patterns of sensitisation to CB1954 broadly followed patterns of promoter activity described in chapter 3 and were strongly correlated with the results of Northern and Western analysis presented in figures 4.3 and 4.4. Analysis of cytotoxicity data revealed 2 types of response, cell lines that did not respond to CB1954 by NTR expression from the hTERC and hTERT promoters (figure 4.5) and cell lines that had a high response (figure 4.6). The cell lines which had low hTERC and hTERT promoter activities in luciferase assays, including 5637, HT29, SK-LU-1 and SUSM-1, did not exhibit significant differences between the cytotoxicity curves for the basic promoter-less vector and for those containing NTR driven by either hTERC or hTERT. In each of these cases, Northern analysis revealed higher intensity signals in the lanes for the CMV promoter and, in-keeping with this, cytotoxicity curves for the CMV promoters were generally significantly different from those of the other promoters.

The IC50 for the basic promoter in 5637 cells was 171.23μM, while IC50 values for the other promoters were 115.55μM (CMV), 110.73μM (hTERC) and 149.03μM (hTERT). Therefore, there was no significant sensitisation to CB1954 by expression of nitroreductase from any of the promoters and this is reflected in the pattern of the kill curve (Figure 4.5) in which the curves for all promoters are tightly packed together. HT29 cells were also not significantly sensitised by any promoter, with the largest shift in IC50 being approximately 2-fold between the basic (227.6 μM) and CMV promoters (109.26).

The remaining cell lines in the group which did not respond to hTERC-NTR or hTERT-NTR / CB1954 treatment (SUSM-1 and SK-LU-1) were all sensitised to CB1954 by NTR expression under control of the CMV promoter, suggesting that the hTERC and hTERT promoters could not drive expression of sufficient levels of nitroreductase to efficiently sensitise these cells. Examination of the kill curves for these 2 ALT cell lines show clear shifts toward lower drug concentrations for the CMV promoter, but not for either telomerase promoter. This is reflected in IC50 values for each promoter. SUSM-1 cells had a mean IC50 for the basic construct of 264.77μM, but only 72.48 μM for the CMV, reflecting an approximately 4-fold sensitisation. Values for hTERC and hTERT were 177.77 μM and 130.82 μM, respectively, indicating approximately 2-fold sensitisation, which was not considered to be significant. Similarly, SK-LU-1 cells showed an approximately 8-fold shift in IC50 between basic (120.1μM) and CMV (14.99μM) constructs, but did not display more than 2-fold sensitisation by hTERC (61.81μM) or hTERT (70.93μM).

4 cancer cell lines, all of which had high promoter activities in luciferase assays (C33-A, GLC4, A2780, Colo320dm) showed strong responses to CB1954 both when nitroreductase was expressed from the CMV promoter, or from either hTERC or hTERT (figure 4.6). C33-A cells had a basic IC50 value of 96.06μM that shifted to 2.01μM for the CMV promoter (approximately 45-fold sensitisation), 15.19μM for the hTERC (approximately 6-fold sensitisation) and 8.59μM for hTERT (approximately 11-fold). Similarly, Colo320dm exhibited marked shifts toward lower CB1954 concentrations when NTR was transfected in the basic construct (191.42μM), under control of the CMV promoter (7.1μM), the hTERC promoter (23.77μM) or of hTERT (13.86μM). These patterns are reflected in the shifted curves evident in figure 4.6.

The remaining 2 cell lines in this group, A2780 and GLC4 had lower basic IC50 values than any of the other cell lines tested (25.76μM for A2780 and 4.66μM for GLC4), but the

expression of NTR from the telomerase promoters could still induce a marked sensitisation to the pro-drug. A2780 cell IC50 values shifted to 1.26μM for the hTERC promoter and 5.2μM for hTERT, while GLC4 cells had IC50 values of less than 1μM for both telomerase promoters (0.53μM, hTERC; 0.68μM, hTERT). These results indicated that the expression of nitroreductase from the hTERC and hTERT promoters could sensitise cell lines to the cytotoxic effect of bioactivated CB1954 in a manner that was dependent on the level of NTR expression and, therefore, on promoter activity.

Interestingly, A549 cells showed no significant sensitisation to CB1954 by either hTERT or CMV promoters, although the hTERC promoter had a strong effect in these cells, lending strength to the argument that the hTERC promoter may provide a useful target in cases where the hTERT promoter is too weak to drive effective transgene expression in specific cells or disease types.

Figure 4.7 gives the summary of sensitisation data for hTERC-NTR and hTERT-NTR constructs across the cell lines assayed. Sensitisation values are derived from the fold difference between IC50 values for the basic, promoter-less vector and for those in which NTR expression is driven by a promoter. Mean values and standard errors were calculated from 3 independent experiments in each case. As described above, the cell lines fall into 2 groups, arranged broadly along the lines of promoter activities detected by luciferase assay (chapter 3): those which are significantly sensitised to the effects of CB1954 by expression of NTR from hTERC and hTERT promoters (cancer cell lines with strong hTERC and hTERT activities), and those which were not (ALT cell lines and cancer cells with low hTERC and hTERT activities).

None of the cell lines with low promoter activity (HT29, SK-LU-1, SUSM-1, 5637) showed more than 2-fold sensitisation to CB1954 after stable transfection with the hTERC-NTR construct. The range in sensitisation was 1.07-fold (HT29) to 1.92-fold (SK-LU-1). Therefore, the hTERC promoter did not drive sufficient NTR expression to significantly sensitise these cells to CB1954. Similarly, the hTERT promoter showed no significant effect on this group of cell, with sensitisation values for all cell lines in the range 0.94-fold (HT-29) and 2.11-fold (SUSM-1). These data strongly suggested that the low capacity for transgene expression from telomerase promoters in these cell lines detected by luciferase assay in chapter 3 limited the expression of nitroreductase and, therefore, the bioactivation and associated toxicity of CB1954.

In contrast, those cell lines shown to have a high promoter activity in chapter 3 (A2780, C33-A, GLC4, Colo320dm) all demonstrated strong sensitisation to the effects of CB1954 when they were stably transfected with hTERC-NTR and hTERT-NTR constructs. A2780 cells showed mean sensitisation values of 19.4-fold for the hTERC promoter and 4.8-fold for the hTERT promoter. In C33-A cells, 6.33-fold sensitisation was achieved using the hTERC promoter and 10.9-fold using hTERT, while these values were 9.54-fold and 8.62-fold (hTERC) and 8.46-fold and 13.89-fold (hTERT) for GLC4 and Colo320dm cells, respectively. A549 cells showed a strong response to CB1954 (6.76-fold sensitisation) when NTR was expressed by the hTERC promoter, but were not significantly sensitised (1.89-fold) in hTERT-NTR transfected cells, suggesting that the hTERC promoter may have the capacity to drive high-level expression of cytotoxic transgenes in certain situations where the hTERT promoter is not strong enough. Thus, hTERC and hTERT promoters drive expression of bacterial nitroreductase in a tumour specific fashion and hTERC-NTR and hTERT-NTR expression constructs can specifically sensitise cancer cell lines with high promoter activities to the cytotoxic effects of the pro-drug CB1954.

4.6 hTERT and hTERC promoter activities are predictive of sensitisation to CB1954.

Northern and Western blot expression data revealed similar results which were correlated with differential sensitivities to CB1954 within a single set of clones when nitroreductase was expressed from different promoters. Figure 4.8 shows the results of northern blot analysis of the expression of NTR for several individual cell lines in addition to representative cytotoxicity curves. Comparison of both parts of the figure for each cell line reveals that the level of nitroreductase expression apparently affected the sensitisation of cells to CB1954.

In C33-A cells, the strongest nitroreductase signal was observed in both Northern and Western blots in the CMV lane, and cells transfected with this construct showed the strongest response to CB1954. The hTERC-NTR and hTERT-NTR bands in C33-A cells were similar and of a lower intensity than that of CMV-NTR, which is well correlated with the cytotoxic effect of CB1954 in these stables. In SK-LU-1 cells, hTERC and hTERT-NTR signals were effectively absent, but a strong band was evident in the CMV lane, reflecting the pattern of cytotoxicity observed (hTERC and hTERT-NTR do not sensitise

these cells to CB1954, while the CMV promoter could drive sufficient nitroreductase expression to generate some 6-fold sensitisation). A549 cells, by contrast, showed a strong band in the hTERC lane and a band of lower intensity in the CMV lane with no detectable bands in either hTERT or basic lanes. Comparison of the kill curve reveals that hTERC directed expression of NTR resulted in the greatest sensitisation to CB1954, with a lesser shift in the curve for the CMV promoter and no significant change from the basic in the case of the hTERT promoter.

These results indicated that differential promoter activities of the hTERC and hTERT promoters in parental cell lines were retained in stable pools of nitroreductase expressing clones, and that the telomerase promoters could be used to drive cell specific differential expression of the nitroreductase gene and specifically sensitise those cells which had high level expression to the effects of CB1954.

4.7 CB1954 sensitivity is retained *in vivo* in a xenograft model of C33-A-NTR and GLC4-NTR.

This section was conducted in collaboration with Dr. Jane Plumb (Plumb et al. 2001).

In order to address the question of whether hTERC-NTR and hTERT-NTR expressing cell lines could be sensitised to CB1954 treatment *in vivo*, 10^7 of each of the C33-A and GLC4 stable cells in 200µl PBS were introduced by subcutaneous injection into the flanks of female athymic nude mice. Six mice were included in each group for analysis. For each of C33a and GLC4, 8 groups were included to allow for analysis of each of the Basic-NTR, CMV-NTR, hTERC-NTR and hTERT-NTR expressing cells, with and without CB1954. After tumour diameter reached at least 5mm, a single injection of 80mg/kg CB1954 (C33-A), 40mg/kg (GLC4), or saline control was administered by tail vein injection. Changes in relative tumour volumes were monitored daily for a period of 7 days and were estimated from calliper measurements with the following formula: volume=d^3 x $\pi/6$. The results are presented in figures 4.9 (a) (C33-A) and (b) (GLC4).

As expected, no difference was observed between the change in tumour volumes of C33-A Basic-NTR over the course of the experiment, whether CB1954 was administered or not. Mean relative tumour volumes both for animals with and without CB1954 increased approximately 2.25-fold over the course of the experiment. By contrast, the mean tumour volumes of animals carrying C33-A CMV-NTR tumours were reduced to 0% of the

starting volumes after a single tail vein administration of 80mg/kg CB1954 on day 0. Tumours, in the control animals, that did not receive CB1954 increased by a factor of 2.47. Thus, a single administration of CB1954 to the CMV-NTR animals caused a complete regression of the detectable tumour mass. Interestingly, both hTERC-NTR and hTERT-NTR expressing tumours also showed a strong response to administration of CB1954, with tumour volumes reduced to 66% and 53%, respectively, of the starting volumes at day 1 while tumours of control animals increased in size by 1.8-fold and 2.2-fold, respectively. These data translate to a difference in mean tumour volumes between treated and untreated animals at the final time point of 2.7-fold for hTERC and 4.1-fold for hTERT. Interestingly, these results were similar to those observed in MTT assays, with the telomerase promoters generating strong and similar responses to administration of CB1954, while the CMV promoter had the strongest response (in this case, resulting in a complete regression).

A second experiment was conducted using animals bearing GLC4 Basic-NTR, CMV-NTR, hTERC-NTR, and hTERT-NTR tumours. The GLC4 Basic-NTR cells showed an intrinsic sensitivity to CB1954 (figure 4.6 and figure 4.9 (b)) that allowed the dose of CB1954 administered to be reduced to 40mg/kg. At this drug concentration, a minimal cytotoxic effect was observed in the Basic-NTR relative to untreated cells, with an increase in tumour volume of approximately 4-fold in the untreated animals and 3-fold in the animals treated with CB1954. CMV-NTR tumour volumes increased by 4.8-fold in untreated animals and by only 1.6-fold in animals given 40mg/kg CB1954. Thus, the difference in tumour volumes at the experimental conclusion was 3-fold. CB1954 treated animals bearing hTERC and hTERT-NTR tumours showed final reductions to 39% and 18% of initial volume, respectively, while untreated tumours grew by 3.7-fold and 5.1-fold. Thus, final changes in tumour volumes of CB1954 treated animals relative to untreated controls were 9.5-fold for the hTERC promoter and 28.3-fold for the hTERT promoter.

Taken together, these results strongly indicated that transduction of cell lines in which the hTERC and hTERT promoters are highly active with hTERC-NTR and hTERT-NTR gene therapy constructs is an effective way to specifically sensitise cancer cells *in vivo* to the cytotoxic effects of CB1954 administration.

4.8 Discussion.

To validate the specificity and efficacy of hTERC and hTERT directed gene therapy, a family of plasmid vectors encoding the NTR gene either in a promoter-less vector, or under the transcriptional control of hTERC, hTERT, or CMV promoters were developed within the laboratory. A panel of cell lines having high and low promoter activities were selected for development of stable cell lines harbouring hTERC-NTR and hTERT-NTR expression constructs. The expression of bacterial nitroreductase within stable pools was evaluated by Northern and Western blotting and the cytotoxic effect of CB1954 bioactivation by NTR was quantified by MTT assay.

Analysis of gene expression showed that nitroreductase was differentially expressed in a promoter dependent manner within sets of stable pools of clones derived from a single parental cell line. In general, the expression of nitroreductase in CMV-NTR transfected cell lines was greater than in hTERC-NTR or hTERT-NTR transfected cells. Cells stably transfected with hTERC-NTR and hTERT-NTR showed similar and lower intensity signals, with hTERC promoter signal generally slightly higher than those of hTERT. No expression was seen in cells stably transfected with the negative control plasmid, pd2-NTR-basic. These results were correlated with the effects on cell sensitisation to the pro-drug CB1954, with high expression levels generally leading to lower IC50 values and, thus, greater cytotoxicity in stable cell lines.

2 groups of cells could be distinguished on the basis of their sensitisation to CB1954 in hTERC-NTR and hTERT-NTR transfected stables. Cells having low promoter activities as defined by luciferase assay in chapter 3 were generally insensitive to the effects of CB1954, while those with higher promoter activities (A2780, GLC4, C33-A, Colo320dm) exhibited shifted cytotoxicity curves and lower IC50 values indicating that cell specific regulation of hTERC and hTERT mediated expression of NTR sensitised these cells to the effects of CB1954.

Moreover, when C33-A cervical carcinoma, and GLC4 small cell lung cancer cells transfected with hTERC and hTERT-NTR constructs were introduced into female athymic nude mice as human tumour xenograft models, a single tail vein injection of 80mg/kg CB1954 to C33-A hTERC-NTR and hTERT-NTR animals was sufficient to reduce tumour volume to 66% and 53%, respectively, of the initial volumes. In case of GLC4 cells, intrinsic sensitivity to CB1954 allowed the concentration to be reduced to 40mg/kg.

Animals bearing hTERC-NTR and hTERT-NTR tumours exhibited tumour volumes reduced to mean values of 39% and 18% of the original volume, while control tumours continued to grow by 3.7-fold (hTERC) and 5.1-fold (hTERT) over the course of the experiment.

A number of factors will combine to influence the efficacy of any gene-targeted approach to cancer therapy. Specific cell populations will be expected to have variable basal sensitivities to the effects of cytotoxic drugs and, therefore, the choice of enzyme/pro-drug combination will have a profound impact on the ability to effectively target tumour cells. The tumour microenvironment may influence the efficiency of delivery of systemically administered drugs or; depending on their mechanisms of action, their cytotoxic effect. Thus, one of the most important characteristics of such a strategy must be the capacity to target cycling and non-cycling tumour cells while leaving normal cells relatively unaffected.

The ability to restrict transcription of nitroreductase and cell sensitisation to CB1954 to cancer cell lines with high telomerase promoter activity by use of the hTERC and hTERT promoters supports the use of these promoters as part of a tumour specific expression system in gene therapy. Other investigators have employed a variety of transgenes including apoptotic mediators, pro-drug activating enzymes, diptheria toxin gene and the noradrenaline transporter for the development of hTERC and hTERT gene therapy systems (Abdul-Ghani et al. 2000; Gu et al. 2000; Koga et al. 2000; Boyd et al. 2001; Koga et al. 2001; Komata et al. 2001; Majumdar et al. 2001). The further development of telomerase directed gene therapy strategies are supported by studies in monolayers and 3 dimensional spheroid tissue culture systems in addition to xenograft models. The data presented in this chapter further support the use of these regulatory sequences as part of a cancer specific transcriptionally directed gene therapy strategy. Incorporation of the NTR gene in the targeting strategy confers a number of attractive features to the model including the potential for targeting non-cycling cells and a well characterised bystander effect. These results provided essential proof of principle to support the further development of hTERC and hTERT directed gene therapy. The hTERC-NTR and hTERT-NTR expression systems were therefore validated for further study using adenoviral vectors as a more realistic model of delivery.

Figure 4.2: Feature maps of the nitroreductase plasmid vectors used in the generation of stable cell lines.

Construction of vectors was performed by Rania Kakani (Plumb et al. 2001), as described in materials and methods. Each vector contains the NTR coding sequence and Kanamycin/Neomycin resistance gene for development of NTR expressing stable cell lines. NTR is driven by either hTERC, hTERT or CMV promoters. A promoterless negative control vector was also included in the panel as a negative control to allow for the evaluation of the efficiency of sensitisation to the pro-drug by each promoter.

Figure 4.3: Northern blot analysis of NTR expression in stable cell lines.

Total RNA was isolated from hTERC-NTR, hTERT-NTR, CMV-NTR and basic-NTR stable cell lines. 25μg total RNA was electrophoresed on a denaturing formaldhyde gel, blotted onto positively charged nylon membrane and probed with the ^{32}-P dCTP labelled 740bp SalI fragment of pd2NTR-hTR. Bands are consistent with the expected NTR transcript size of approximately 1kbp. Upper blots, lanes 1-4, 5637 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lanes 5-8, A2780 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lanes 9-12, C33-A hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lanes 13-16, A549 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lower blots, lanes 1-4, Colo320dm hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lanes 5-8, SK-LU-1 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lanes 9-12, SUSM-1 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. The control probe for the 18s RNA gene is shown in the lower panel of each blot.

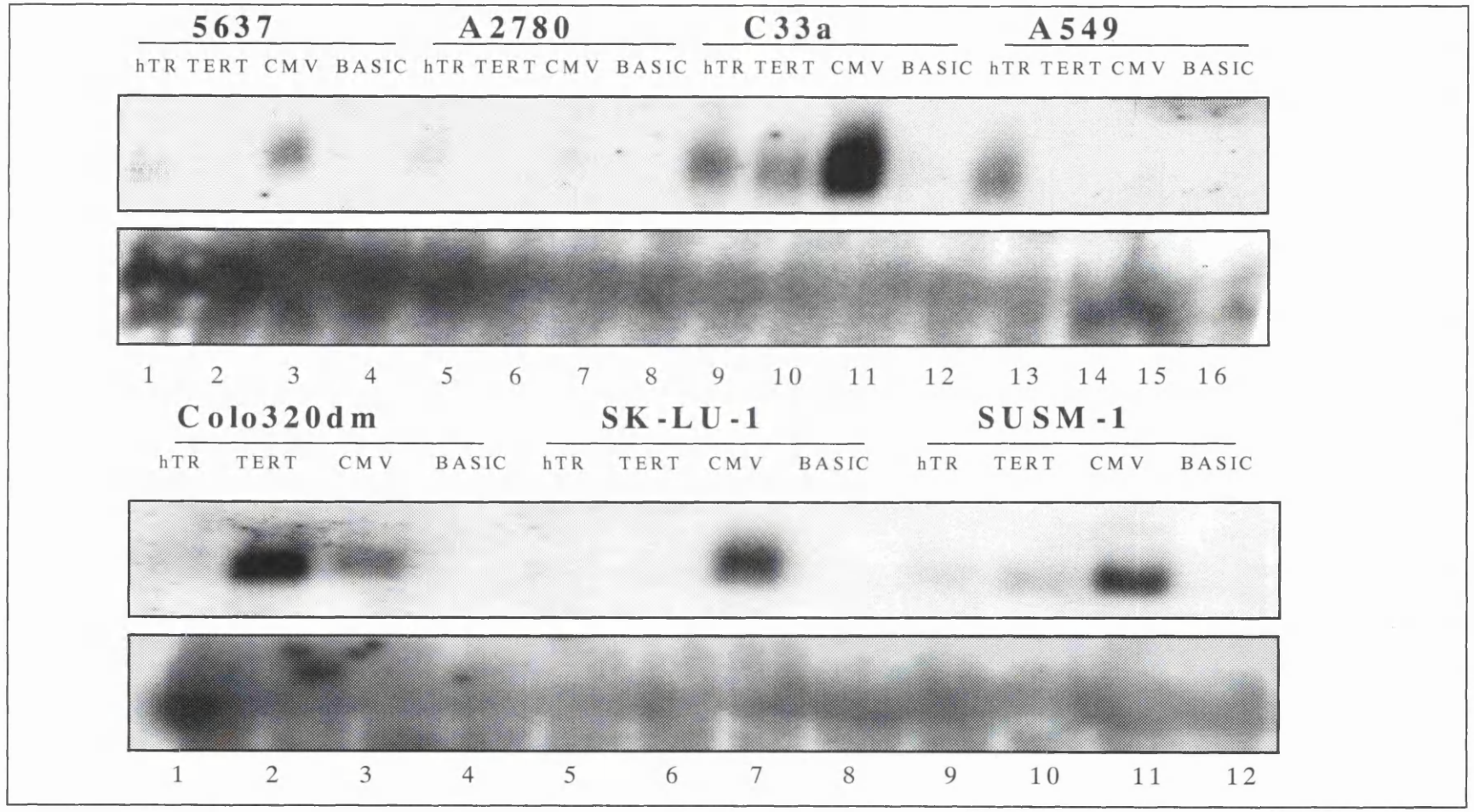
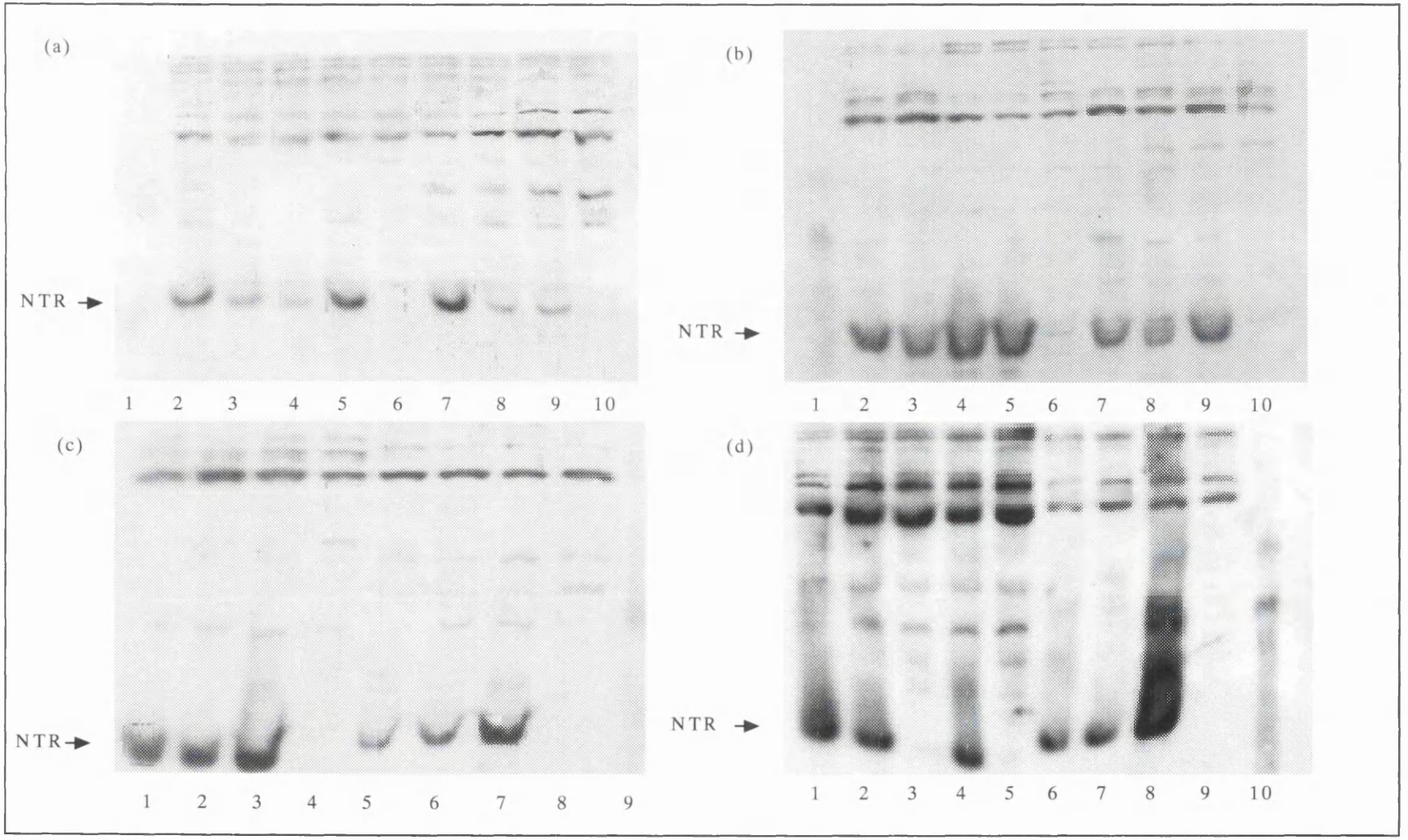

5637
A2780
C33a
A549
hTR TERT CMV BASIC hTR TERT CMV BASIC hTR TERT CMV BASIC hTR TERT CMV BASIC
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Colo320dm
SK-LU-1
SUSM-1
hTR TERT CMV BASIC hTR TERT CMV BASIC hTR TERT CMV BASIC
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4.4: Western blot analysis of NTR expression in stable cell lines.

Cell lines transfected with NTR gene therapy vectors were probed using the rabbit anti-NTR antibody R36 and a commercial HRP-conjugated anti-rabbit antibody. Bands were consistent with the reported approximate molecular weight of 24kDa. (a) HT29 and A549 stables. Lane 1, Benchmark protein standard (Gibco). Lane 2, C33-A hTERC-NTR control. Lanes 3-6, HT29 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lanes 7-10, A549 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. (b) Colo320dm and 5637 stables. Lane 1, Benchmark protein standard (Gibco). Lane 2, C33-A hTERC-NTR control. Lanes 3-6, Colo320dm hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lanes 7-10, 5637 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. (c) C33-A and SUSM-1 stables. Lanes 1-4, C33-A hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lanes 5-8, SUSM-1 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lane 9, Benchmark protein standard (Gibco). (d) A2780 and SK-LU-1 stables. Lane 1, C33-A hTERC-NTR control. Lanes 2-5, A2780 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lanes 6-9, SK-LU-1 hTERC-NTR, hTERT-NTR, CMV-NTR and Basic-NTR stables. Lane 10, Benchmark protein standard (Gibco). Observed relative band intensities were consistent with the results of northern blot analysis.


(a)
NTR
1 2 3 4 5 6 7 8 9 10
(b)
NTR
1 2 3 4 5 6 7 8 9 10
(c)
NTR
1 2 3 4 5 6 7 8 9
(d)
NTR
1 2 3 4 5 6 7 8 9 10

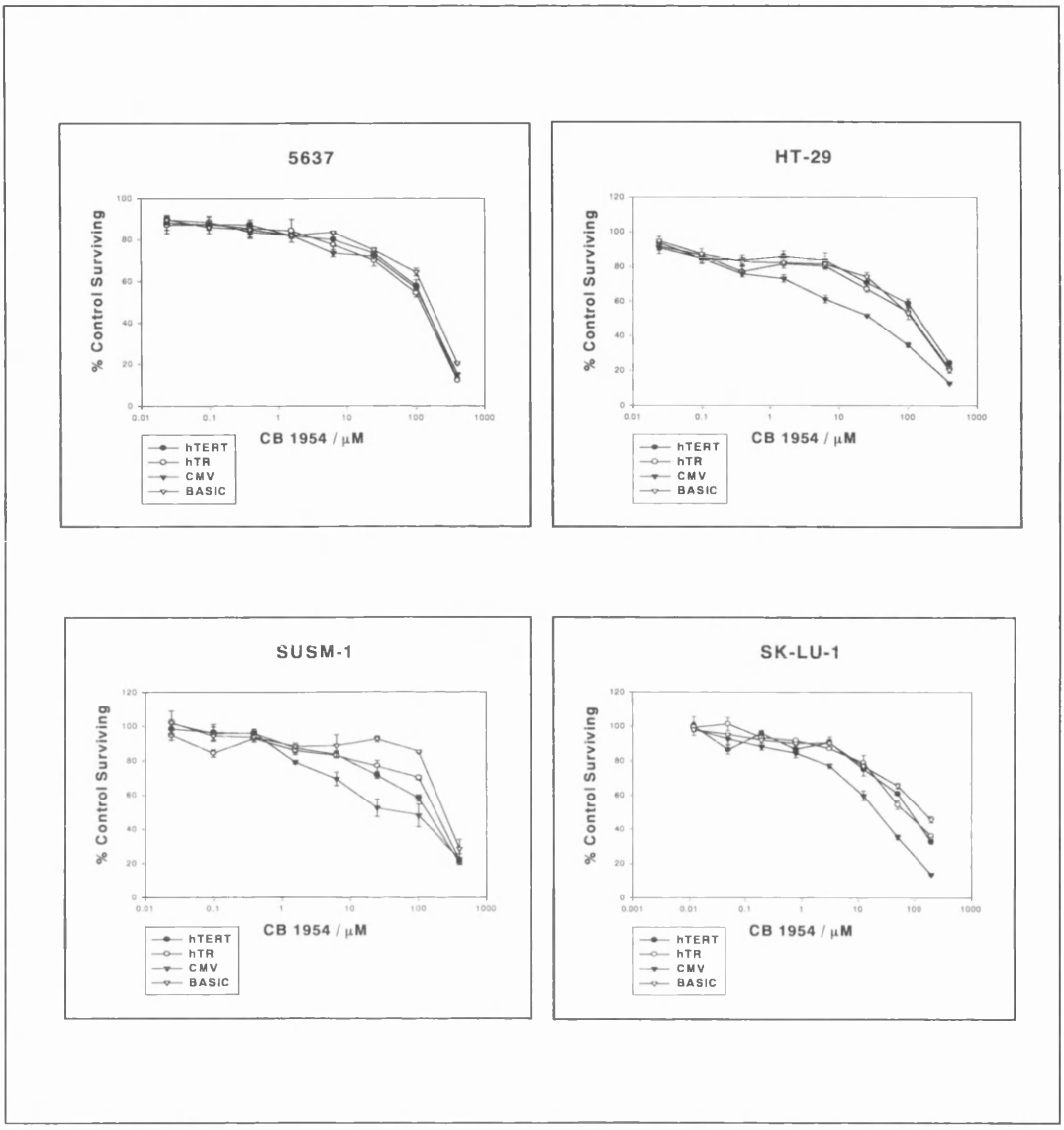


Figure 4.5: Cytotoxicity curves of the cell lines that were not sensitised to CB1954.

The immortal cell lines 5637, SUSM-1, HT29 and SK-LU-1 were not significantly sensitised to CB1954 by expression of NTR from hTERC (open circles) and hTERT (closed circles) promoters. Each point represents the mean and standard error of cell density estimated by MTT assay from triplicate plates and expressed as a percentage of control (untreated) cells. All assays were repeated at least 3 times. The strongest promoter in these cell lines is CMV (closed triangles). The curves for hTERC and hTERT promoters are not significantly shifted away from those of the basic, promoterless vector (open triangles).

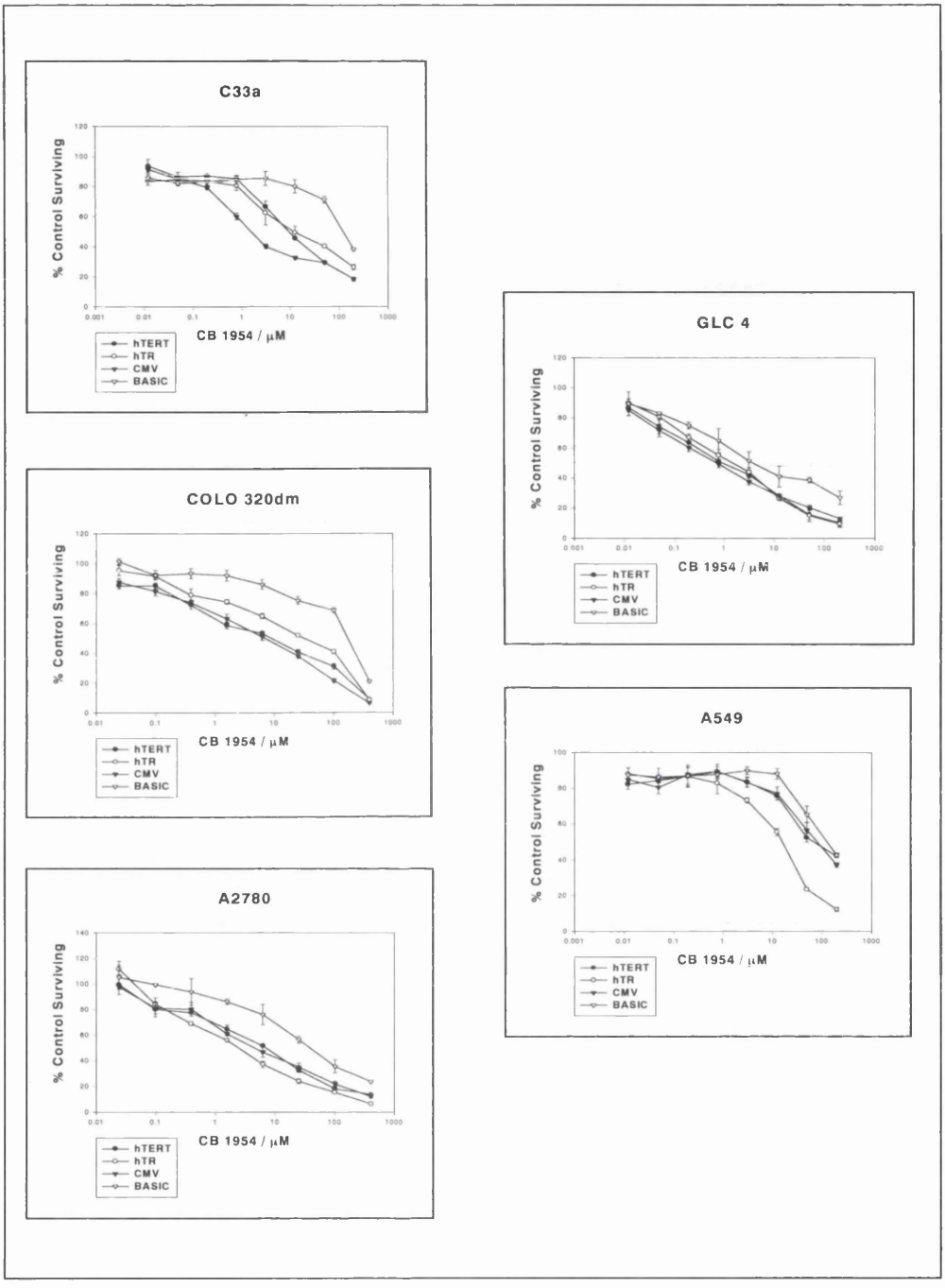
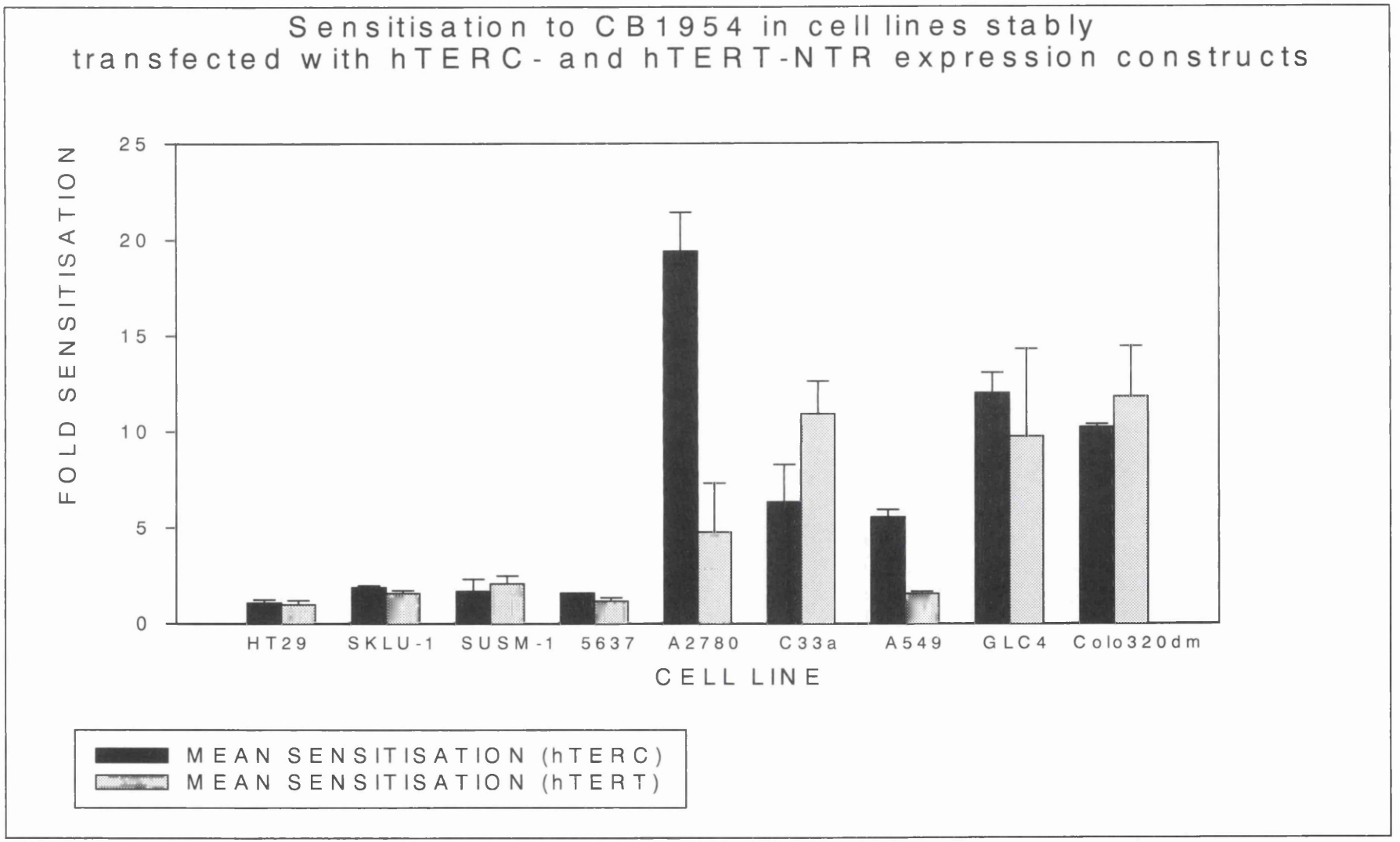


Figure 4.6: Cytotoxicity curves of the cell lines that showed significant sensitisation to CB1954 by expression of hTERC-NTR and hTERT-NTR.

C33-A, A2780, A549, GLC4 and Colo320dm cells showed significant sensitisation to CB1954 when NTR was expressed from one or both of the hTERC (open circles) and hTERT (closed circles) promoters. Each point represents the mean and standard error of cell density estimated by MTT assay from triplicate plates and expressed as a percentage of control (untreated) cells. All assays were repeated at least 3 times. Data shown are representative cytotoxicity curves. In these cell lines, hTERC and hTERT showed activity that was able to drive sufficient expression of NTR to sensitise the cells to CB1954. This is shown by the shift in the cytotoxicity curves away from that of the basic promoterless vector (open triangles). The CMV promoter showed variable activity between these cells.

Figure 4.7: Mean sensitisation to CB1954 by expression of hTERC-NTR and hTERT-NTR in all stable cell lines.

Sensitisation values are taken to be the fold difference between mean IC50 values derived from triplicate plates for basic-NTR transfected cells and those transfected with hTERC-NTR or hTERT-NTR plasmids and are calculated from the mean sensitisation values derived from 3 independent experiments. IC50 measurements within individual experiments were analysed using Softmax 2.32 software and are the mean values across 3 plates of the drug concentration necessary to give a 50% reduction in cell density. Only the cell lines with high telomerase promoter activities in chapter 3 are significantly sensitised to CB1954.


Sensitisation to CB1954 in cell lines stably transfected with hTERC- and hTERT-NTR expression constructs
FOLD SENSITISATION
25
20
15
10
5
0
HT29
SKLU-1
SUSM-1
5637
A2780
C33a
A549
GLC4
Colo320dm
CELL LINE
MEAN SENSITISATION (hTERC)
MEAN SENSITISATION (hTERT)

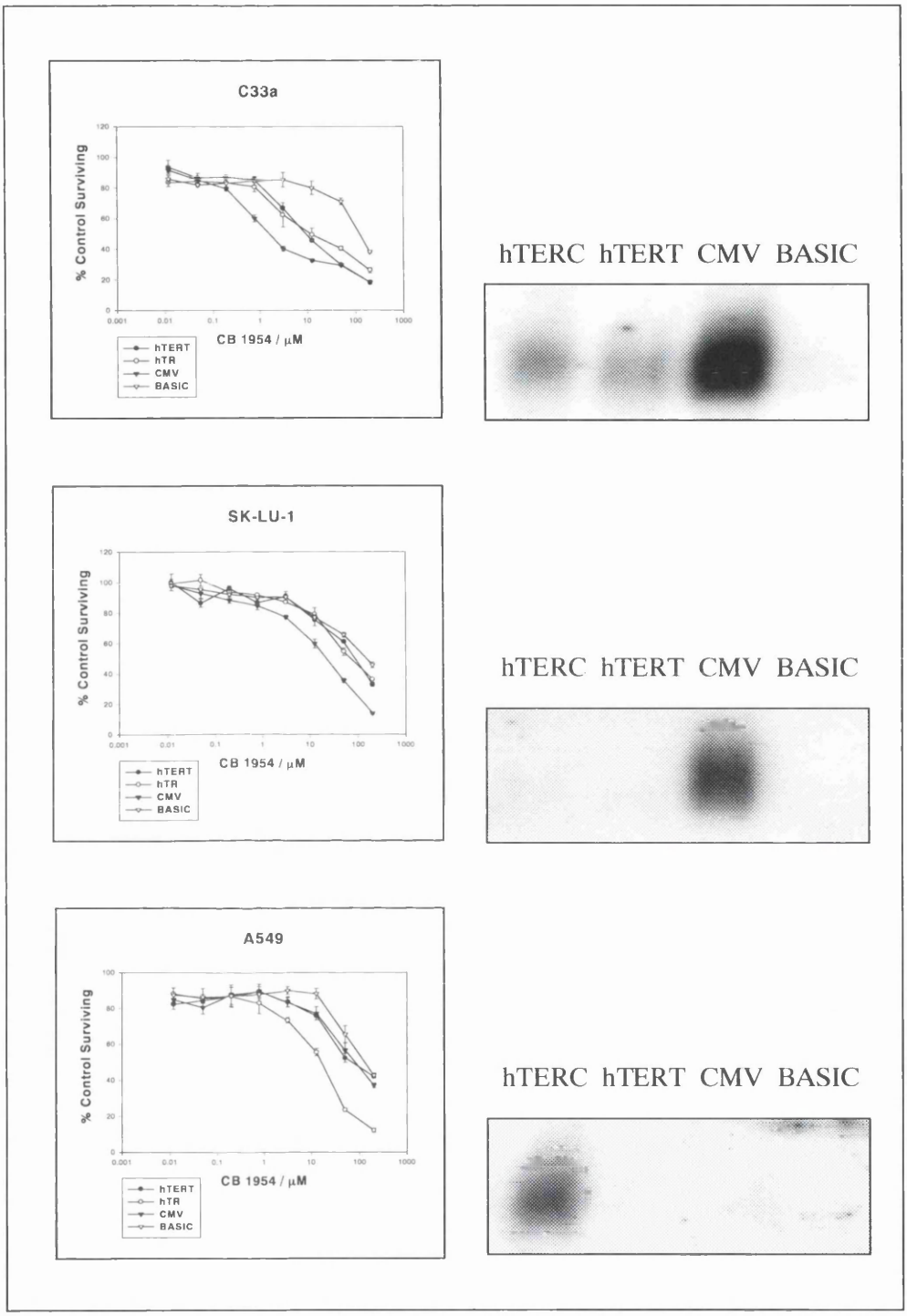


Figure 4.8: Promoter activities predict sensitisation to CB1954 in NTR expressing stable cell lines.

Stable cell lines transfected with basic-NTR, CMV-NTR, hTERC-NTR, or hTERT-NTR were exposed to CB1954 for 24 hours and the cytotoxic effect was quantified by MTT assay as previously described. Cytotoxicicty curves are shown in the left panels. Panels on the right show the levels of NTR detected in Northern blots (figure 4.3) for each pool of stables. High expression of NTR was correlated with sensitisation to CB1954 manifested by a shift in one or more of the cytotoxicity curves away from the basic (open triangles) curve. Both hTERC and hTERT showed high expression in C33-A cells that was lower than that of CMV, reflecting the pattern of the curve. Similarly, CMV was the only promoter capable of driving significant expression resulting in cytotoxicity in SK-LU-1 cells, while in A549 cells the only strong promoter was hTERC.

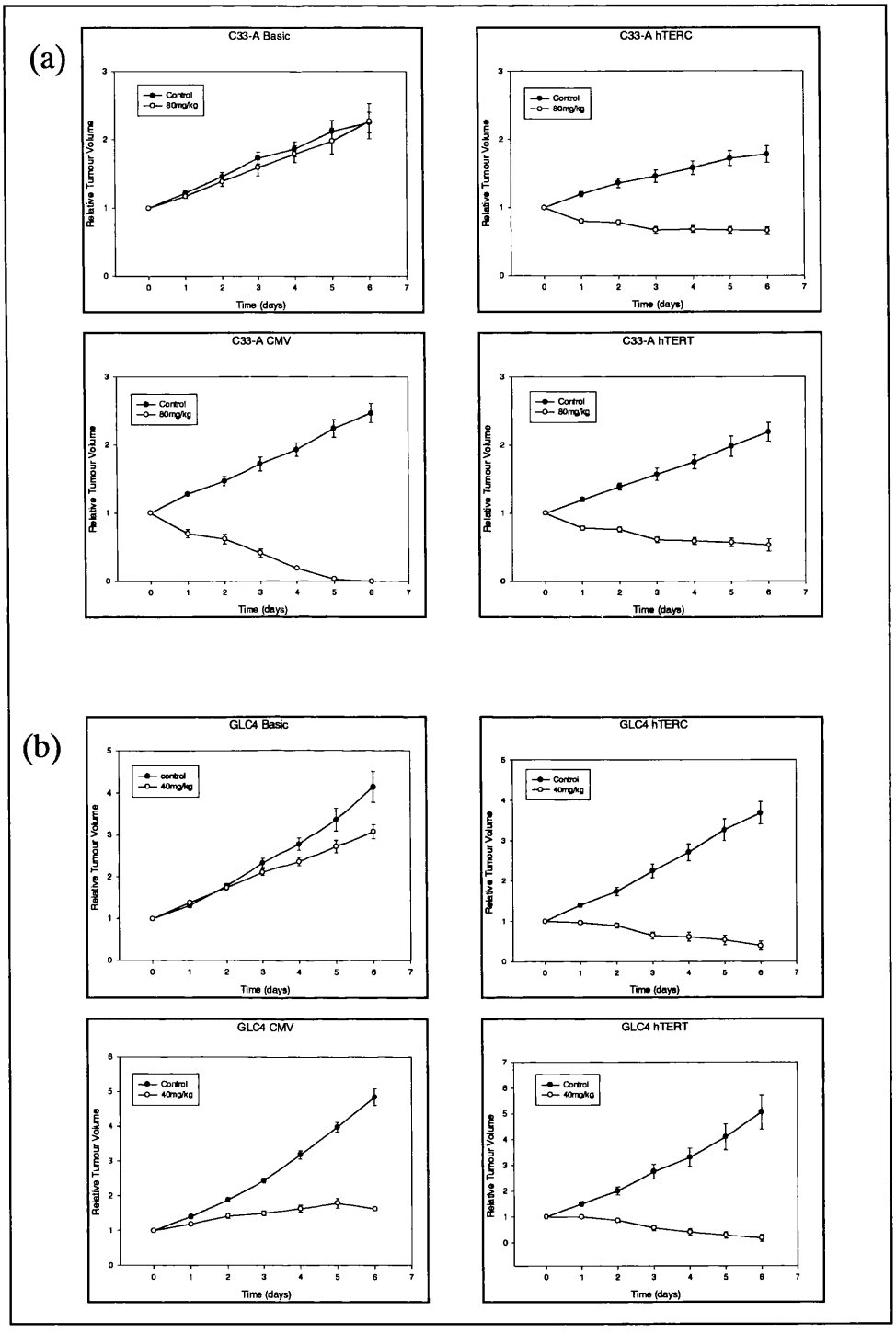


Figure 4.9: Reduction of tumour volume in GLC4-NTR and C33-A-NTR xenografts after i.v. injection of CB1954.

Each of the stable cell lines (a) C33-A and (b) GLC4 Basic-NTR, CMV-NTR, hTERC-NTR and hTERT-NTR were introduced into the flanks of female athymic nude mice. When tumour diameters reached approximately 5mm, animals were administered a single tail vein injection of 80mg/kg (C33-A) or 40 mg/kg (GLC4) of CB1954. Tumour volumes were monitored daily for 6 days after administration of CB1954. Each point represents the mean and standard error derived from 6 mice per group.

CELL LINE	BASIC (s.e.) μM	CMV (s.e.) μM	hTERC (s.e.) μM	hTERT (s.e.) μM
HT29	227.6 (57.68)	109.26 (41.77)	213.57 (49.6)	242.2 (53.56)
SK-LU-1	120.07 (25.73)	14.99 (6.68)	61.81 (11.9)	70.93 (11.94)
SUSM-1	264.77 (22.88)	72.48 (21.1)	177.77 (24.77)	130.82 (25.74)
WI-38	76.84 (21.78)	55.44 (17.1)	60.62 (5.68)	77.09 (10.91)
5637	171.23 (4.45)	115.55 (8.41)	110.73 (3.99)	149.03 (9.36)
A2780	25.76 (15.15)	3.65 (1.34)	1.26 (0.65)	5.2 (0.38)
C33-A	96.06 (29.55)	2.01 (0.259)	15.19(0.02)	8.59 (1.38)
GLC4	4.66 (0.81)	0.7 (0.23)	0.53 (0.09)	0.68 (0.25)
Colo320dm	191.42 (56.51)	7.1 (2.87)	23.77 (6.84)	13.86 (3.43)
A549	125.49 (20.81)	81.12 (10.9)	19.12 (3.38)	67.01 (6.03)

Table 4.2: Mean μM IC50 values for the 4 constructs in stable cell lines.

NTR expressing stable cell lines were treated for 24 hours with a titration of CB1954. Mean values and standard errors (s.e.) are derived from 3 independent experiments. For a single experiment, IC50 values were calculated from the mean value of triplicate measurements of the concentration of CB1954 necessary to give a 50% reduction in cell density. Data were analysed with the Softmax 2.32 microplate analysis software.

CHAPTER 5

ADENOVIRUS MEDIATED DELIVERY OF hTERC AND hTERT-NITROREDUCTASE GENE THERAPY VECTORS

- Adenovirus is an efficient vehicle for gene delivery to cell lines in this model.
- Cloning and characterisation of Ad-hTERC-NTR and Ad-hTERT-NTR gene therapy virus vectors.
- Promoter dependent expression of an NTR splice variant.
- Ad-hTERC-NTR and Ad-hTERT-NTR sensitise human cancer cells to CB1954 *in vitro and in vivo.*

5 ADENOVIRUS MEDIATED DELIVERY OF hTERC AND hTERT-NITROREDUCTASE GENE THERAPY VECTORS.

5.1 Abstract

In order to model delivery of hTERC-NTR and hTERT-NTR expression constructs to human cancer cells *in vitro*, the constructs were cloned into the E1 region of an E1/E3 deleted adenovirus using the Ad-easy cloning system obtained from Q-biogene (He et al. 1998) to generate the adenoviruses Ad-hTERC-NTR and Ad-hTERT-NTR. Analysis of NTR expression in human cervical carcinoma and mortal human foetal lung fibroblast cells infected with Ad-hTERC-NTR and Ad-hTERT-NTR revealed the expected cell specific, promoter dependent patterns of NTR expression. However, northern blotting seemed to reveal a longer transcript in hTERT-NTR virus infected cells than in the Ad-hTERC-NTR infected cells. To clarify the length and sequence of the transcribed RNA species, cDNAs were generated and sequenced, revealing a splice variant with an 187bp deletion from the expected transcript sequence both for hTERC-NTR and hTERT-NTR. The deletion is predicted to encode an in-frame 22 amino acid truncation of the NTR protein with an additional 5 amino acid mutation. The deletion was not predicted to abrogate the function of NTR, and to confirm that functional nitroreductase was expressed in a cancer cell specific manner, 3 cancer and 2 mortal cell strains were infected with the hTERC-NTR and hTERT-NTR viruses and subjected to cell survival assays after challenge with CB1954. The results indicated that the cells were sensitised to CB1954 in a promoter-dependent manner, with a cervical carcinoma cell line and an ovarian adenocarcinoma cell line, both of which exhibit high promoter activity, significantly sensitised to CB1954. The remaining cancer and mortal cell strains, which have low hTERC and hTERT promoter activities, were not sensitised to the effects of CB1954. Sensitisation to CB1954 was dependent also on infection efficiency. Thus, adenovirus vectors harbouring hTERC-NTR and hTERT-NTR expression constructs sensitise human cancer cells to the cytotoxic effects of activated CB1954 in a manner which is dependent partly on promoter activity and partly on infectivity.

5.2 Introduction

Adenoviruses are currently the vectors of choice for a broad range of gene delivery systems in gene therapy. Among the attractive features of adenovirus vectors are a broad tissue tropism and the ability to infect both cycling and non-cycling cells with a high efficiency ((Mashhour et al. 1994) and references therein). Additionally, well characterised systems have been in use for a number of years which permit the high titre production of replication defective vectors deleted in the E1 region of the viral genome (first generation vectors), which is essential for efficient early gene transcription, by trans-complementation in cell lines stably expressing E1 gene products (Graham et al. 1977; Imler et al. 1996).

In the classical model of adenovirus internalisation and nuclear trafficking, an initial high affinity interaction occurs between residues of the terminal knob domain of the trimeric viral capsid fibre protein and the primary cellular receptor, hCAR, the human Coxsackie and Adenovirus Receptor (Bergelson et al. 1997; Kirby et al. 1999; Roelvink et al. 1999; Kirby et al. 2000). Subsequent interactions between cellular $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and protruding RGD motifs in the virus capsid protein penton base mediate endocytotic internalisation in a pathway dependent on Dynamin (Wickham et al. 1993; Wang et al. 1998). While other cellular adenovirus receptors, such as MHC class I heavy chain, have been implicated in non-CAR dependent internalisation pathways (Hong et al. 1997), hCAR dependent internalisation is considered to be the primary internalisation pathway in human cancer cells when both hCAR and MHC class I heavy chain are expressed (McDonald et al. 1999). Indeed, CAR expression has been correlated with adenovirus permissiveness or refraction in a number of systems including ovarian cell lines, mouse hepatocytes *in vivo*, and a model of adenovirus gene delivery to differentiated versus non-differentiated airway epithelium (Zabner et al. 1996; Zabner et al. 1997; Walters et al. 1999; You et al. 2001).

Penetration of the endosome occurs swiftly following endocytosis and prior to endolysosomal fusion in a pathway that involves both a function of the cytoplasmic tail of integrin β_5 and the activation of the pH dependent adenovirus protease on endosomal acidification (Wickham et al. 1994; Greber et al. 1996; Wang et al. 2000). The combined action of these events mediates both virus uncoating and membrane permeation leading to adenovirus release to the cytosol. The virus subsequently traffics to the nucleus by a microtubule dependent mechanism (Suomalainen et al. 1999; Leopold et al. 2000) and makes an interaction with nuclear pores via the hexon capsid protein (Greber et al. 1997).

Trafficking to the nucleus and subsequent transgene expression is rapid, requiring around 1 hour for 99% of cell-surface bound virus to reach the nucleus and around 24 hours for maximal transgene expression (Leopold et al. 1998).

In the nucleus, the alternatively spliced products of the E1a gene mediate the initiation of transcription of other viral and cellular genes including the viral E2 products which encode the polymerase and DNA binding proteins that are essential for virus replication. Transcriptional modulation by E1a gene products occurs by multiple independent mechanisms including displacement of the cellular transcription factor E2F-1 from negative regulation by pRB facilitating the transcriptional up-regulation of S-phase components (reviewed in Flint et al. 1997). Thus, the wild type adenovirus E1 gene products mediate changes to the cellular environment favourable for productive infection. Hence, E1 deleted adenoviruses are defective in the genes necessary for efficient replication and introduction of transgenes of interest into the E1 region allows for highly efficient transduction of target cell populations with genes of interest.

Replication defective adenoviruses are therefore regarded to be excellent gene delivery vehicles for use *in vitro* and *in vivo*. Indeed, the on-line clinical trials database of the Journal of Gene Medicine reports that adenovirus vector clinical trials account for 27.7% of current trials in gene transfer (164 of 596 worldwide trials recorded in the database) (Wiley & sons (http://www.wiley.com/legacy/whileychi/genmed/clinical). However, while a number of tissue types have been successfully targeted, the efficiency of first generation adenoviral vectors as gene delivery vehicles for the transduction of tissues *in vivo* is limited by at least three major factors.

Firstly, the immunological hurdle to adenovirus gene therapy is considerable. Adenovector capsid components are highly immunogenic, inducing widespread activation of cellular immune responses that may damage the target tissue and can also limit persistence of transgene expression (Yang et al. 1996; Kafri et al. 1998; Molinier-Frenkel et al. 2000). Additionally, antigenic transgenes may also elicit an immune response (Yang et al. 1996). An important additional problem in humans is the high frequency of previous exposure to wild-type adenovirus infections: antibodies to serotypes 1, 2, and 5 are present in around 40-60% of children (reviewed in Horowitz 1996), and this may limit the efficiency of gene transfer even on the first delivery of recombinant vector (Stallwood et al. 2000). Strategies developed to overcome this problem include coating the viral capsid with non-immunogenic polymers (Fisher et al. 2001).

Additionally, endogenous gene expression of E1 deleted adenovirus vectors, while severely limited, is not absolutely abolished. The E4 orf6/7 gene product can mediate low level transcriptional activation of late gene products leading to enhanced immunogenicity and low-level replication (O'Connor et al. 2000). Strategies developed to overcome this problem initially focussed on the development of second-generation vectors incorporating deletions of both E1 and E4 genes (Gao et al. 1996), or E1 and E2 genes (Gorziglia et al. 1996). However, more recent developments in technology for vector manipulation have seen the high titre production of helper dependent, or "gutless" vectors, that have essentially the entire wild-type viral genome deleted with the exception of the inverted terminal repeat and the packaging signal (Hardy et al. 1997), reviewed in (Morsy et al. 1999). These vectors allow for the incorporation of larger or multiple transgenes and display reduced immunogenicity manifested by enhanced transgene persistence *in vivo* (Schiedner et al. 1998).

The efficiency of delivery to target tissues is largely dependent on expression of hCAR. Among the tissues to which adenovirus mediated gene delivery has been inefficient due to low or undetectable expression of hCAR are differentiated airway epithelium (Walters et al. 1999), in addition to alveolar macrophages (Kaner et al. 1999) and the de-differentiated cells of human tumours such as some bladder cancer cell lines (Li et al. 1999) and some ovarian cancer cell lines (You et al. 2001). The recent identification of hCAR as a transmembrane component of tight junctions may help to explain the inefficiency of transfer to some polarised epithelial surfaces *in vivo* as the receptor may be sequestered in tight junctions (Cohen et al. 2001). Moreover, recent work indicates that the CAR transcript is alternatively spliced, giving rise to a second major variant deleted in the transmembrane domain that may act as a soluble receptor to sequester adenovirus particles. Several additional deleted and truncated variants are also detectable only by nested PCR, although the significance as concerns the fibre binding function is unknown. Interestingly, skeletal muscle was shown to express only the smallest of the truncated variants (Thoelen et al. 2001). The last several years have seen major advances in the genetic and antibody mediated manipulation of adenovirus surface proteins which have enabled retargeting of binding specificity of the fibre to alternative cell surface markers such as the folate receptor, epithelial cell adhesion molecule, fibroblast growth factor receptor and epidermal growth factor receptor (Douglas et al. 1996; Gu et al. 1999; Haisma et al. 1999; Dmitriev et al. 2000). This kind of approach may be expected to overcome the limitations to adenovirus gene transfer imposed by the hCAR expression profile, and may additionally enable the development of vectors incorporating multiple layers of target specificity.

Several groups have reported the use of adenovectors to deliver pro-drug activating enzymes to cancer cells, such as viral Thymidine Kinase, Cytosine Deaminase and, more recently, nitroreductase. Most studies place these genes under the control either of commonly used control promoters such as SV40 or CMV or of tissue specific promoters such as PSA or CEA. While most studies to date remain at the pre-clinical stage, a number of clinical protocols have been proposed (Alvarez et al. 1997; Crystal et al. 1997; Morris et al. 2000).

In this chapter, a reporter adenovirus expressing the E.Coli LacZ gene under the control of the CMV promoter (Ad-CMV-LacZ) is used to demonstrate the efficiency of adenovirus mediated gene transfer to cell lines characterised in the preceding chapters. Ad-CMV-LacZ efficiently transduced most cell lines and is therefore selected for further study as a potential vehicle for transduction of hTERC-NTR and hTERT-NTR expression constructs into cancer cell lines. The hTERC-NTR and hTERT-NTR expression constructs previously demonstrated to specifically sensitise cancer cells in a promoter dependent fashion to the effects of the pro-drug CB1954 are cloned into the E1 region of an E1/E3 deleted adenovirus genome using the commercially available Adeasy system (He et al. 1998) to generate the vectors Ad-hTERC-NTR and Ad-hTERT-NTR. Recombinant adenoviruses were amplified by Q-Biogene custom services.

Expression of NTR is characterised by northern blotting RNA extracted from a cervical carcinoma cell line infected with Ad-hTERC-NTR and Ad-hTERT-NTR and data are presented indicating that NTR mRNA expression is initiated at the correct transcriptional start sites of the hTERC and hTERT promoters within an adenovirus backbone. NTR mRNA was polyadenylated at a site within the adenovirus right arm homology region, but the major mRNA species expressed from both hTERC and hTERT promoters was an alternative splicing product with a 187bp deletion, in which the terminal 66bp of the NTR coding sequence are removed and the rest of the sequence is fused with a stop codon in the viral right arm homology domain. The splice variant encoded an in-frame 22 amino acid truncation of the NTR protein with an additional 5 amino acid mutation, but this was not predicted to affect the function of NTR.

Western blotting detection of NTR in protein extracts of a cervical carcinoma cell line and a mortal foetal lung fibroblast cell strain indicated that a protein species of approximately the correct apparent molecular weight (24kDa) was expressed in a promoter dependent and cell specific manner, yielding high level expression from both telomerase promoters only

in the cancer cell line. The effects of NTR expression in relation to the sensitisation of adenovirus transduced cell lines to CB1954 are investigated and results are presented to indicate that adenovirus mediated delivery of hTERC-NTR and hTERT-NTR to normal and cancer cell lines can specifically sensitise cancer cells to the effects of CB1954 in a manner dependent both on promoter activity and transduction efficiency. Moreover, direct intra-tumour injection of human cervical carcinoma xenografts grown in female athymic nude mice with Ad-hTERC-NTR and Ad-hTERT-NTR followed by tail vein injection with 80mg/kg CB1954 resulted in a potent anti-tumour effect with no obvious general toxicity in the animals. Therefore, adenovirus vectors harbouring hTERC-NTR and hTERT-NTR expression constructs sensitise human cancer cells to the cytotoxic effects of activated CB1954 both *in vitro* and *in vivo*.

5.3 Evaluation of the efficiency of delivery of adenovirus vectors to normal and cancer cell lines.

One of the most common ways to evaluate adenovirus infectivity in cells is by the use of a reporter virus harbouring a transgene such as GFP or LacZ, whose product can easily be detected in infected cells. In order to evaluate the replication defective adenovirus system as an adequate gene delivery system for hTERC-NTR and hTERT-NTR gene therapy, a commercially available CMV-LacZ adenovirus was employed.

13 cancer, mortal and ALT cell lines included in earlier analyses for hTERC and hTERT promoter activity and sensitivity to CB1954 in stable cell line models of hTERC and hTERT-nitroreductase gene therapy were infected for 1 hour at 37°C with a titration of CMV-Lac Z adenovirus (0, 1, 10, or 100 plaque forming units (p.f.u.) per cell) in 100µl. After 1 hour, the cells were incubated overnight in low serum and the following day the cells were fixed and stained overnight with X-gal at pH 7.4 to detect blue (infected) cells. Cells were photographed and those cells staining blue were counted and expressed as a percentage of total cells in each of 5 random fields for each concentration of virus. Approximately 1000 cells were counted in total for each multiplicity of infection and all experiments were conducted at least twice. The mean infectivity for each cell line, derived from two independent experiments, is presented in figure 5.1 and representative photomicrographs of adenovirus infected cells are presented in figure 5.2.

Figure 5.1 confirms that adenovirus is a highly efficient gene delivery vehicle for incorporation in the hTERC and hTERT gene therapy model: while no blue cells were seen

in any of the mock infected cell lines, the Lac Z adenovirus could transduce several cell lines with 100% efficiency at a multiplicity of infection of 100 p.f.u. per cell. 5637, SUSM-1, A549, IMR-90, BJ-1hTERT, and WI-38 cells were all transduced in a dose-dependent fashion, reaching 100% infectivity at 100 p.f.u. per cell, with minimum infectivity at low viral doses of 1 p.f.u. per cell of between 10% (A549) and 23% (SUSM-1). That WI-38 cells could be so efficiently transduced is of paramount importance since these cells could not be developed as stable cell lines for evaluation of CB1954 induced cytotoxicity in hTERC and hTERT gene therapy, and they provide an essential negative control by which to evaluate the cancer cell specificity of this approach.

A number of other cell lines were less effectively transduced, including the cancer cell lines C33-A, A2780, and GLC4. The percentages of C33-A infected at the 3 virus concentrations was 13% at 1 p.f.u., 43% at 10 p.f.u., reaching a maximal infection of 64% at 100 p.f.u., while A2780 infection efficiencies ranged between 3.5% at 1 p.f.u. and only 43.1% at 100 p.f.u per cell. The colorectal adenocarcinoma cell line Colo320dm could be infected at efficiencies of 23% (1 p.f.u.), 49% (10 p.f.u.) and 90% (100 p.f.u.) although, as described below, these cells were susceptible to widespread acute toxicity which may have affected the result. GLC4 cells were among the least efficiently infected of all cell lines, showing less than 1% infectivity at both 1 and 10 p.f.u. per cell and reaching a maximal value of only 4.6% infected cells at 100 p.f.u. The testicular teratoma line SuSa, were also refractive to the virus, with less than 2% infected cells at either 1 or 10 p.f.u. per cell. It is interesting to note, since the efficacy of hTERC and hTERT-NTR constructs delivered by an adenovirus will depend not only on promoter activity in cell lines but also on transduction efficiency, that the cancer cell lines which displayed greatest sensitisation to CB1954 in the preceding chapter were all less effectively transduced by adenovirus than the cell lines which did not respond.

At the highest multiplicity of infection, acute toxicity was observed in several cell lines. The infection efficiency of SuSa testicular teratoma cells could not be determined at 100 p.f.u. per cell, as no cells remained attached to the wells after overnight incubation in low serum. A similar reduction in cell density, although not as pronounced, was observed in Colo320dm cells; following overnight incubation prior to staining, the density of cells anchored to the growth surface was reduced to approximately 10% of the density of mock infected cells. The infectivity of the virus in the surviving fraction of Colo320dm cells has been quantified, although it is acknowledged that acute toxicity may mask the true value. C33-A cells exhibited a comparatively low degree of vector-induced toxicity (cell densities

reduced to around 70% of control at 100 p.f.u.), which could be reduced further in the assays described below by infection with an upper limit of 50 p.f.u. per cell. No significant toxicity was observed in any other cell line.

5.4 Cloning and amplification of the Ad-hTERC-NTR and Ad-hTERT-NTR gene therapy vectors using the Adeasy system.

A number of commercially available systems can be employed to facilitate the cloning of transgenes into the E1 region of the adenovirus genome. In this study, the Adeasy system, which relies on homologous recombination of the adenoviral genome with corresponding sequences in a specifically engineered "transfer vector" within a recA+ bacterial strain has been employed (He et al. 1998). Figures 5.3 and 5.4 show the cloning strategy that was employed in the generation of recombinant Ad-hTERC-NTR and Ad-hTERT-NTR.

The 740bp SalI fragment of pd2NTR-hTERC, containing the complete coding sequence of nitroreductase, was isolated by restriction digest, gel electrophoresis and gel extraction and was cloned into the SalI site of the transfer vector pShuttle to form the intermediate transfer vector pShuNT. Next, the 889bp HindIII and 606bp XhoI fragments of pd2NTR-hTERC and pd2NTR-hTERT, respectively, were isolated and cloned into the HindIII and XhoI sites of pShuNT to generate the transfer vectors pShuNT-hTERC and pShuNT-hTERT, containing the NTR coding sequence under the control of hTERC and hTERT promoters. The constructs were linearised by digestion with PmeI, which cleaves between the left and right arm homology regions of the closed circular plasmid, releasing these in the correct orientation for interaction with the corresponding regions of pAdeasy-1.

1μg of each linearised transfer vector was co-transformed with 100ng pAdeasy-1 in the BJ5183 E.Coli strain by electroporation in a 2mm cuvette at 2.8KV and plated out overnight to allow the growth of transformed colonies. This bacterial strain supports homologous recombination by virtue of the presence of the recA protein. Selected colonies were inoculated into L-broth and allowed to grow overnight then miniprep DNA was extracted and potential recombinant plasmids were transformed into DH5α for stable amplification of the plasmid. Presence of the recombined transfer vector in the adenoviral backbone was confirmed by restriction digest and the orientation of the promoter in relation to NTR was confirmed by sequencing with the primers Shunt1f and Shunt1r.

Recombinant adenoviruses were amplified on the HEK-293 cell line, which produces E1a gene products *in trans*. Amplification and purification of the adenoviruses was performed by the custom service of Q-biogene. 293 cells were initially transfected with 5μg recombinant adenoviral DNA. 50μl DNA (0.1μg/μl) was mixed with 169μl dH_2O and 5μl 2M $CaCl_2$. An Additional 26μl $CaCl_2$ was added after mixing and a second tube containing 250μl HBS (Hepes Buffered Saline) was prepared. Air was bubbled through the HBS with a pipette and during this time, the DNA/$CaCl_2$ mixture was added. Cells were removed from incubation and the transfection mixture was added drop-wise to the medium. Cells were incubated overnight, then the transfection solution was removed and the cells were rinsed in PBS. Cells were then incubated for 14 days to allow for the formation of a cytopathic effect (CPE). After this stage, small-scale amplification was performed using an initial volume of 0.1ml of crude virus from cell lysate supernatant to infect 10^5 cells. Virus was released from cells by 3 cycles of freeze/thawing between -20°C and 37°C. After several rounds of amplification using aliquots of up to 45ml crude virus released in tissue culture supernatant to infect up to $3x10^8$ 293 cells, the large-scale adenovirus preparation was performed by freeze/thawing to release virus and purification by $CsCl_2$ banding. Quantification of the viral titrations in Viral Particles (V.P.) was performed by measurements of optical density ($O.D._{260}$) and measurements of Plaque Forming Units (P.F.U.) were performed by plaque assay.

VECTOR	VIRAL PARTICLES/ml	PLAQUE FORMING UNITS/ml
Ad-hTERC-NTR	$2.50x10^{11}$	$1.60x10^{10}$
Ad-hTERT-NTR	$1.86x10^{11}$	$2.85x10^{10}$

Table 5.1: Quantification of adenovirus particle titre by O.D. and plaque assay

Adenovirus gene therapy vectors were amplified on 293 cells as described in materials and methods. Quantification of viral particles was determined at $O.D._{260}$ and infectious units were calculated by plaque assay.

5.5 Characterisation of the expression of NTR in Ad-hTERC-NTR and Ad-hTERT-NTR infected cells.

5.5.1 Alternative splicing of NTR transcripts expressed in C33-A

In order to confirm the promoter dependent expression of functional nitroreductase in cancer cells, 75 cm^2 flasks of C33-A cells were infected for 1 hour at 37°C at a multiplicity of infection of 50 p.f.u. per cell with each of the Ad-hTERC-NTR and Ad-hTERT-NTR viruses. 48 hours post-infection, corresponding to the day on which pro-drug would be administered in an *in vitro* cytotoxicity assay, RNA or protein was extracted from the cells to be used in assays of gene expression. Figure 5.5 shows the results of northern blot analysis of the expression of nitroreductase in C33-A cells. Cells infected with Ad-hTERC-NTR and Ad-hTERT-NTR (lanes 2 and 3) produced strong signals with the hTERC promoter construct generating a more intense signal than hTERT. Both hTERC and hTERT promoters generated stronger signals than equivalent amounts of RNA extracted from the C33-A-hTERC-NTR stable cell line (lane 1), indicating that the efficiency of gene transfer was good even though not all cells would have been transduced at this multiplicity of infection (figure 5.1 & 5.2). Mock infected cell RNA (lane 4) did not contain any detectable NTR transcript. These results indicated that NTR was expressed in C33-A cells following adenovirus transduction in a promoter dependent manner. The size of both transcripts was greater than that of the NTR transcript expressed in C33-A-hTERC-NTR cells, in-keeping with polyadenylation of the transcript at a signal within the adenovirus right arm homology region. Unexpectedly, however, the hTERT-NTR transcript migrated at a size approximately 100-200 bases longer than the hTERC-NTR transcript.

In order to precisely define the length and sequence of the transcripts, 5' and 3' RACE reactions were performed using cDNA transcribed from total RNA extracted from Ad-hTERC-NTR and Ad-hTERT-NTR infected C33-A cells using the SmartRACE kit (Clontech). Figure 5.6 shows gel photographs of the PCR reactions for amplification of the 5' (lane 1 (a) and (b)) and 3' ends (lane 6 (a) and (b)) of the (a) Ad-hTERC-NTR and (b) Ad-hTERT-NTR transcripts. The other lanes represent positive and negative internal controls. Amplification of cDNA generated from Ad-hTERC-NTR infected cells using

primers specific for the 5' end of NTR produced a single, specific band of a size consistent with the expected 262bp product of the 5' amplification (lane 1), as measured against a 123bp DNA ladder. The Ad-hTERT-NTR 5' reaction produced a single weak product, consistent with the expected size of 244bp (lane 1). It should be noted that several groups have reported variation in the transcriptional start site of the hTERT promoter.

The product of the 3'RACE reaction from Ad-hTERC-NTR infected cells (lane 6 (a)) was, unexpectedly, approximately 200bp shorter than the predicted product of approximately 1200bp, while Ad-hTERT-NTR 3' RACE reaction (lane 6 (b)) unexpectedly produced a doublet with one large band migrating at the expected size of approximately 1200bp and an extra band some 200bp shorter. In order to precisely define the sequence of each transcript, reaction products generated from 5' and 3' RACE were directly cloned into pCRII-TOPO vector (InVitrogen) which allows direct cloning of PCR products, and grown in DH5α cells for DNA maxipreparation of clones and sequencing. Sequence analysis and contig assembly of the fragments was performed in Vector NTI suite.

Alignment of the sequences of the hTERC-NTR 5'end RACE product with the predicted insert of hTERC-NTR expression cassette (figure 5.7 (a)) confirmed the location of the hTERC transcriptional start site proposed by Feng et al (Feng et al. 1995) and indicated that the transcription of NTR from the hTERC promoter-NTR expression construct is initiated correctly within the adenovirus backbone. Moreover, the transcriptional start site defined by the product of the hTERT 5' RACE reaction (figure 5.7 (b)) lies 64bp upstream from the hTERT start codon. This is consistent with previous reports that place the hTERT transcriptional start in the region of 60 to 112bp upstream of the ATG (Cong et al. 1999; Takakura et al. 1999; Wick et al. 1999). Hence, transcription from both hTERC and hTERT promoters was correctly initiated within the adenovirus expression vectors.

Assembly and alignment of the complete 5' and 3' sequence contig of the long transcript identified in the Ad-hTERT-NTR 3' RACE reaction revealed that the transcript sequence was identical to the predicted sequence for the NTR transcript. (Figure 5.7(c)). However, the complete sequences of the short 3' RACE reactions of both Ad-hTERC-NTR and Ad-hTERT-NTR transcripts revealed a 187bp deletion from the predicted sequence that removes the terminal 66bp of the NTR coding sequence, in addition to a portion of the downstream viral sequence (figure 5.7 (a) and (b)).

In order to determine at which stage during viral cloning, amplification, or infection the deletions had occurred, PCR primers AdNTSeq5a and AdDelR were designed to amplify a product that includes the deleted region and in which the deleted region would be easily distinguishable from the full length product. In a full length DNA, the amplification was designed to give rise to a 762bp product, but reactions containing the deletion amplify a 575bp product. For each of Ad-hTERC-NTR and Ad-hTERT-NTR, separate PCR reactions were performed using the plasmid DNA that was originally transfected into 293 cells to generate the virus, DNA extracted from the viral particles using Lyse-n-Go reagent, and cDNA generated from virus infected C33-A cells. Figure 5.8 shows the products of the amplification reactions. Both the plasmid (lanes 2 and 5) and virus (lanes 3 and 6) DNAs of both Ad-hTERC-NTR (lanes 2-4) and Ad-hTERT-NTR (lanes 5-7) amplified a single full length product, indicating that the deletion was not present at any stage prior to or during the virus amplification. Amplification products generated from infected C33-A cDNA, however, contained 3 identical discrete bands. The largest band migrated at a size consistent with the full-length product, while the smallest band migrated at a size consistent with the presence of the 187bp deletion and was also the major product both in Ad-hTERC-NTR and Ad-hTERT-NTR reactions. An intermediate band of approximately 700bp was also identified.

Since both the deleted sequences in the short transcripts and the PCR reaction products of both Ad-hTERC-NTR and Ad-hTERT-NTR were identical, the results suggested the action of a non-random recombination event, such as alternative splicing. In order to address this possibility, the sequence of the full length transcript of hTERT-NTR (figure 5.7(a)) was used in a search for potential splice donor/acceptor sites using the SPL search facility at the Sanger Centre website (http://genomic.sanger.ac.uk/gf/gf.shtml). The results are displayed in the results screen copied into figure 5.9. Boxed numbers 687 and 873 (highlighted in the sequence of figure 5.7 (a)) represent the putative donor/acceptor sites that correspond exactly to the deleted region of the short transcripts (figures 5.7 (b) and (c)). Therefore, the major NTR mRNA expressed in C33-A after infection with both Ad-hTERC-NTR and Ad-hTERT-NTR is a splice variant encoding a 187bp deletion.

5.5.2 *In silico characterisation of the product of the short NTR splice variant*

Analysis of the predicted open reading frames in Vector NTI suite revealed that the deleted transcript codes for an in-frame truncation of the NTR protein that removes the terminal 22

amino acids. The translated product is predicted to contain an additional 5 amino acid mutation (^{191}VGHHS - QPPPP) compared with the published sequence, due to fusion of the nitroreductase coding sequence with a portion of the viral right arm homology domain which contains the stop codon UGA (Figure 5.10). In order to assess whether the truncated NTR would be expected to give rise to an inactive phenotype, firstly a search of the SWISSPROT database on the NCBI QBLAST server was conducted using the missing and mutated sequence to determine if the deleted residues possess homology to any other functional proteins. The results of the search indicated that the amino acid sequence has homology only with four other bacterial nitroreductase species (figure 5.11). Thus, these residues are not conserved in known protein species other than nitroreductases and are unlikely to be involved in similar catalytic function of proteins that are functionally homologous such as DT-diaphorase.

Using the nnpredict secondary structural prediction tool for comparison between NTR and the other nitroreductase sequences identified in the BLAST search did not reveal conservation of structural elements in this region (http://www.cmpharm.ucsf.edu/cgi-bin/nnpredict.pl) (figure 5.12). Moreover, the mutated region is distal from the active site of electron transfer to CB1954 and to the entrance by which CB1954 is predicted to access this site. However, It should be noted that two deleted residues, namely R^{207} and K^{205} may interact with the protruding phosphate group of the internally bound flavin mononucleotide electron donor and contribute to the stabilisation of its conformation within the binding pocket (Parkinson et al. 2000) (figure 5.10). Several other distal residues also orient FMN within the binding pocket. Thus, while the site of catalysis is unaffected, theoretically the binding of the FMN cofactor may not be optimal. However, the functional studies described below do not support the idea that the function of NTR was adversely affected.

Western blot analysis of 20 μg protein extracted from Ad-hTERC-NTR and Ad-hTERT-NTR infected C33-A and WI-38, shown in figure 5.13, revealed a single protein species of approximately the correct apparent molecular size in lanes 2 and 3, corresponding to the Ad-hTERC-NTR and Ad-hTERT-NTR infected C33-A. No signal was detected in the mock-infected lane of either cell line (lane 4, C33-A; lane 7, WI-38), or in the Ad-hTERT-NTR infected WI-38 (lane 6), but a weak signal was detected in WI-38 infected with Ad-hTERC-NTR (lane 5). Together, these results indicated that a truncated nitroreductase species was transcribed and translated from the adenoviral vectors in the expected promoter-dependent and cell-specific manner and confirmed that the cell-specific regulation of the telomerase promoters is retained in an adenoviral backbone.

5.6 Ad-hTERC-NTR and Ad-hTERT-NTR efficiently sensitise cancer cell lines to the effects of CB1954 in a promoter dependent and dose-dependent fashion.

In order to determine whether the function of nitroreductase was affected by the truncation, and whether the promoter specific responses to CB1954 observed in stable cell lines (Plumb et al. 2001, Chapter 4) are retained in parental cell lines expressing nitroreductase exogenously introduced by Ad-hTERC and Ad-hTERT vectors, 3 cancer and 2 mortal cell lines were infected with both 10 and 50 p.f.u. per cell of each of Ad-LacZ, Ad-hTERC-NTR, and Ad-hTERT-NTR for 1 hour at 37°C in 6-well plates. After infection, the cells were incubated in growth medium for a further 2 hours then trypsinised and plated out into 96-well plates (NTR infected cells) for MTT assay, or re-plated into 6-well plates (LacZ infected cells). Ad-hTERC-NTR and Ad-hTERT-NTR infected cells were allowed to proliferate for 2 days prior to 24 hour challenge with CB1954. On the same day, cells infected with LacZ were fixed and stained to give an indication of the percentage of cells infected with the adenoviral vectors on the day of drug challenge. All experiments were repeated at least 3 times.

Figures 5.14 and 5.15 show representative cytotoxicity curves for the cell lines tested and IC50 values for all cell lines with each promoter at each multiplicity of infection are given in table 5.2. A clear promoter-dependent and dose-dependent sensitising effect was seen only in C33-A and A2780 cells (figure 5.14), while other cell lines were unaffected by the combination of Ad-hTERC-NTR or Ad-hTERT-NTR infection and treatment with CB1954 (figure 5.15). Thus, the responses of cell lines to Ad-NTR and CB1954 treatment fell into two groups: those that were sensitised and those that were unaffected.

The summary of the IC50 values after CB1954 treatment for cells infected with Ad-hTERC-NTR and Ad-hTERT-NTR vectors at 10 and 50 p.f.u per cell is given in table 5.2. Values are derived from the results of 3 independent experiments for each cell line. C33-A cells showed the greatest response to both vectors. The basal IC50 of the uninfected cell line was 176.13μM, falling to 33.43μM and 9.76μM after infection with Ad-hTERC-NTR and 63.13μM and 35.86μM when infected with Ad-hTERT-NTR. Thus, adenovirus vectors harbouring the telomerase-nitroreductase expression cassettes sensitised C33-A cells to the effects of CB1954 in a promoter dependent and dose-dependent fashion. The shift in IC50 values is reflected in the pattern of the cytotoxicity curve for C33-A cells

presented in figure 5.14 (a): the concentration curves for cells infected with the viruses are shifted toward a lower drug concentration in a promoter and dose dependent manner, with the hTERC promoter generating the strongest response. The only other cell line tested that was significantly sensitised to CB1954 was the ovarian adenocarcinoma cell line A2780. A2780 had a basal IC50 value of 28.5μM and, therefore, were intrinsically one of the most sensitive cell lines assayed. Nevertheless, introduction of Ad-hTERC-NTR resulted in decreased IC50 values of 10.69μM and 3.85μM at 10 and 50 p.f.u, respectively, and introduction Ad-hTERT-NTR resulted in IC50 values of 17.73μM and 10.86μM at 10 and 50 p.f.u per cell. Thus, Ad-hTERC-NTR resulted in the greatest sensitisation, reflected in the dose-dependent shift of the concentration curve in figure 5.14 (b), while Ad-hTERT-NTR resulted in a modest sensitisation of approximately 2.8-fold at the highest infectious dose.

A caveat of telomerase directed gene therapy is that it must be tumour specific. In order to determine the capacity of the constructs to generate high-level expression of NTR and sensitisation to CB1954 specifically in cancer cells, two mortal cell strains were assayed. These were WI-38 foetal lung fibroblasts, and HMEC normal human mammary epithelial cells. In contrast to the strong sensitisation observed in C33-A and A2780, the two mortal cell lines tested were not sensitised to the effects of CB1954 by introduction of Ad-hTERC-NTR and Ad-hTERT-NTR. Mock infected WI-38 had an IC50 of 153.63μM that was not significantly changed either by introduction of Ad-hTERC-NTR at 10 p.f.u. per cell (178.09μM) and 50 p.f.u per cell (164.72μM), or by infection with Ad-hTERT-NTR at 10 p.f.u per cell (148.6μM) and 50 p.f.u. per cell (174.12μM). Thus, the hTERC and hTERT promoters did not drive sufficient expression of NTR to sensitise WI-38 fibroblasts to CB1954 (see also western blot in figure 5.13). Additionally, the normal mammary epithelial cells (HMEC) were not sensitised to CB1954. HMEC had a basal IC50 of 31.67μM and were therefore comparatively sensitive to CB1954 prior to transduction with Ad-hTERC-NTR and Ad-hTERT-NTR. Expression of NTR from the hTERC promoter resulted in IC50 values of 45.38μM at 10 p.f.u per cell and 36.02μM at 50 p.f.u. per cell, while the values for cell infected with Ad-hTERT-NTR were 27.76μM (10 p.f.u.) and 29.65μM (50 p.f.u.). These data indicate that normal mammary epithelial cells are not significantly affected by hTERC and hTERT promoter mediated expression of NTR.

No sensitisation was expected by introduction of Ad-hTERC-NTR or Ad-hTERT-NTR into the other cancer cell line tested, 5637 bladder carcinoma cells, as these cells had low promoter activity in chapter 3 and were not sensitised to CB1954 by expression of NTR

from hTERC and hTERT promoters in stable cell line models. According to expectation, the IC50 values for 5637 were not significantly altered by introduction of the viruses, shifting only from 102.86μM (mock infected) to 93.55μM (hTERC, 10 p.f.u.), 73.36μM (hTERC, 50p.f.u.), 112.61 (hTERT, 10p.f.u.), and 103.17μM (hTERT, 50p.f.u.). The lack of sensitisation to CB1954 is demonstrated by the shape of the cytotoxicity curve in figure 5.16, in which the concentration curves for all treatments are tightly packed together with no dose dependent shifts.

These data indicated that the Ad-hTERC-NTR and Ad-hTERT-NTR vectors could transduce cell lines with the NTR expression constructs and that the function of nitroreductase was not adversely affected by the truncation characterised in the previous section. NTR retained its ability to activate CB1954 and to sensitise cells to the cytotoxic effects of activated CB1954. The degree of cytotoxicity was dependent on the levels of expression of NTR and, hence, on the hTERC and hTERT promoter activities in adenovirus infected cell lines. This is supported by western blot analysis of NTR protein expression levels in Ad-hTERC-NTR and Ad-hTERT-NTR infected C33-A cells and WI-38 cells (figure 5.13), by the cytotoxicity data for infected mortal and cancer cell lines (figures 5.14 and 5.15) and by the promoter activity and cytotoxicity data presented in the preceding chapters. C33-A cells, which have high hTERC and hTERT promoter activities (chapter 3 and chapter 4) expressed high levels of the truncated NTR that were sufficient to sensitise the cells to CB1954 *in vitro*, while WI-38 cells infected at an equivalent multiplicity of infection expressed low (hTERC) and undetectable (hTERT) levels of NTR and were not affected by Ad-hTERC-NTR and Ad-hTERT-NTR infection followed by CB1954 challenge.

5.7 Telomerase-nitroreductase vectors sensitise cancer cells to CB1954 in a promoter dependent and infectivity dependent manner.

The efficiency of any gene targeted enzyme/pro-drug activation approach to cancer therapy will depend on a number of factors including promoter activity, efficiency of transgene transduction within the target cell population, basal susceptibility to the effects of the drug and bystander effects. Thus, an important factor to be addressed is the relationship between the sensitising effects of the Ad-NTR vectors and the efficiency of transduction. In the earlier assessment of adenovirus as a gene delivery vehicle (section

5.3), cells were infected with a titration of Ad-CMV-Lac Z and incubated overnight in low serum to limit cell division prior to staining. In the cytotoxicity assays described above, however, the cells are incubated for 2 days under conditions conducive to division to ensure that they are in the exponential phase of growth at the time of drug addition. Thus, the cells initially infected with virus will have undergone mitosis. Since not all of the cells will have been infected and since adenovirus is not integrated into the host genome, it is likely that an increasing proportion of the daughter cells in a cycling culture will not harbour the expression constructs. In order to address this issue, in parallel with Ad-hTERC-NTR and Ad-hTERT-NTR infection, cells were infected with Ad-CMV-LacZ at equal multiplicities of infection to the gene therapy vectors. Ad-CMV-LacZ infected cells were replated into 6-well plates in parallel with cells plated out for MTT assay and were fixed and stained 48 hours post-infection, on the same day as CB1954 challenge, to give an indication of the proportion of cells infected on the day of drug administration. The results of a representative experiment, including infectivity, IC50 and sensitisation data are given in figure 5.16, while figure 5.17 shows the collation of the infectivity and sensitisation data for all cell lines assayed.

In C33-A cells, infection with Ad-hTERC-NTR resulted in a mean 7.51-fold (10 p.f.u.) and 24.08-fold (50 p.f.u.) sensitisation to CB1954, while infection with Ad-hTERT-NTR resulted in only 2.02-fold (10 p.f.u.) and 7.36-fold (50 p.f.u.). Interestingly, Ad-hTERC-NTR infected C33-A were more sensitive overall to CB1954 than the equivalent stable cell line, despite transduction of only 59.18% (10 p.f.u.) and 80.33% (50 p.f.u.) of cells. In contrast, WI-38 cells, which were more easily infected than C33-A (64.95% at 10 p.f.u., and 97.81% at 50 p.f.u.) were not sensitised to CB1954 either by Ad-hTERC-NTR (0.91-fold and 0.94-fold sensitisation at 10 and 50 p.f.u.) or by Ad-hTERT-NTR (1.09-fold and 0.99-fold sensitisation at 10 and 50 p.f.u.). This indicates firstly, that the sensitising effect of the adenoviral gene therapy vectors was dependent in part on promoter activity and that cell lines with low promoter activity are not sensitised to CB1954 even when essentially all of the cells are transduced. Additionally, the dose dependent increase in sensitisation observed in C33-A cells indicates that the efficiency of gene transfer is an important consideration in evaluating the efficacy of the approach.

Further evidence for the importance of transduction efficiency is observable in the comparison between A2780-NTR stable cell lines and Ad-NTR transduced A2780. A2780-NTR stable cell lines were strongly sensitised to CB1954 in chapter 4, but the effect following infection with the NTR viruses was less pronounced. Cells infected with Ad-

hTERC-NTR were sensitised to the drug by 2.69-fold (10 p.f.u.) and 9.15-fold (50 p.f.u.). Infection of A2780 with the hTERT-NTR virus resulted in sensitisation values of only 1.42-fold and 2.5-fold, which were not considered to be significant. The low values observed for A2780 cells presumably reflected the low efficiencies of infection at both 10 p.f.u. per cell (16.28%) and 50 p.f.u. per cell (33.56%).

Interestingly, while HMEC and 5637 cells were not significantly sensitised to CB1954 even by infection with 50 p.f.u. per cell Ad-hTERC-NTR, infection efficiencies were relatively good in these lines. Sensitisation values for cell lines infected with 50 p.f.u. per cell Ad-hTERC-NTR were 1.45-fold (5637) and 0.87-fold (HMEC). Transduction with 50 p.f.u. of the Ad-CMV-Lac Z virus resulted in infection efficiencies of 94.5% (5637) and 66.21 % (HMEC). Thus, 3 cell lines were infected at a similar or better efficiency than C33-A and A2780, yet the low promoter activities of these cell lines were insufficient to sensitise the cells to CB1954. These data are encouraging and demonstrate that Ad-hTERC-NTR and Ad-hTERT-NTR vectors could efficiently sensitise cancer cells to the effects of CB1954 in both a promoter- dependent and dose-dependent manner.

5.8 Ad-hTERC-NTR and Ad-hTERT-NTR gene therapy vectors sensitise human cervical carcinoma cells to CB1954 *in vivo.*

This experiment was performed in collaboration with Dr. Jane A. Plumb, CRC Dept. Medical Oncology.

In order to assess the sensitisation of model human tumours to Ad-hTERC-NTR and Ad-hTERT-NTR vector administration followed by CB1954 *in vivo*, 10^7 C33-A cells in 200µl PBS were injected subcutaneously into the flanks of athymic female nude mice. A total of 7 groups of mice representing untreated controls, CB1954 only, hTERC-NTR and hTERT-NTR viruses with and without CB1954, and a CMV-LacZ group to address the efficiency of gene transfer *in vivo*. A total of 6 mice bearing 1 tumour each were included in each group. Tumour volumes were monitored until measured tumour diameter reached at least 5mm, at which time the mice were randomly distributed into groups of 6 and a single intra-tumoural injection of $4x10^8$ p.f.u. Ad-hTERC-NTR, Ad-hTERT-NTR and CMV-LacZ in 100µl volume was administered to the tumours of appropriate groups of animals. 24hrs after infusion of the virus, a single injection of 80mg/kg CB1954 was administered to mice

bearing tumours infected with the NTR viruses by tail vein injection. Daily calliper measurements of were performed for a further 7 days and tumour volumes were estimated from the measurements (volume=d^3 x $\pi/6$). LacZ tumours were harvested for staining on the day of drug injection.

Figure 5.18 shows the mean change in tumour volumes over 7 days from untreated xenografts, those of animals injected with drug only, animals whose tumours were injected with virus only with no additional drug, or tumours in animals given intra-tumoural Ad-hTERC-NTR or Ad-hTERT-NTR on day 0, followed by 80mg/kg CB1954 on day 1. The tumours in flanks of control animals that either were untreated, injected intravenously with 80mg/kg CB1954 without virus, or given a single intratumour injection of AD-hTERT-NTR or Ad-hTERC-NTR without CB1954, increased in volume at a similar rate, approximately doubling in size over the seven days (range of change in volume 1.97-fold to 2.16-fold). By contrast, the mean volumes of tumours that were injected i.t. with Ad-hTERC-NTR or Ad-hTERT-NTR on day 0, followed by 80mg/kg CB1954 i.v. on day 1, were arrested. The mean change in tumour volume of these animals was just 1.13-fold for Ad-hTERC-NTR and 1.22-fold for Ad-hTERT-NTR. Thus, a single injection of telomerase-nitroreductase gene therapy vectors coupled to a single CB1954 administration resulted in a 40% reduction in tumour volume for the hTERT-NTR virus and a 43% reduction in volume for the hTERC-NTR virus. These data indicated that Ad-hTERC-NTR and Ad-hTERT-NTR had a selective and efficacious anti-tumour effect *in vitro* and *in vivo* and strongly support the further development of a telomerase-nitroreductase gene therapy system.

5.9 Discussion.

In the preceding chapters, a panel of human cancer cell lines and mortal cell strains have been characterised for their relative capacity to drive transgene expression from the telomerase hTERC and hTERT promoters and for the ability of hTERC-NTR and hTERT-NTR expression vectors to specifically sensitise cancer cells to CB1954 in a promoter dependent manner when all cells within a population carry the constructs. A major limitation in current gene therapy applications is the efficiency and specificity of transgene delivery to target cell populations. While a number of viral and non-viral vector systems are currently under development, adenoviruses have been widely used in models of gene therapy due to the high efficiency of gene delivery that can be achieved in diverse cell types.

To validate the use of adenovirus vectors for the delivery of hTERC-NTR and hTERT-NTR expression constructs, a panel of thirteen human cell lines was infected with a CMV-LacZ reporter adenovirus and stained with X-Gal to evaluate transduction efficiencies. The results indicated that adenovirus could infect most cell lines with a high efficiency, reaching 100% infectivity at high multiplicities of infection (100p.f.u. per cell) in six cell lines, with maximal efficiencies ranging between 43% and 90% for five other cell lines. The use of adenovirus as a vehicle for the delivery of hTERC-NTR and hTERT-NTR constructs to human cancer cells was therefore validated by these assays. However, two cell lines, including the small cell lung cancer line GLC4 which had a high promoter activity and responded well to NTR/CB1954 in the preceding chapters, were almost completely refractive to the virus. For this reason, these cell lines were not included in the rest of the model.

To model a realistic strategy for delivery of the gene therapy constructs to cells, hTERC-NTR and hTERT-NTR expression constructs from the pd2-NTR panel of plasmids described in chapter 4 were sub-cloned into the deleted E1 region of a first-generation adenovirus backbone using the commercially available Adeasy system ((He et al. 1998)). Amplification and purification of recombinant Ad-hTERC-NTR and Ad-hTERT-NTR adenovirus vectors was performed by the custom service of Q-biogene.

Northern blot analysis of expressed NTR transcripts from a human cervical carcinoma cell line infected with Ad-hTERC-NTR and Ad-hTERT-NTR indicated that the NTR mRNAs were expressed to a high level in a promoter dependent manner with the hTERC promoter stronger than hTERT when cells were infected with equivalent infectious doses. However, the transcripts migrated at different sizes. In order to characterise the transcripts more fully, cDNA was synthesised from total RNA extracted from Ad-hTERC-NTR and Ad-hTERT-NTR infected C33-A cells and PCR reactions specific for the 5'and 3' ends of the transcripts were performed. The single product of the 3' end amplification of the Ad-hTERC-NTR transcript and the two products of the 3' amplification of the Ad-hTERT-NTR transcripts were sequenced and revealed a 187bp deletion only present in expressed cDNA that corresponded to a putative splicing variant. The mutation encoded by the splicing variant translates to a truncated NTR species, but is not predicted to abrogate the function of NTR, although some effects on nitroreduction efficiency cannot be ruled out. The 5' ends of the products of both Ad-hTERC-NTR and Ad-hTERT-NTR are in-keeping with previous results, indicating that the correct transcriptional start sites of the hTERC and hTERT promoters are maintained within the adenovirus backbone.

Infection of a panel of five mortal and cancer cell lines with Ad-hTERC-NTR and Ad-hTERT-NTR resulted in significant sensitisation to CB1954 in two cancer cell lines: an ovarian adenocarcinoma line and a cervical carcinoma line. No other cell lines were significantly affected by transduction with Ad-hTERC-NTR and Ad-hTERT-NTR. Sensitisation of these cell lines was in-keeping with data presented in previous chapters and indicated that the expression of NTR following transduction with the Ad-NTR gene therapy vectors could specifically sensitise human cancer cells to CB1954 in a manner that is dependent in part on promoter activity and in part on infectivity. This is supported by western blot analysis of NTR expressed in the mortal foetal lung fibroblast cell strain WI-38 compared with the cervical carcinoma cell line C33-A showing low to undetectable activity of both promoters in the mortal cell strain, but high level expression in the cancer cell line.

The data presented in this chapter and in those preceding therefore support the virus mediated delivery of hTERC-NTR and hTERT-NTR expression constructs combined with CB1954 as a valid therapeutic approach for cytotoxic gene therapy of human cancer.

Figure 5.1: Mean adenovirus infection efficiency in cancer, mortal and ALT cell lines.

Cell lines were either mock infected, or infected with a titration of Ad-CMV-LacZ at 1, 10, or 100p.f.u./cell for 1 hour. Cells were then incubated overnight in medium containing 2% serum and the next day were fixed in glutaraldehyde. Cells were stained overnight at neutral pH and the proportion of infected cells was estimated by counting the proportion of blue cells in 5 random fields at X20 objective (a total of 500-1000 cells). Data shown are the means and standard error derived from 2 independent experiments in each cell line.

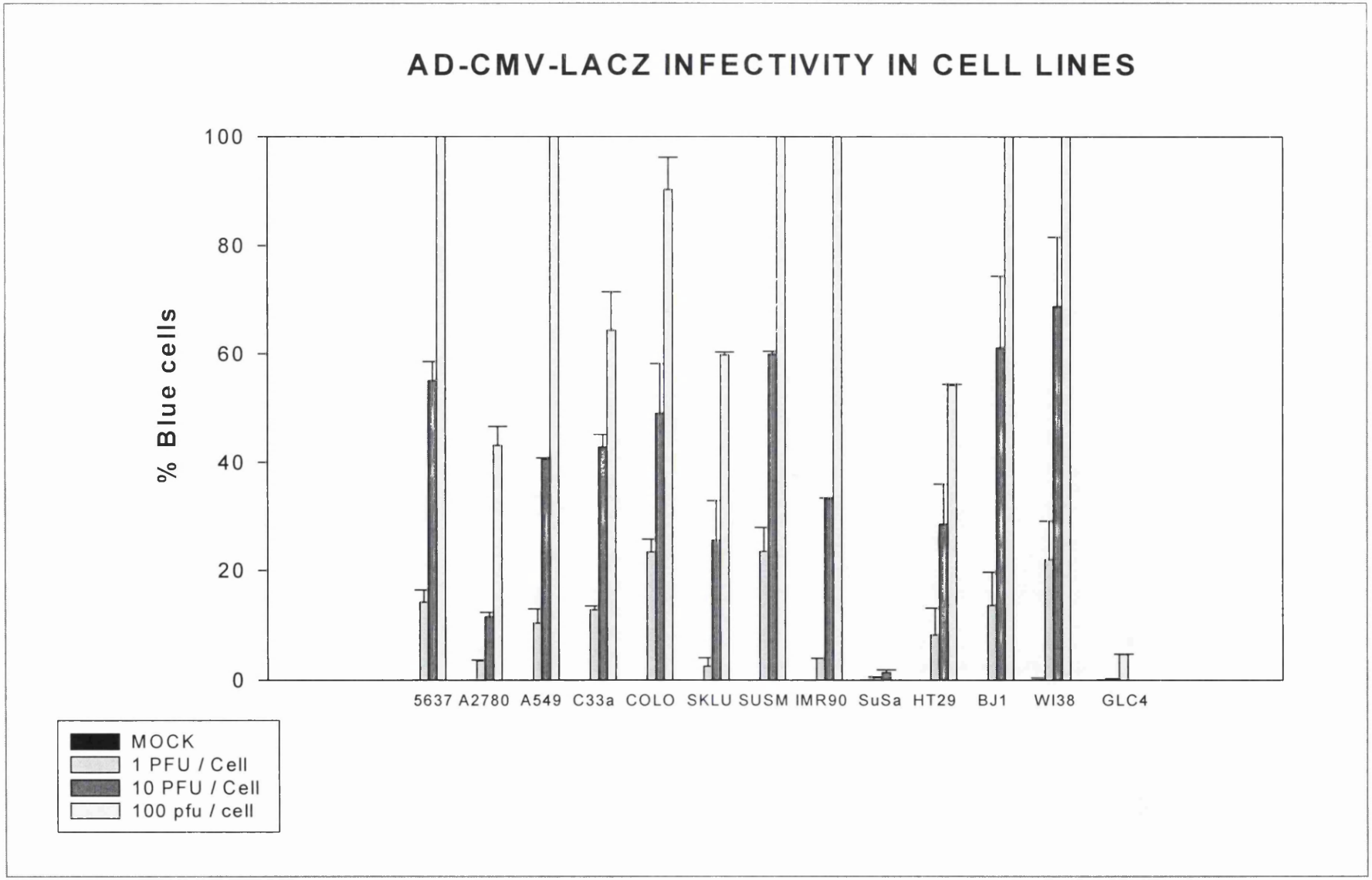
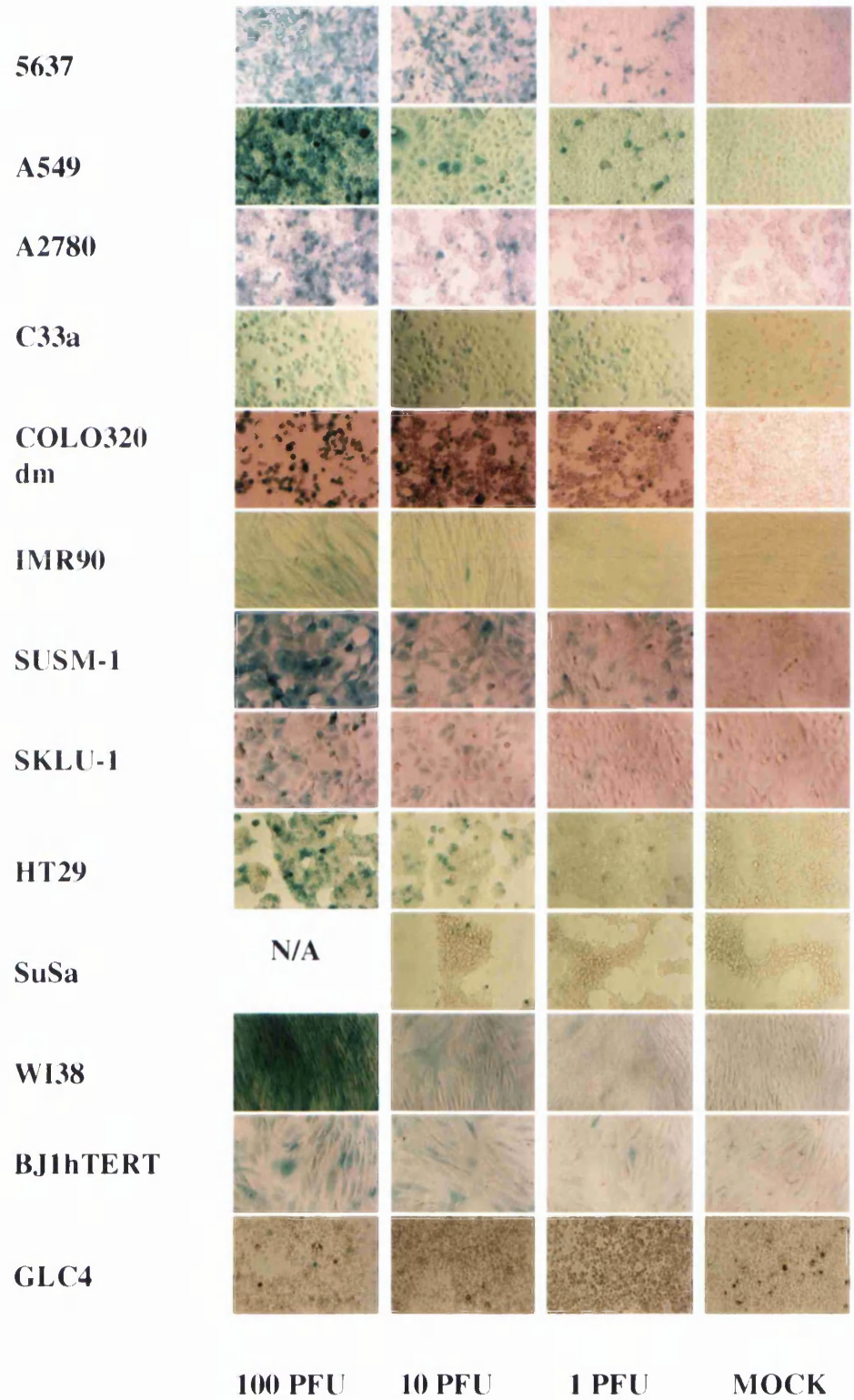

AD-CMV-LACZ INFECTIVITY IN CELL LINES
% Blue cells
100
80
60
40
20
0
5637
A2780
A549
C33a
COLO
SKLU
SUSM
IMR90
SuSa
HT29
BJ1
WI38
GLC4
MOCK
1 PFU / Cell
10 PFU / Cell
100 pfu / cell

Figure 5.2: Representative photomicrographs of Ad-CMV-LacZ infected cell lines.

Cells were either mock infected, or infected with a titration of Ad-CMV-LacZ at 1, 10, or 100p.f.u./cell for 1 hour at 37°C. The cells were incubated overnight and the following day were fixed in glutaraldehyde and stained overnight with X-Gal. Photomicrographs were taken with a digital camera and the proportion of infected cells was estimated by counting 5 random fields at X20 objective. In total, approximately 500-1000 cells were counted for each treatment. All experiments were repeated at least twice.

ADENOVIRUS INFECTIVITY IN CELL LINES



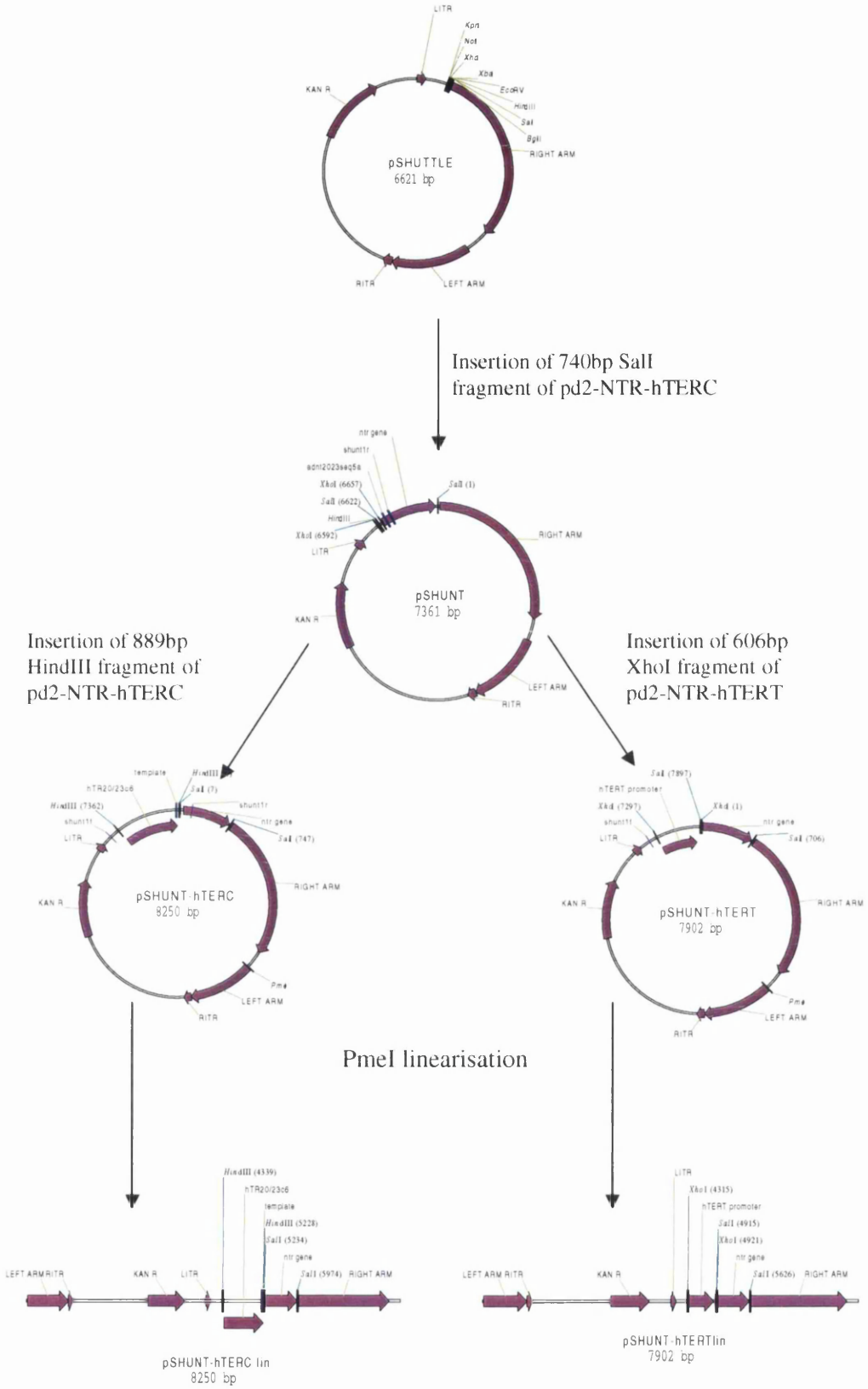


Figure 5.3: Cloning Ad-NTR gene therapy vectors (1): generation of intermediate transfer vectors.

The 740bp SalI fragment of pd2NTR-hTERC was ligated in to pShuttle to generate the vector pShunt, which contains the NTR coding sequence. The 889bp HindIII fragment of pd2-NTR-hTERC and the 606bp XhoI fragment of pd2-NTR-hTERT were cloned into pShunt to generate the plasmids pSHUNT-hTERC and –hTERT. These intermediate transfer vectors were linearised with PmeI for recombination into pAdeasy-1.

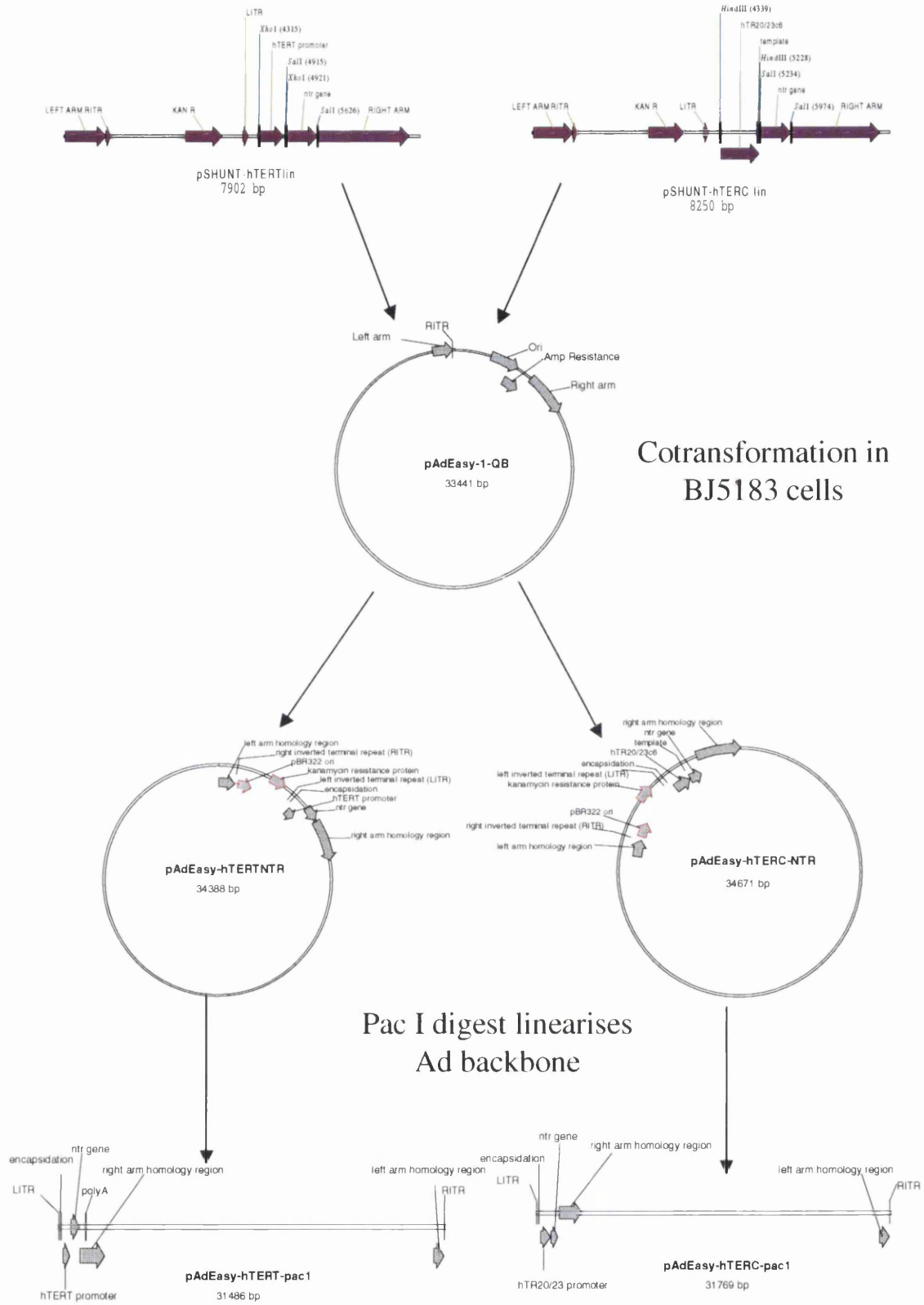


Figure 5.4: Cloning Ad-NTR gene therapy vectors (2): generation of recombinant adenovirus genomes.

Linear transfer vectors pSHUNT-hTERC and –hTERT were cotransformed with pAdeasy-1 in BJ5183 cells. Potential recombinants were selected and screened by restriction digest and sequencing with the primers Shunt1f and Shunt1r. Recombinants were linearised and transfected into HEK-293 cells for large scale adenovirus preparation.

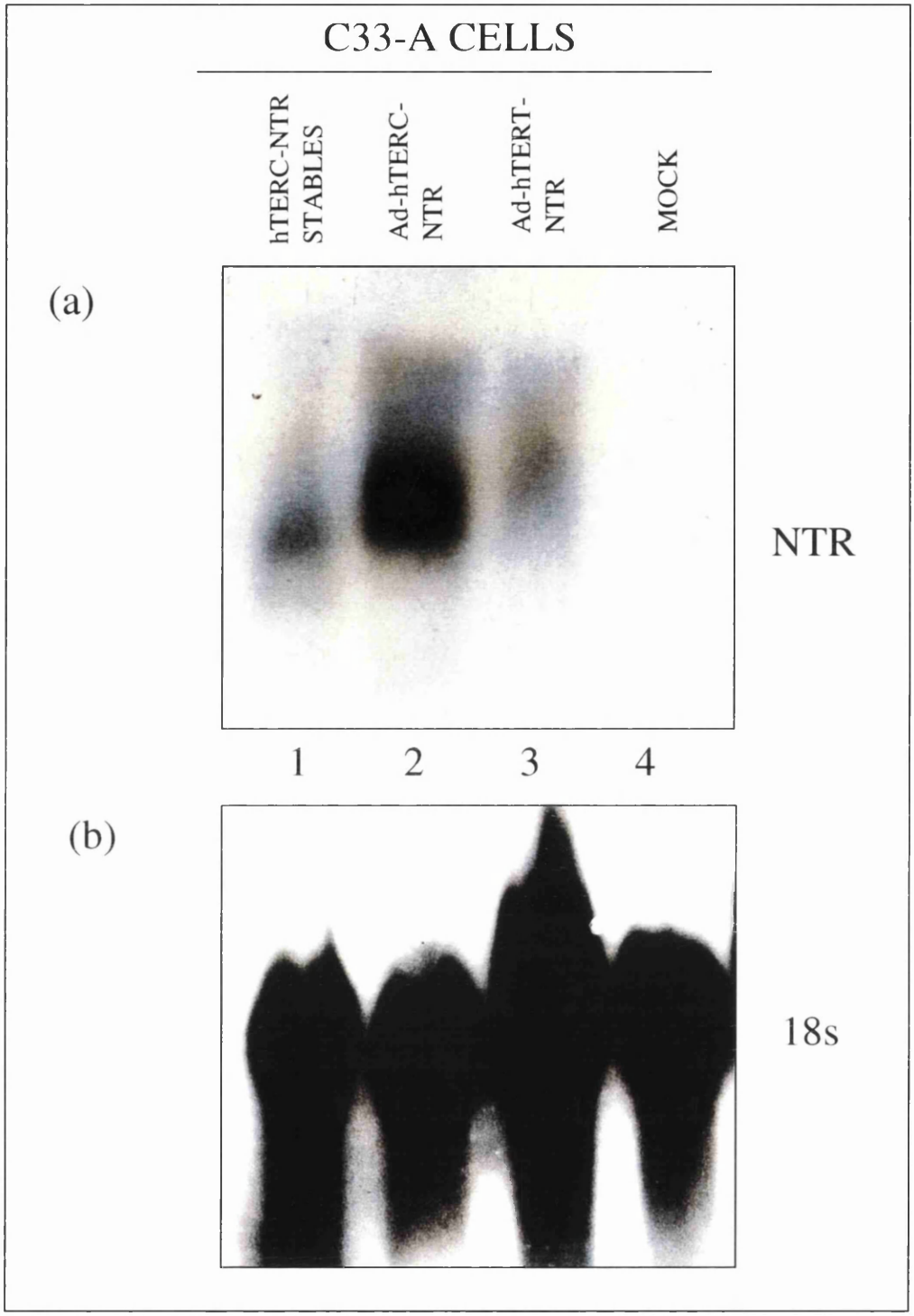


Figure 5.5: Northern blot analysis of NTR expression in Ad-hTERC-NTR and Ad-hTERT-NTR infected cervical carcinoma cells.

C33-A cells were mock infected, or infected with 50p.f.u./cell of Ad-hTERC-NTR or Ad-hTERT-NTR. RNA was extracted and 25μg was electrophoresed and blotted onto nylon filters. Filters were probed with the 32P-dCTP labelled 740bp SalI fragment of pd2-NTR-hTERC (a), or mouse 18s rRNA gene (b). Lane 1, C33-A-hTERC-NTR stable cell line RNA. Lanes 2 and 3, Ad-hTERC-NTR and Ad-hTERT-NTR infected C33-A cells. Lane 4, mock infected C33-A cells. The hTERC promoter gave the strongest signal in C33-A cells.

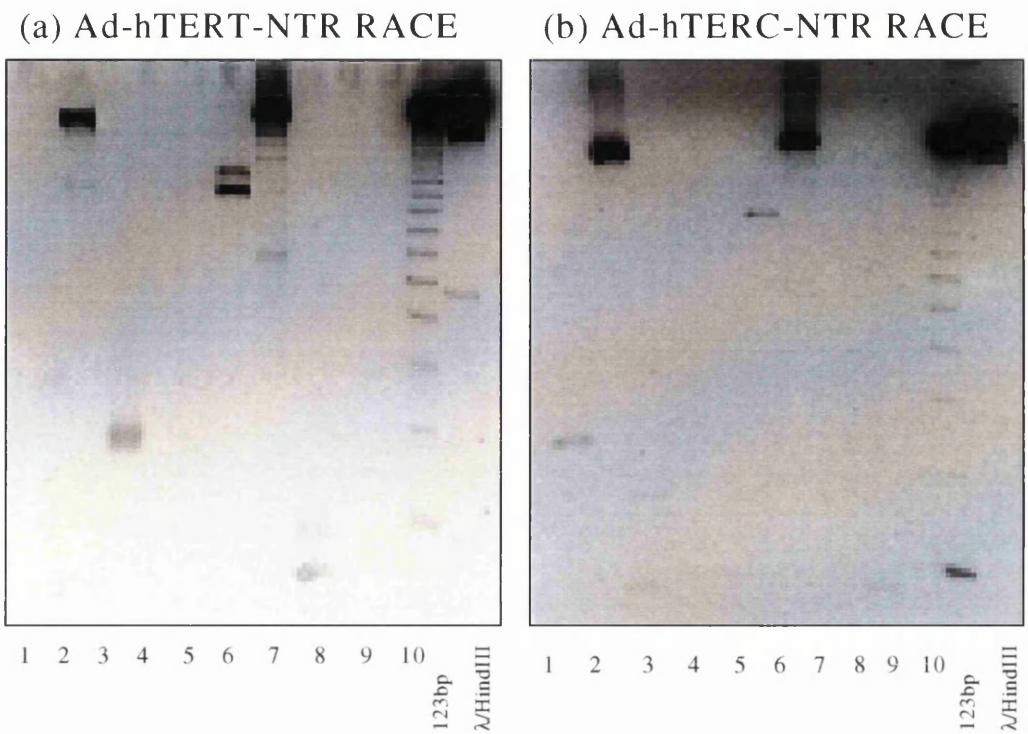


Figure 5.6: 5' and 3' RACE reactions for amplification of ends of Ad-hTERC-NTR and Ad-hTERT-NTR cDNAs.

cDNA synthesis and amplification of 5' and 3' ends of cDNAs was performed using Clontech SMART-RACE kit. The reactions generate both 5' and 3' ready cDNAs, each of which has a synthetic sequence added at the 5' and 3' ends, respectively, allowing annealing of a "universal primer mix" outside the transcript sequence and, hence, amplification of the entire end. 1μg total RNA was used for the initial generation of cDNA and 2.5μl cDNA was used in each PCR reaction. Amplification of the 5' and 3' ends of NTR transcripts was performed using the primers shunt1r (5' amplification) and AdNTseq5a (3' amplification) in addition to the 5' or 3' universal primer mix supplied with kit that anneals to a synthetic sequence added to the cDNA during first strand synthesis. Lanes are identical in both panels. Lane 1, 5' end amplification. Lane 6, 3' end amplification. Lanes 3 and 8, control amplification using both ADNTseq5a and shunt1r. Lanes 2 and 7, positive control amplifications of transferrin receptor. Lanes 9 and 10, negative controls using one primer only. Some non-specific bands were evident in the control reactions, but the NTR 5' and 3' PCR yielded discrete products.

(a)

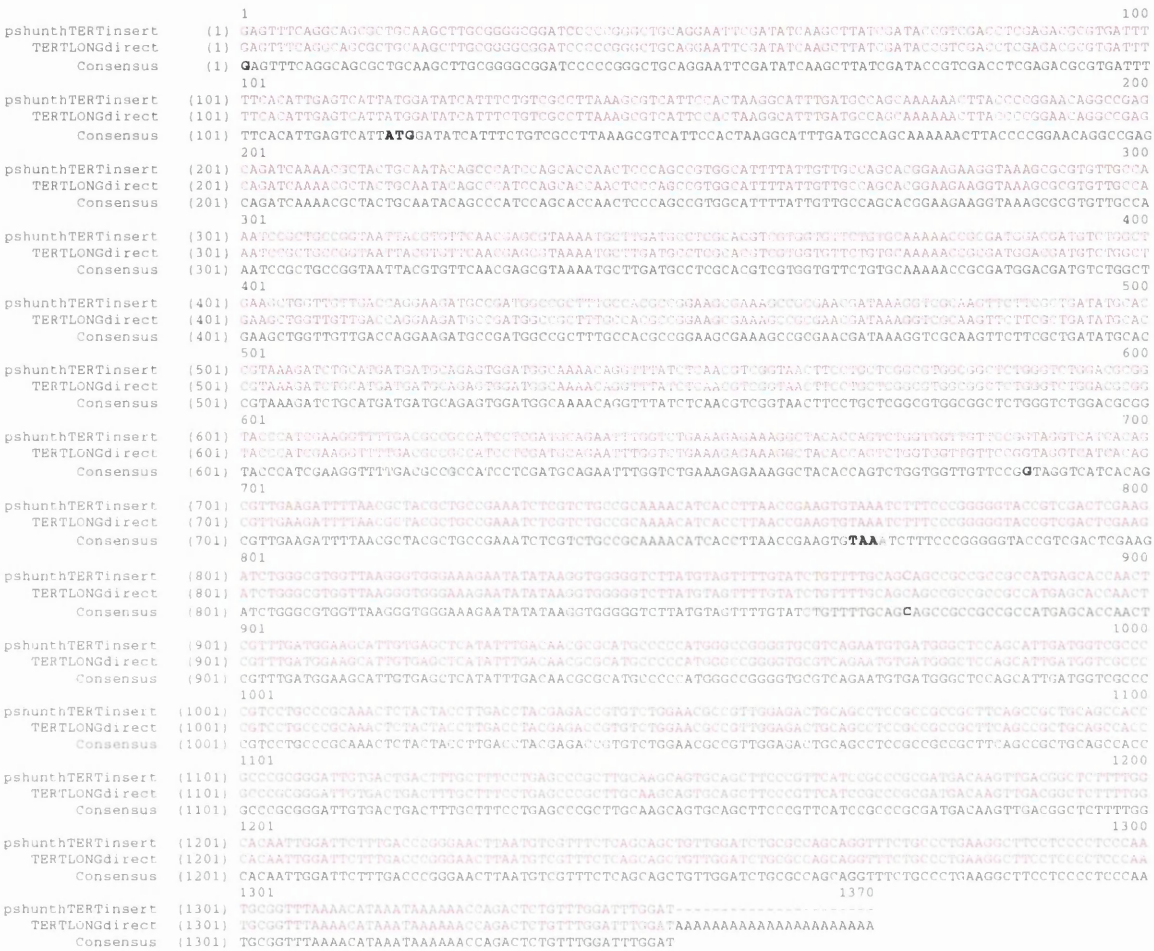
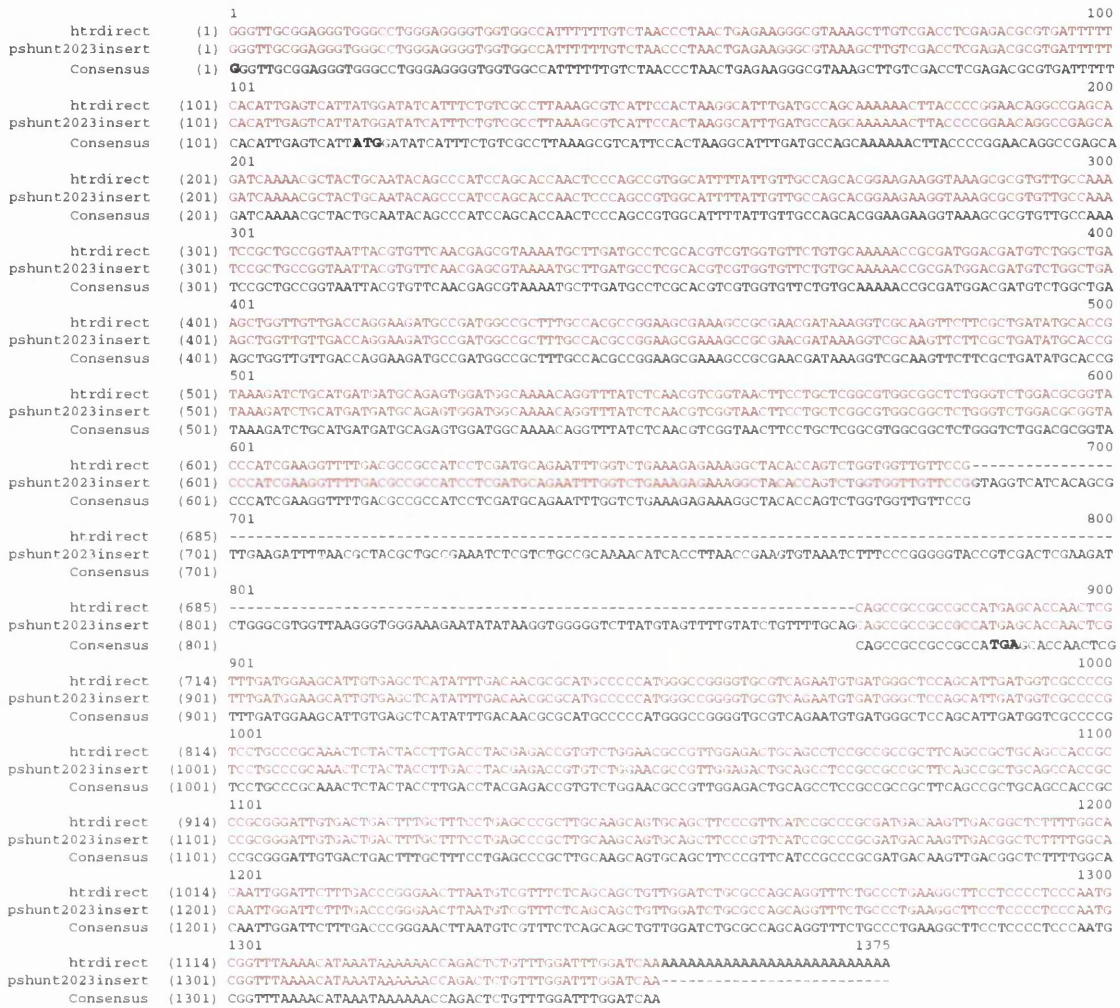


Figure 5.7: Sequences of Ad-hTERC-NTR and Ad-hTERT-NTR transcripts expressed in C33-A cells.

Products of the 5' and 3' RACE reactions were directly cloned into pCR-II for sequencing by dideoxy chain termination using the M13 primer pair and Adtranscript1 (3' end). Analysis of sequences and contigs was performed using Vector NTI 6. (a) shows the full sequence of the long transcript identified in hTERT RACE reactions corresponding to a full length NTR transcript, while (b) and (c) (next pages) show the sequences of the short transcripts for hTERC and hTERT respectively. Both short transcripts have a 187bp deletion corresponding to a splice variant. The transcriptional start sites, start codons, the true NTR stop codon TAA (a) and the TGA stop codons in the adenovirus backbone in (b) and (c) are shown in enlarged bold characters. Additionally, the putative splice donor and acceptor sites at nt687 and 873 are highlighted in (a).

(b)



(c)


1 100
pshunthTERTinsert (1) GAGTTTCAGGCAGCGCTGCAAGCTTGCGGGGCGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGACGCGTGATTT
TERTshortdirect (1) GAGTTTCAGGCAGCGCTGCAAGCTTGCGGGGCGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGACGCGTGATTT
Consensus (1) GAGTTTCAGGCAGCGCTGCAAGCTTGCGGGGCGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGACGCGTGATTT
101 200
pshunthTERTinsert (101) TTCACATTGAGTCATTATGGATATCATTTCTGTCGCCTTAAAGCGTCATTCCACTAAGGCATTTGATGCCAGCAAAAAACTTACCCCGGAACAGGCCGAG
TERTshortdirect (101) TTCACATTGAGTCATTATGGATATCATTTCTGTCGCCTTAAAGCGTCATTCCACTAAGGCATTTGATGCCAGCAAAAAACTTACCCCGGAACAGGCCGAG
Consensus (101) TTCACATTGAGTCATTATGGATATCATTTCTGTCGCCTTAAAGCGTCATTCCACTAAGGCATTTGATGCCAGCAAAAAACTTACCCCGGAACAGGCCGAG
201 300
pshunthTERTinsert (201) CAGATCAAAACGCTACTGCAATACAGCCCATCCAGCACCAACTCCCAGCCGTGGCATTTTATTGTTGCCAGCACGGAAGAAGGTAAAGCGCGTGTTGCCA
TERTshortdirect (201) CAGATCAAAACGCTACTGCAATACAGCCCATCCAGCACCAACTCCCAGCCGTGGCATTTTATTGTTGCCAGCACGGAAGAAGGTAAAGCGCGTGTTGCCA
Consensus (201) CAGATCAAAACGCTACTGCAATACAGCCCATCCAGCACCAACTCCCAGCCGTGGCATTTTATTGTTGCCAGCACGGAAGAAGGTAAAGCGCGTGTTGCCA
301 400
pshunthTERTinsert (301) AATCCGCTGCCGGTAATTACGTGTTCAACGAGCGTAAAATGCTTGATGCCTCGCACGTCGTGGTGTTCTGTGCAAAAACCGCGATGGACGATGTCTGGCT
TERTshortdirect (301) AATCCGCTGCCGGTAATTACGTGTTCAACGAGCGTAAAATGCTTGATGCCTCGCACGTCGTGGTGTTCTGTGCAAAAACCGCGATGGACGATGTCTGGCT
Consensus (301) AATCCGCTGCCGGTAATTACGTGTTCAACGAGCGTAAAATGCTTGATGCCTCGCACGTCGTGGTGTTCTGTGCAAAAACCGCGATGGACGATGTCTGGCT
401 500
pshunthTERTinsert (401) GAAGCTGGTTGTTGACCAGGAAGATGCCGATGGCCGCTTTGCCACGCCGGAAGCGAAAGCCGCGAACGATAAAGGTCGCAAGTTCTTCGCTGATATGCAC
TERTshortdirect (401) GAAGCTGGTTGTTGACCAGGAAGATGCCGATGGCCGCTTTGCCACGCCGGAAGCGAAAGCCGCGAACGATAAAGGTCGCAAGTTCTTCGCTGATATGCAC
Consensus (401) GAAGCTGGTTGTTGACCAGGAAGATGCCGATGGCCGCTTTGCCACGCCGGAAGCGAAAGCCGCGAACGATAAAGGTCGCAAGTTCTTCGCTGATATGCAC
501 600
pshunthTERTinsert (501) CGTAAAGATCTGCATGATGATGCAGAGTGGATGGCAAAACAGGTTTATCTCAACGTCGGTAACTTCCTGCTCGGCGTGGCGGCTCTGGGTCTGGACGCGG
TERTshortdirect (501) CGTAAAGATCTGCATGATGATGCAGAGTGGATGGCAAAACAGGTTTATCTCAACGTCGGTAACTTCCTGCTCGGCGTGGCGGCTCTGGGTCTGGACGCGG
Consensus (501) CGTAAAGATCTGCATGATGATGCAGAGTGGATGGCAAAACAGGTTTATCTCAACGTCGGTAACTTCCTGCTCGGCGTGGCGGCTCTGGGTCTGGACGCGG
601 700
pshunthTERTinsert (601) TACCCATCGAAGGTTTTGACGCCGCCATCCTCGATGCAGAATTTGGTCTGAAAGAGAAAGGCTACACCAGTCTGGTGGTTGTTCCGGTAGGTCATCACAG
TERTshortdirect (601) TACCCATCGAAGGTTTTGACGCCGCCATCCTCGATGCAGAATTTGGTCTGAAAGAGAAAGGCTACACCAGTCTGGTGGTTGTTCCG----------
Consensus (601) TACCCATCGAAGGTTTTGACGCCGCCATCCTCGATGCAGAATTTGGTCTGAAAGAGAAAGGCTACACCAGTCTGGTGGTTGTTCCG
701 800
pshunthTERTinsert (701) CGTTGAAGATTTTAACGCTACGCTGCCGAAATCTCGTCTGCCGCAAAACATCACCTTAACCGAAGTGTAAATCTTTCCCGGGGGTACCGTCGACTCGAAG
TERTshortdirect (687) ----------
Consensus (701)
801 900
pshunthTERTinsert (801) ATCTGGGCGTGGTTAAGGGTGGGAAAGAATATATAAGGTGGGGGTCTTATGTAGTTTTGTATCTGTTTTGCAGCAGCCGCCGCCGCCATGAGCACCAACT
TERTshortdirect (687) ----------CAGCCGCCGCCGCCATGAGCACCAACT
Consensus (801) CAGCCGCCGCCGCCATGAGCACCAACT
901 1000
pshunthTERTinsert (901) CGTTTGATGGAAGCATTGTGAGCTCATATTTGACAACGCGCATGCCCCCATGGGCCGGGGTGCGTCAGAATGTGATGGGCTCCAGCATTGATGGTCGCCC
TERTshortdirect (714) CGTTTGATGGAAGCATTGTGAGCTCATATTTGACAACGCGCATGCCCCCATGGGCCGGGGTGCGTCAGAATGTGATGGGCTCCAGCATTGATGGTCGCCC
Consensus (901) CGTTTGATGGAAGCATTGTGAGCTCATATTTGACAACGCGCATGCCCCCATGGGCCGGGGTGCGTCAGAATGTGATGGGCTCCAGCATTGATGGTCGCCC
1001 1100
pshunthTERTinsert (1001) CGTCCTGCCCGCAAACTCTACTACCTTGACCTACGAGACCGTGTCTGGAACGCCGTTGGAGACTGCAGCCTCCGCCGCCGCTTCAGCCGCTGCAGCCACC
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Consensus (1001) CGTCCTGCCCGCAAACTCTACTACCTTGACCTACGAGACCGTGTCTGGAACGCCGTTGGAGACTGCAGCCTCCGCCGCCGCTTCAGCCGCTGCAGCCACC
1101 1200
pshunthTERTinsert (1101) GCCCGCGGGATTGTGACTGACTTTGCTTTCCTGAGCCCGCTTGCAAGCAGTGCAGCTTCCCGTTCATCCGCCCGCGATGACAAGTTGACGGCTCTTTTGG
TERTshortdirect (914) GCCCGCGGGATTGTGACTGACTTTGCTTTCCTGAGCCCGCTTGCAAGCAGTGCAGCTTCCCGTTCATCCGCCCGCGATGACAAGTTGACGGCTCTTTTGG
Consensus (1101) GCCCGCGGGATTGTGACTGACTTTGCTTTCCTGAGCCCGCTTGCAAGCAGTGCAGCTTCCCGTTCATCCGCCCGCGATGACAAGTTGACGGCTCTTTTGG
1201 1300
pshunthTERTinsert (1201) CACAATTGGATTCTTTGACCCGGGAACTTAATGTCGTTTCTCAGCAGCTGTTGGATCTGCGCCAGCAGGTTTCTGCCCTGAAGGCTTCCTCCCCTCCCAA
TERTshortdirect (1014) CACAATTGGATTCTTTGACCCGGGAACTTAATGTCGTTTCTCAGCAGCTGTTGGATCTGCGCCAGCAGGTTTCTGCCCTGAAGGCTTCCTCCCCTCCCAA
Consensus (1201) CACAATTGGATTCTTTGACCCGGGAACTTAATGTCGTTTCTCAGCAGCTGTTGGATCTGCGCCAGCAGGTTTCTGCCCTGAAGGCTTCCTCCCCTCCCAA
1301 1380
pshunthTERTinsert (1301) TGCGGTTTAAAACATAAATAAAAAACCAGACTCTGTTTGGATTTGGAT----------
TERTshortdirect (1114) TGCGGTTTAAAACATAAATAAAAAACCAGACTCTGTTTGGATTTGGATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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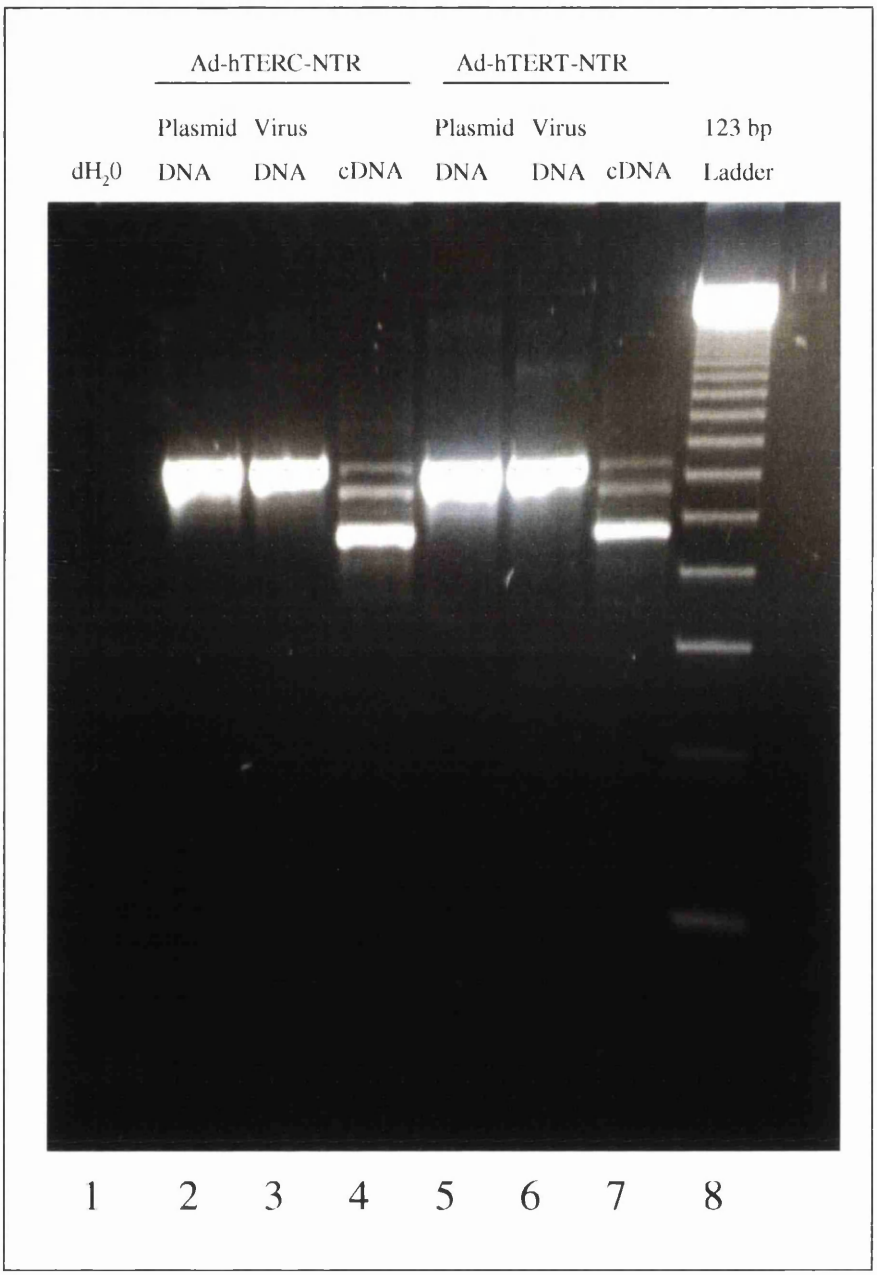
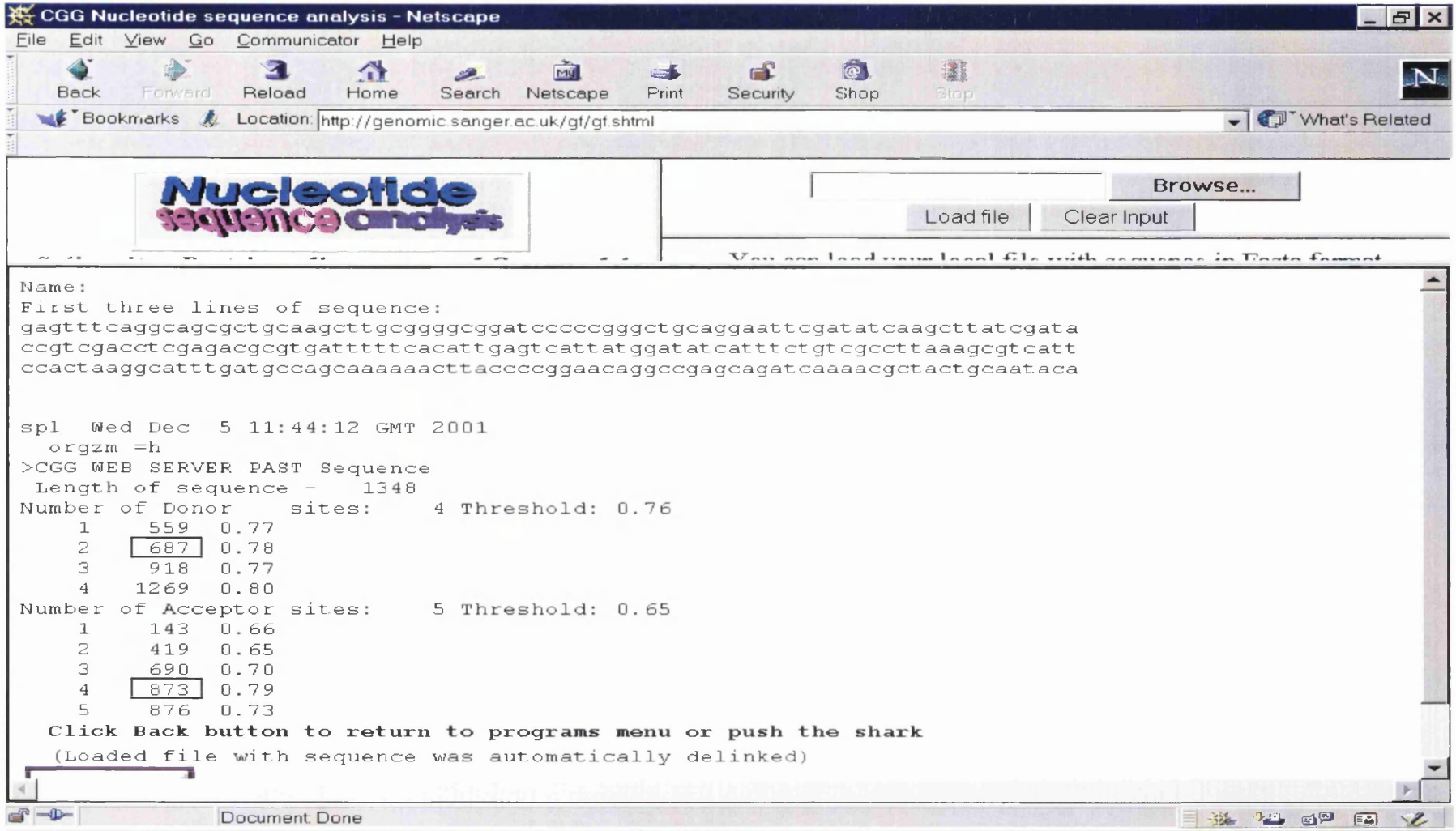


Figure 5.8: Amplification across deleted transcript region using virus DNA, plasmid DNA and cDNA.

In order to determine the source of the deletion in the short NTR transcripts, PCR reactions were performed on the DNAs generated at each stage of the virus cloning (adenovirus genomic plasmids, extracted viral DNA and expressed cDNA), using the primers AdNTseq5a and AdDelr that generate a 762bp amplicon if the full length product is present, but a 575bp amplicon if the deletion is present. The deletion, in addition to two other minor products including the full length product, was present only in the expressed cDNA, suggesting the involvement of a mechanism such as alternative splicing.

Figure 5.9: The short NTR expression product in C33-A cells is an alternative splice variant.

In order to determine the reason for the expression of several transcripts in C33-A cells, the sequence of thee full length hTERT transcript (expected sequence, figure 5.7a) was submitted for a search of splice donor/acceptor sites via the SPL search facility on the website of the Sanger centre. The search results indicated that the deleted region was flanked by splicing donor/acceptor sites corresponding precisely to the location of the deletion (compare highlighted nucleotides 687 and 873 in figure 5.7a with the boundaries of the deleted region in figure 5.7b and c). The sites at nucleotides 687 and 873 are indicated by boxes in the screen shown in the figure.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What's Related
Nucleotide sequence analysis
Browse...
Load file
Clear Input
Name:
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2 687 0.78
3 918 0.77
4 1269 0.80
Number of Acceptor sites: 5 Threshold: 0.65
1 143 0.66
2 419 0.65
3 690 0.70
4 873 0.79
5 876 0.73
Click Back button to return to programs menu or push the shark
(Loaded file with sequence was automatically delinked)
Document: Done

```
                                    1                                                  50
                NTRtranslate    (1) MDIISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHFIV
           TERTSHORTtranslate   (1) MDIISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHFIV
Translation of TERTLONGdirect   (1) MDIISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHFIV
                hTRtranslate    (1) MDIISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHFIV
                   Consensus    (1) MDIISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHFIV
                                    51                                                 100
                NTRtranslate   (51) ASTEEGKARVAKSAAGNYVFNERKMLDASHVVVFCAKTAMDDVWLKLVVD
           TERTSHORTtranslate  (51) ASTEEGKARVAKSAAGNYVFNERKMLDASHVVVFCAKTAMDDVWLKLVVD
Translation of TERTLONGdirect  (51) ASTEEGKARVAKSAAGNYVFNERKMLDASHVVVFCAKTAMDDVWLKLVVD
                hTRtranslate   (51) ASTEEGKARVAKSAAGNYVFNERKMLDASHVVVFCAKTAMDDVWLKLVVD
                   Consensus   (51) ASTEEGKARVAKSAAGNYVFNERKMLDASHVVVFCAKTAMDDVWLKLVVD
                                   101                                                 150
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           TERTSHORTtranslate (101) QEDADGRFATPEAKAANDKGRKFFADMHRKDLHDDAEWMAKQVYLNVGNF
Translation of TERTLONGdirect (101) QEDADGRFATPEAKAANDKGRKFFADMHRKDLHDDAEWMAKQVYLNVGNF
                hTRtranslate  (101) QEDADGRFATPEAKAANDKGRKFFADMHRKDLHDDAEWMAKQVYLNVGNF
                   Consensus  (101) QEDADGRFATPEAKAANDKGRKFFADMHRKDLHDDAEWMAKQVYLNVGNF
                                   151                                                 200
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           TERTSHORTtranslate (151) LLGVAALGLDAVPIEGFDAAILDAEFGLKEKGYTSLVVVPQPPPP-----
Translation of TERTLONGdirect (151) LLGVAALGLDAVPIEGFDAAILDAEFGLKEKGYTSLVVVPVGHHSVEDFN
                hTRtranslate  (151) LLGVAALGLDAVPIEGFDAAILDAEFGLKEKGYTSLVVVPQPPPP-----
                   Consensus  (151) LLGVAALGLDAVPIEGFDAAILDAEFGLKEKGYTSLVVVPVPPPSVEDFN
                                   201          217
                NTRtranslate  (201) ATLPKSRLPQNITLTEV
           TERTSHORTtranslate (196) -----------------
Translation of TERTLONGdirect (201) ATLPKSRLPQNITLTEV
                hTRtranslate  (196) -----------------
                   Consensus  (201) ATLPKSRLPQNITLTEV
```

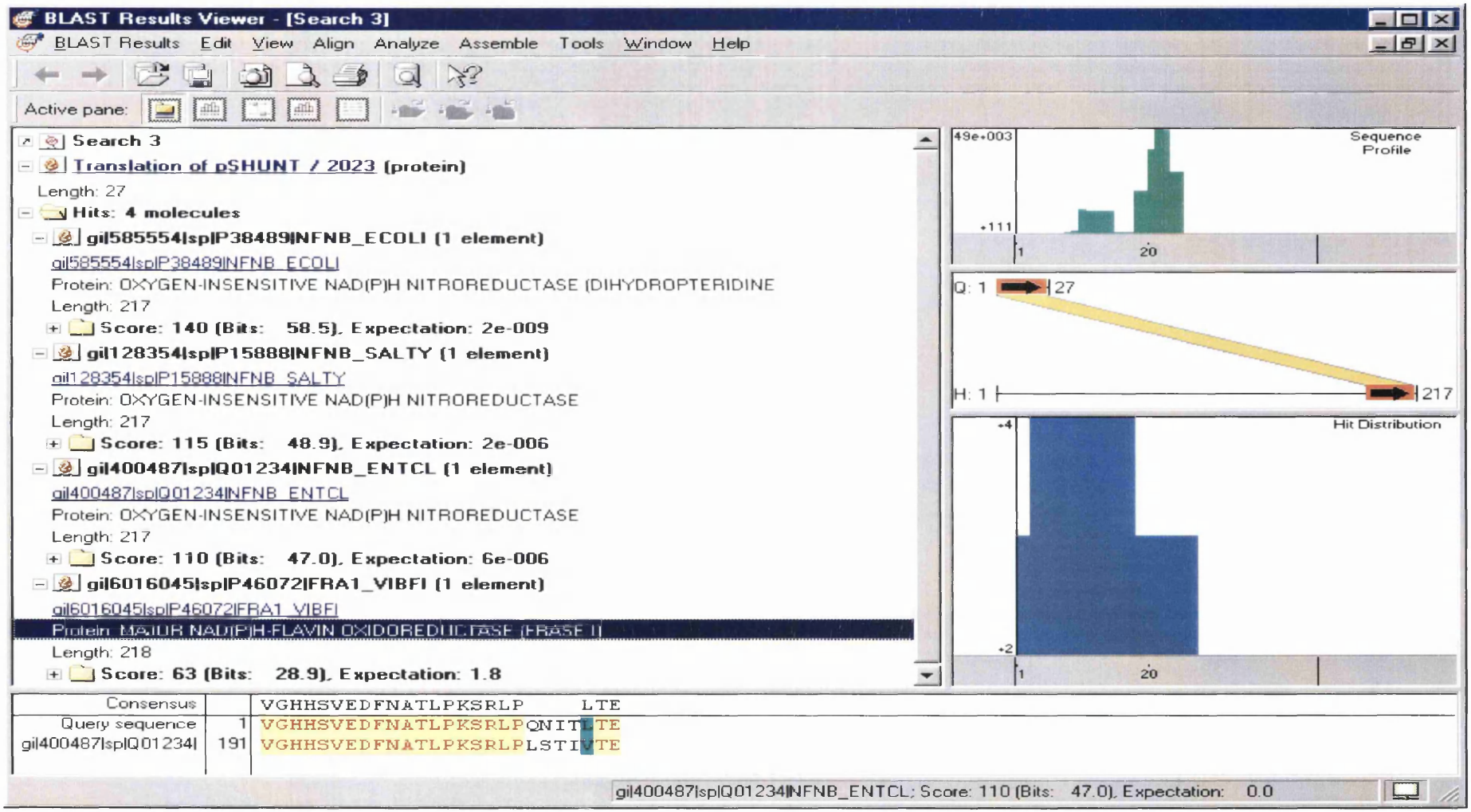
Figure 5.10: The NTR splice variant encodes a truncated protein.

Analysis of the expected products of translation of the short NTR transcripts in Vector NTI 6 indicated that the expected protein had a 22 amino acid C-terminal truncation (sequences "TERT short translate" and "hTR translate" in the figure above). The deleted region is shown by blue lettering. Additionally, the truncated protein has a 5 amino acid mutation shown in black lettering. The true sequence (VGHHS) (sequences "NTR translate", and "translation of TERT long direct") is replaced by the sequence QPPPP. The final amino acid of the mutant sequence is shown in green. In the representation shown in the next page, single residues involved in the catalytic interaction with FMN are indicated in blue and those residues involved in FMN interaction that are in the deleted region (also in blue) are indicated by green letters.


```
  1    MDIISVALKR HSTKAFDASK KLTPEQAEQI KTLLQYSPSS TNSQPWHFIV
 51    ASTEEGKARV AKSAAGNYVF NERKMLDASH VVVFCAKTAM DDVWLKLVVD
101    QEDADGRFAT PEAKAANDKG RKFFADMHRK DLHDDAEWMA KQVYLNVGNF
151    LLGVAALGLD AVPIEGFDAA ILDAEFGLKE KGYTSLVVVP VGHHSVEDFN
201    ATLPKSRLPQ NITLTEV
```

Figure 5.11: BLAST search for protein regions homologous to the mutated residues of Ad-NTR.

The 27 deleted and mutated residues were submitted for a BLAST search using Vector NTI 6 online facilities in order to determine whether other protein species with similar function show homology in this region. The search identified 3 other nitroreductase species that are closely related to E.Coli NTR, but did not identify any proteins such as DT-Diaphorase that perform a similar catalytic function. Thus, these residues are not likely to be involved in catalysis.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Active pane:
Search 3
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gi|585554|sp|P38489|NFNB_ECOLI
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Length: 217
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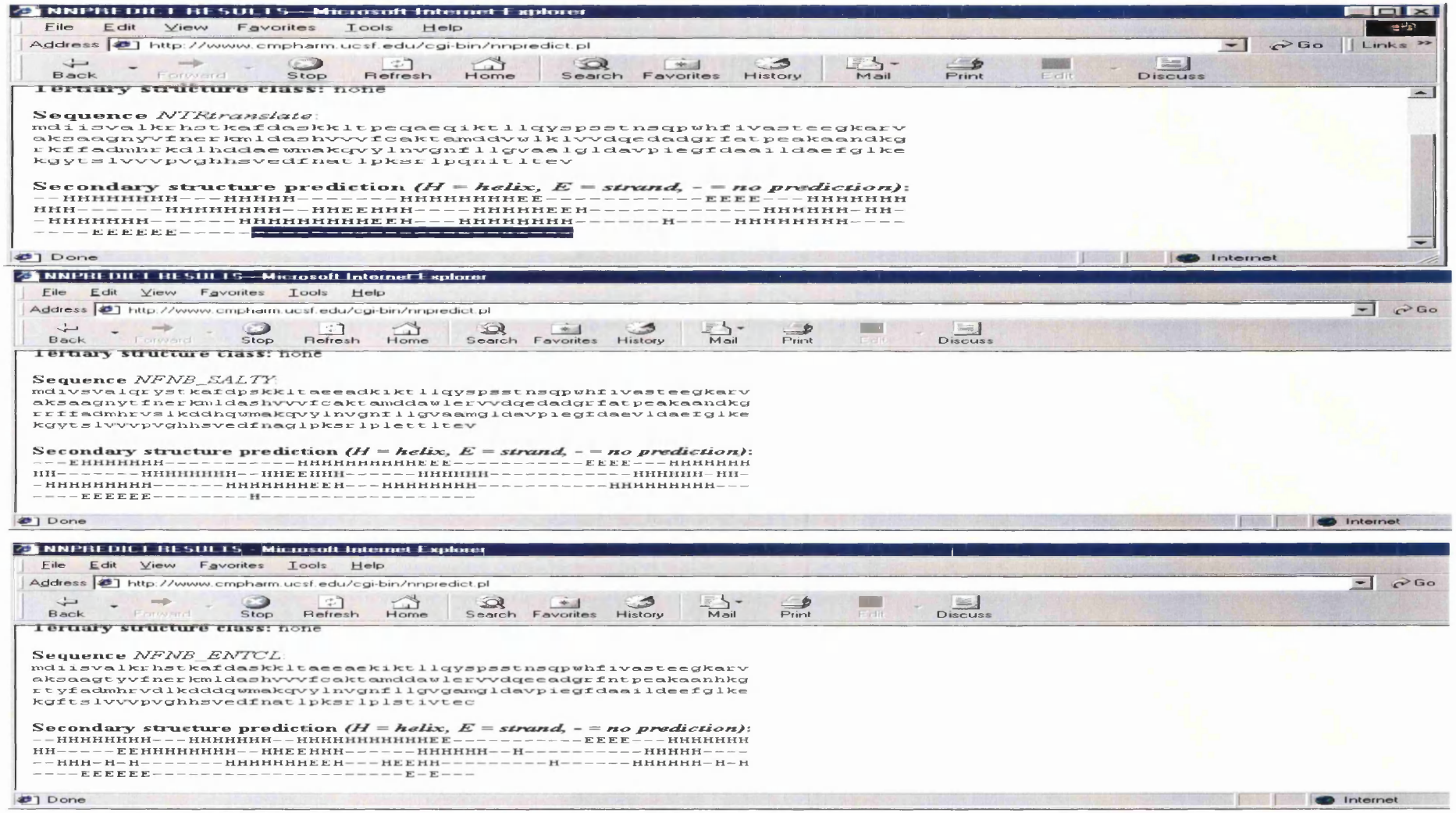
Protein: MAJOR NAD(P)H-FLAVIN OXIDOREDUCTASE (FRASE II
Length: 218
Score: 63 (Bits: 28.9), Expectation: 1.8
49e+003
+111
Sequence Profile
1
20
Q: 1
27
H: 1
217
+4
+2
Hit Distribution
1
20
Consensus VGHHSVEDFNATLPKSRLP LTE
Query sequence 1 VGHHSVEDFNATLPKSRLPQNITLTE
gi|400487|sp|Q01234| 191 VGHHSVEDFNATLPKSRLPLSTIVTE
gi|400487|sp|Q01234|NFNB_ENTCL; Score: 110 (Bits: 47.0), Expectation: 0.0

Figure 5.12: Secondary structure predictions for 3 nitroreductase species.

The sequences of the 3 most closely related NTR species identified in the BLAST search were submitted for secondary structural predictions via the online nnpredict facility of Vector NTI 6. The top panel gives the sequence of E.Coli NTR and the mutated and deleted region is highlighted in blue. In the screen-shot, a run of H corresponds to a predicted helix, while a run of E corresponds to a strand. A dash indicates no prediction. There was no conservation of predicted secondary structural features across the species in this region, while other regions are predicted to form long tracts of helices that appear quite well conserved.


NNPREDICT RESULTS—Microsoft Internet Explorer
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kgytslvvvpvghhsvedfnatlpksrlpqnitltev
Secondary structure prediction (H = helix, E = strand, - = no prediction):
Done
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NNPREDICT RESULTS—Microsoft Internet Explorer
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NNPREDICT RESULTS - Microsoft Internet Explorer
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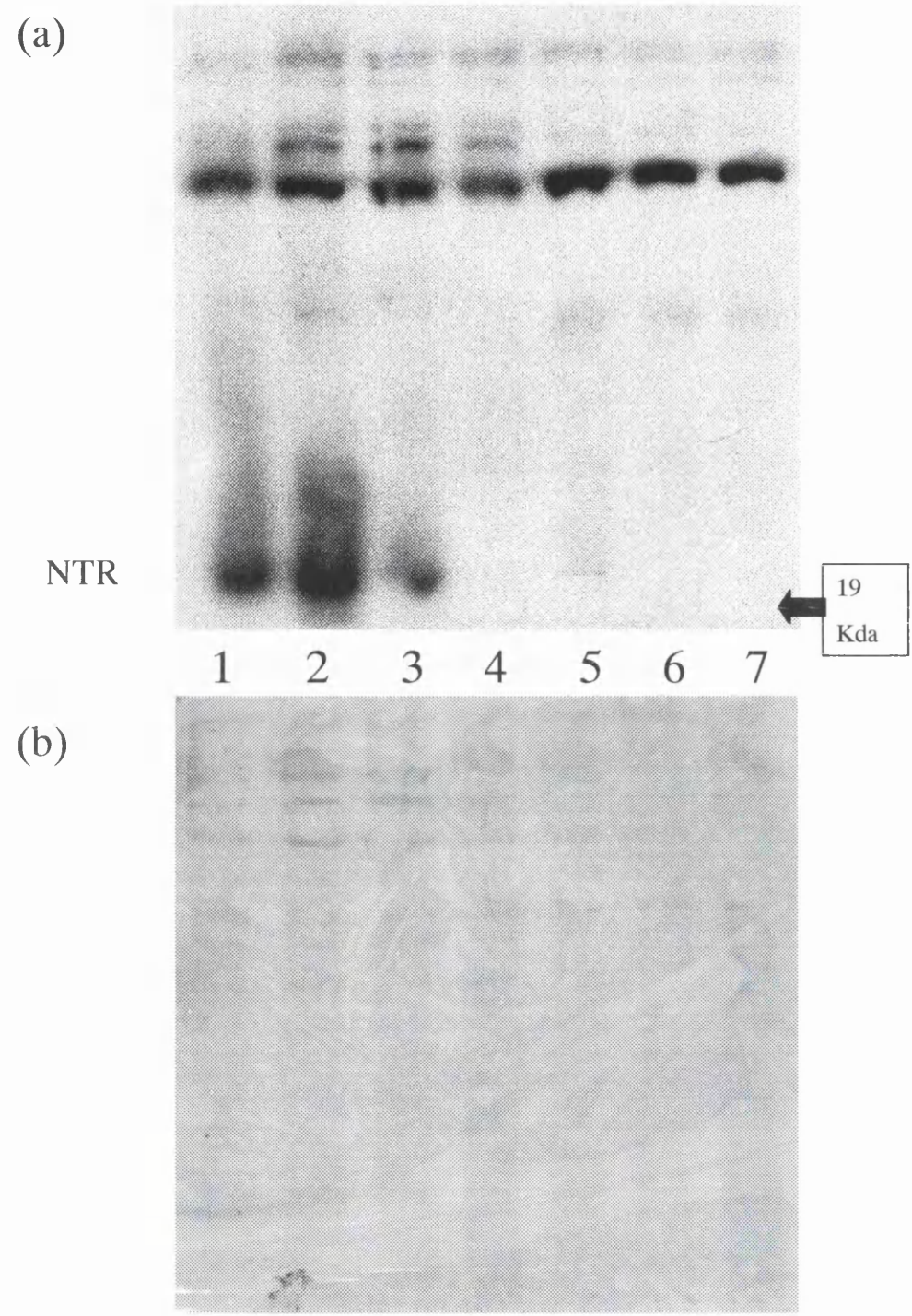


Figure 5.13: Western blot analysis of expressed NTR in WI-38 and C33-A cells.

Protein was extracted from cells infected with 50p.f.u./cell of NTR viruses. 20μg protein was run out by SDS-PAGE and blotted onto nitrocellulose filters. Filters were probed with the rabbit anti-NTR antibody R36, then with the HRP-conjugated anti-rabbit secondary. Lane 1, C33-A-hTERC-NTR stable cell line protein. Lanes 2, 3, 4, C33-A cells infected with Ad-hTERC-NTR (2), Ad-hTERT-NTR (3), or mock infected. The results are consistent with the band intensities observed in northern blots. Lanes 5, 6, 7, WI-38 cells infected with Ad-hTERC-NTR (5), Ad-hTERT-NTR (6) or mock infected (7). Control membrane stained with amido black is shown in (b). The band intensities indicated that, while WI-38 cells were more infectable than C33-A (Figures 5.1 and 5.2), the hTERC and hTERT promoters could not drive high level NTR expression in these cells. In contrast, hTERC and hTERT drove high level NTR expression in C33-A.

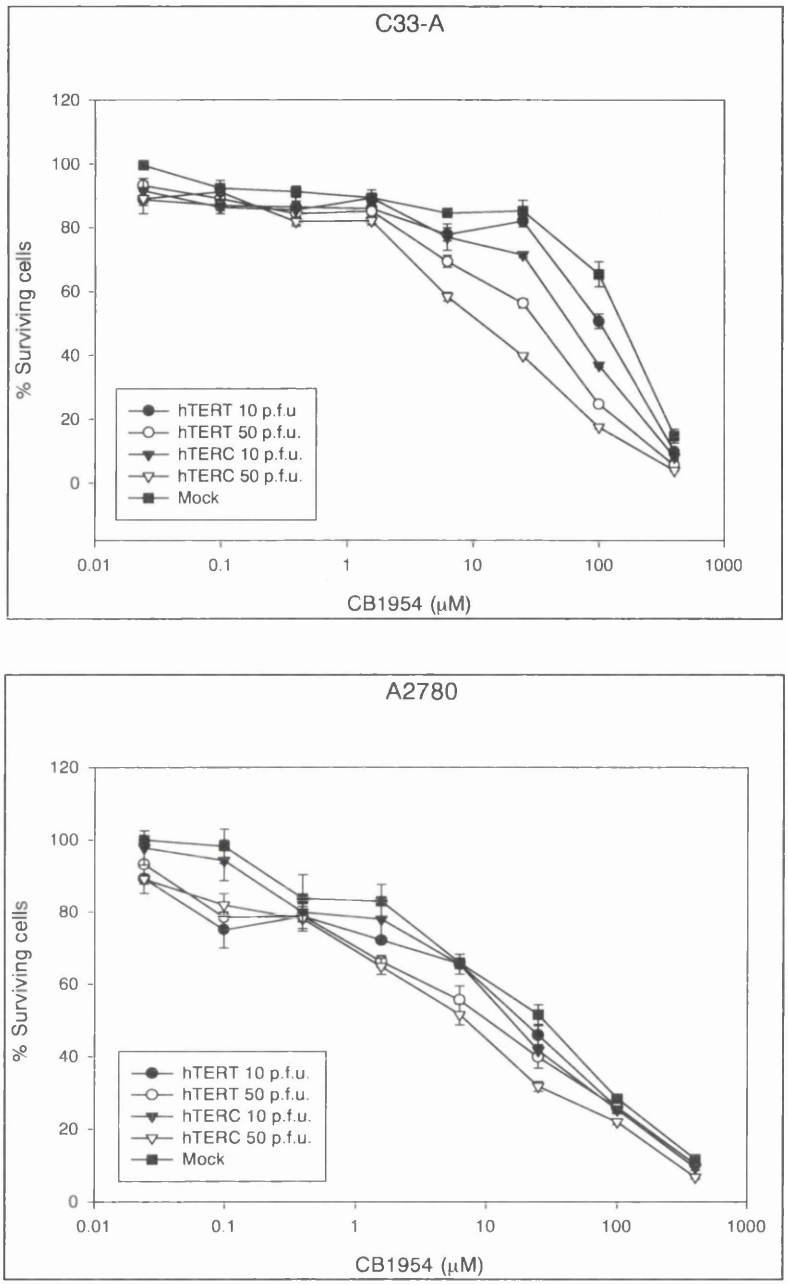


Figure 5.14: Cervical carcinoma and ovarian adenocarcinoma cells are efficiently sensitised to CB1954 after infection with Ad-hTERC-NTR and Ad-hTERT-NTR.

C33-A and A2780 cells were either mock infected or infected with a titration of 10 and 50p.f.u./cell of each of Ad-hTERC-NTR, Ad-hTERT-NTR, or the control virus Ad-CMV-LacZ. Cells were infected for 1 hour then replated into 96 well plates for MTT assay and incubated for 2 days. The cells were challenged with a titration of CB1954 for 24 hours then allowed to recover for a further 3 days prior to MTT assay. Each data point is the mean and standard error derived from triplicate plates for a representative experiment. All experiments were repeated at least 3 times. Data analysis was performed using the Softmax 2.32 microtitre plate analysis software. The sensitisation of these cell lines to CB1954 is manifested by a virus dose dependent shift of the curves away from the mock infected curve (filled squares).

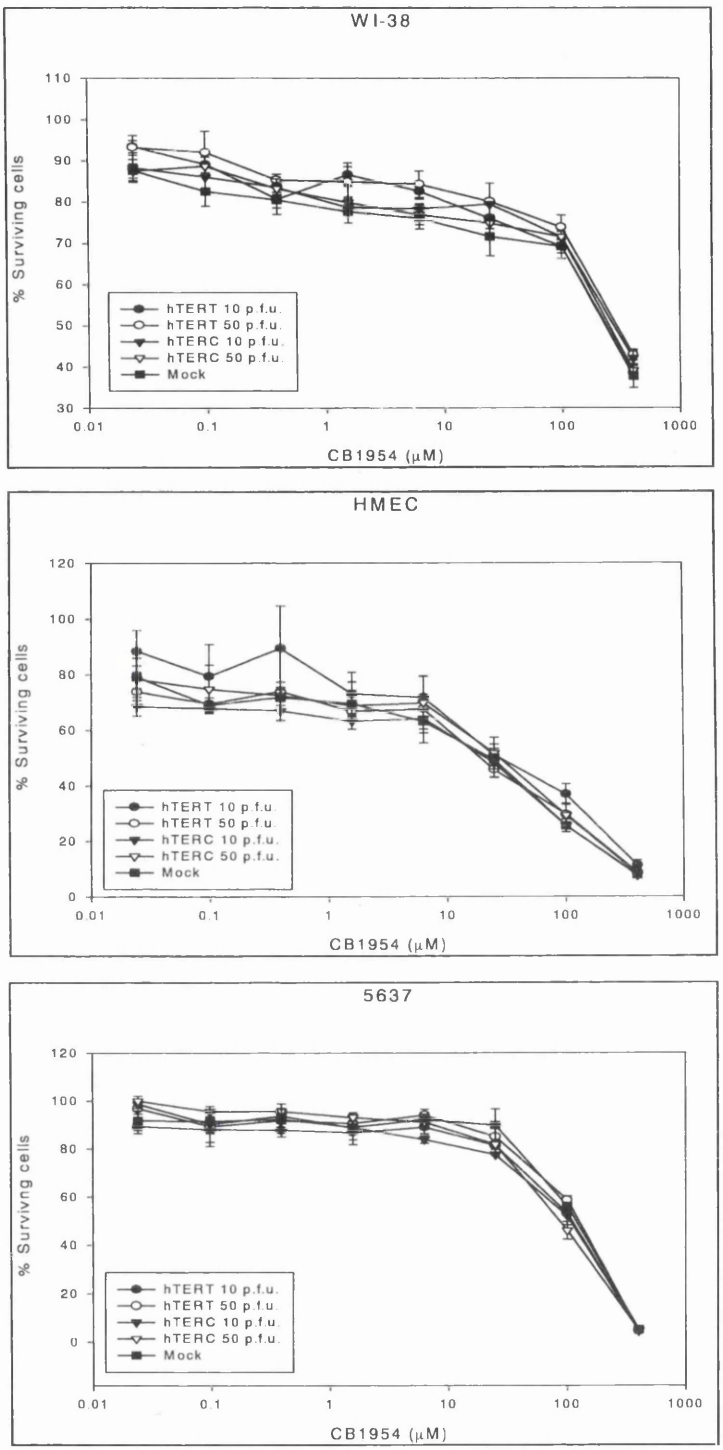
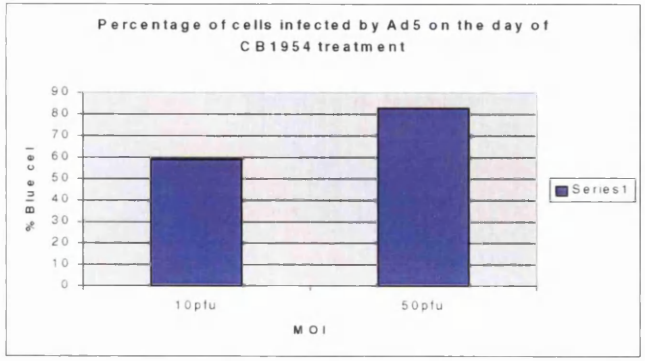


Figure 5.15: Cytotoxicity curves of the cell lines that are not sensitised to CB1954 by transduction with Ad-hTERC-NTR and Ad-hTERT-NTR.

WI-38, 5637 and HMEC cells were either mock infected or infected with a titration of 10 and 50p.f.u./cell of each of Ad-hTERC-NTR, Ad-hTERT-NTR, or the control virus Ad-CMV-LacZ. Cells were infected for 1 hour then replated into 96 well plates for MTT assay and incubated for 2 days. The cells were challenged with a titration of CB1954 for 24 hours then allowed to recover for a further 3 days prior to MTT assay. Each data point is the mean and standard error derived from triplicate plates for a representative experiment. All experiments were repeated at least 3 times. Data analysis was performed using the Softmax 2.32 microtitre plate analysis software. The lack of sensitisation of these cell lines to CB1954 is manifested by curves for adenovirus infected cells that are tightly packed together with the mock infected curve (filled squares).

Figure 5.16: Representative parallel cytotoxicity and infectivity assay in C33-A cells.

In order to assess the dual dependence of sensitisation to CB1954 on promoter activity and infectivity, all cells tested were infected with a titration of 10 or 50p.f.u. per cell of each of the NTR viruses in addition to the CMV-LacZ virus. On the day of drug addition, the LacZ infected cells were stained with X-Gal to give an indication of the proportion of infected cells on the day of drug administration. The figure shows the summary of a single representative experiment, including the percentage of infected cells and photomicrographs of X-Gal stained cells, in addition to the cytotoxicity curves, IC50 values and the derivation of the sensitisation value from the IC50 values. All experiments were repeated at least 3 times and cell lines expected to have low promoter activities were assayed in parallel with a cell line having high promoter activity.



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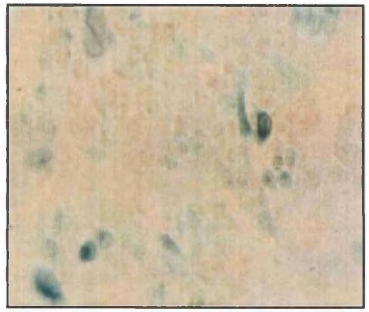

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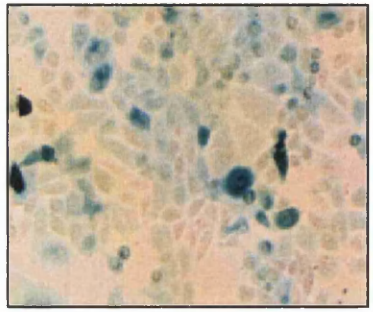
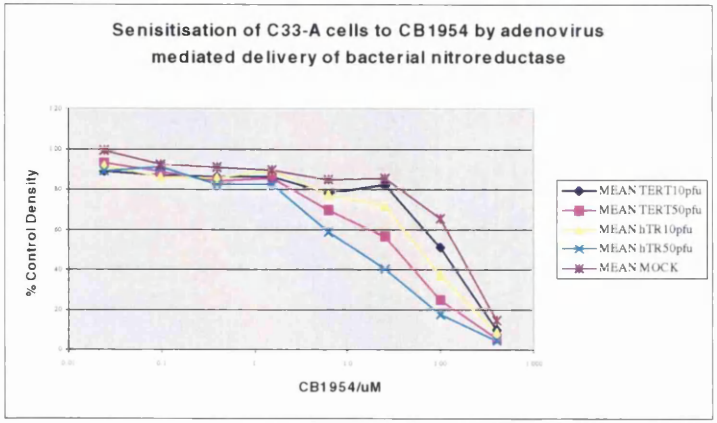
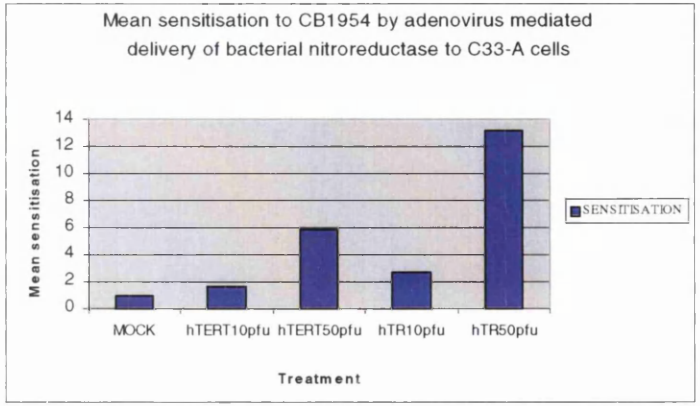




CONSTRUCT	IC50	S.ERROR	SENSITISATION
MOCK	171.03	19.07	1
hTERT10pfu	106.93	5.31	1.6
hTERT50pfu	29.35	1.12	5.83
hTR10pfu	62.29	0.88	2.75
hTR50pfu	13.01	0.4	13.15



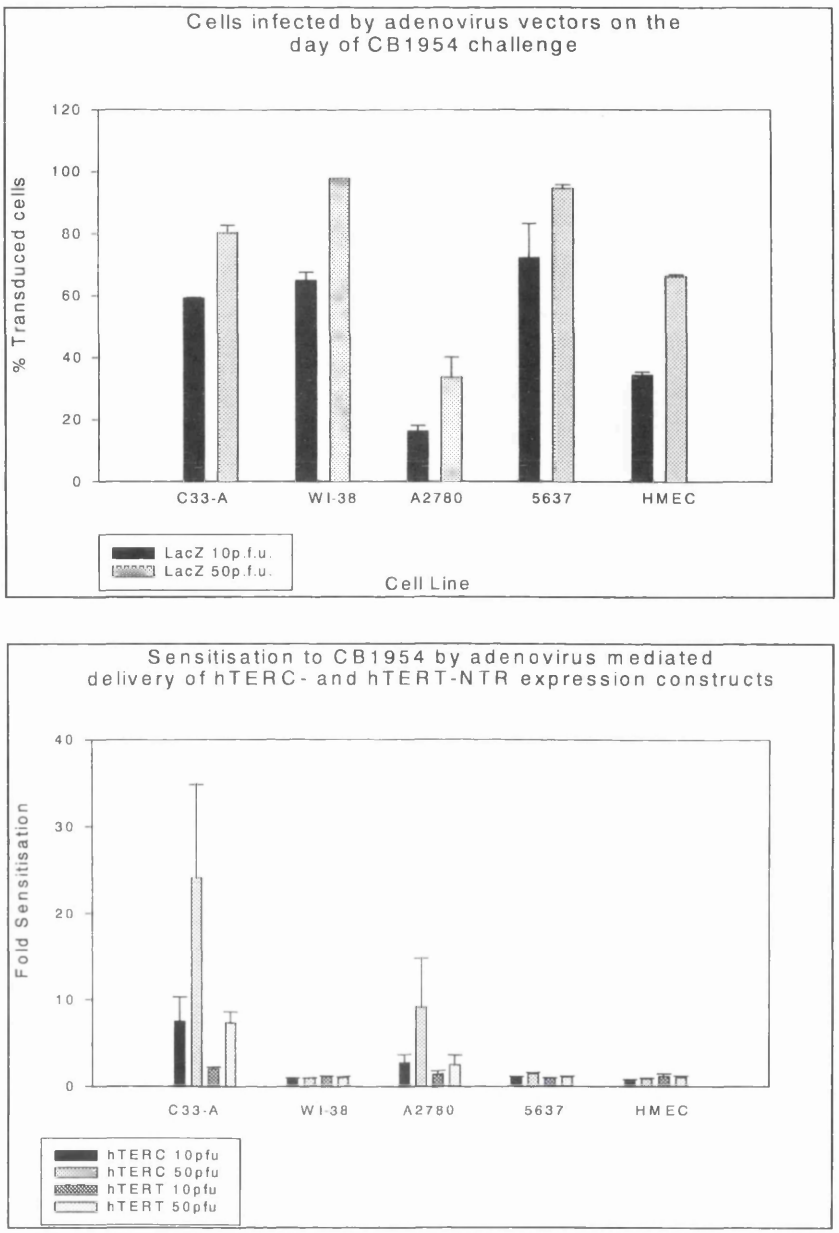


Figure 5.17: Summary of infectivity and sensitisation in cell lines infected with Ad-hTERC-NTR and Ad-hTERT-NTR.

Cell lines were either mock infected, or infected with 10 or 50p.f.u./cell of either Ad-hTERC-NTR, Ad-hTERT-NTR, or Ad-CMV-LacZ for 1 hour. After infection, cells were replated and MTT or infectivity assays were performed as described earlier. Infectivity values in the upper panel are the means and standard errors derived from 2 independent experiments in each cell line and independent values were derived from the mean proportion of X-Gal stained cells in 5 random fields at X20 objective (approximately 500-1000 cells). IC50 values for CB1954 cytotoxicity in individual experiments are derived from the mean concentration of drug, taken across triplicate plates, necessary to reduce the cell density to 50% of control (untreated) cells. The sensitisation value in individual experiments is taken to be the fold difference between the IC50 of the mock infected cells and the IC50 value of the curve for any single treatment. All cytotoxicity experiments were repeated at least 3 times and the data given in the lower panel are the means and standard errors derived from 3 independent experiments.

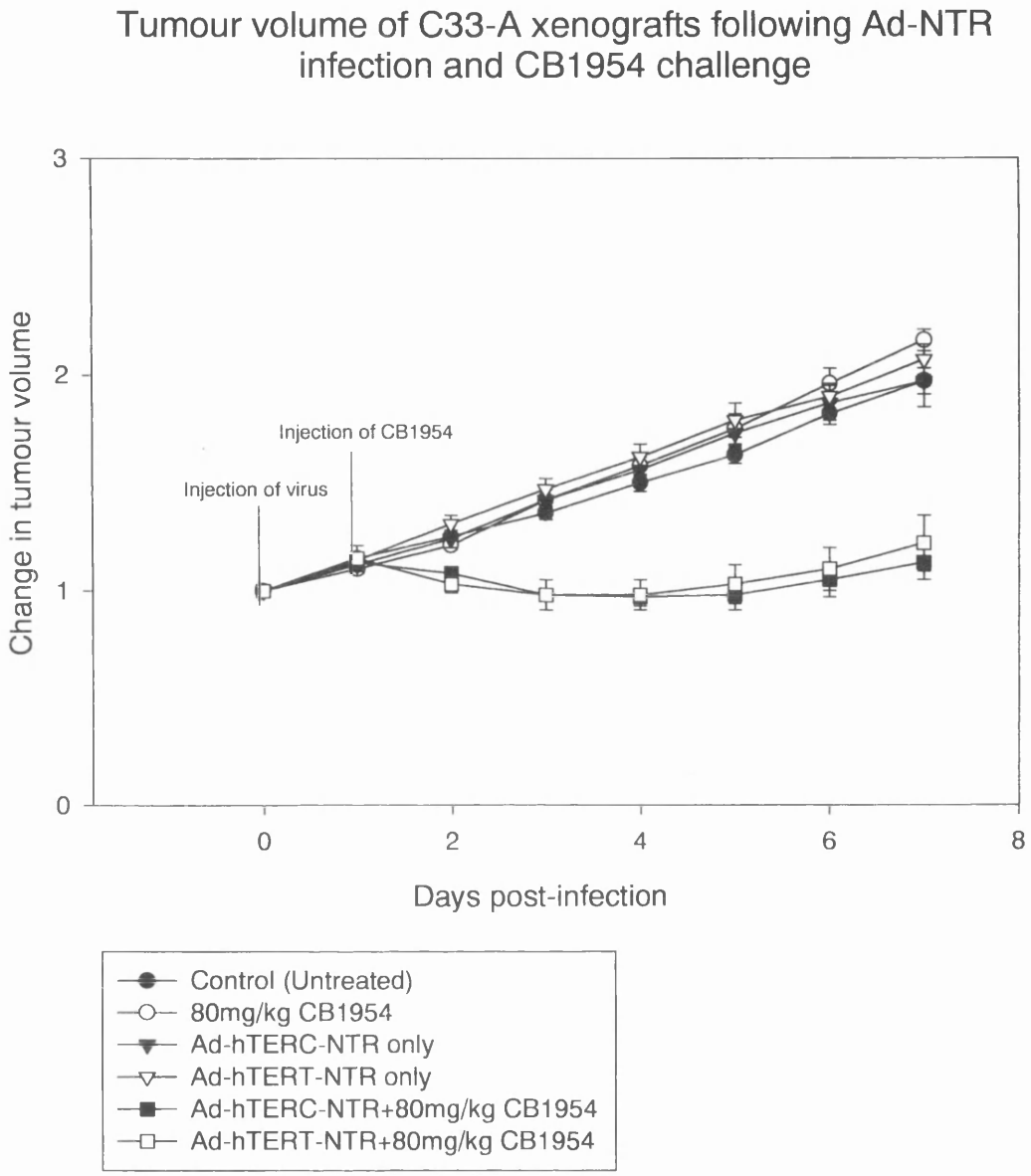


Figure 5.18: Ad-hTERC-NTR and Ad-hTERT-NTR sensitise human cervical carcinoma cells to CB1954 induced cytotoxicity *in vivo*.

10^7 C33-A cells per mouse were injected subcutaneously into the flanks of 6 groups of 6 female athymic nude mice and allowed to develop for 14 days until tumour diameters were approximately 5mm. At this time (day 0), 4 groups of mice were injected intra-tumourally with a total of $4x10^8$pf.u. either of Ad-hTERC-NTR or Ad-hTERT-NTR (2 groups for each virus). The following day (day 1), 3 groups (1 drug only group and 1 each of the virus injected groups) were intravenously injected with 80mg/kg CB1954 and the mean tumour volumes of all groups were monitored daily for 7 days. Results given are the mean tumour volumes and standard errors at each time point derived from 6 mice per group by the formula volume=$d^3x\ \pi/6$. A clear reduction in tumour volume over the course of the experiment is evident in the groups injected with both virus and drug, but not in any of the control groups.

CELL LINE	MOCK IC50 (s.e.) μM	hTERC 10 P.F.U. IC50 (s.e.) μM	hTERC 50 P.F.U. IC50 (s.e.) μM	hTERT 10 P.F.U. IC50 (s.e.) μM	hTERT 50 P.F.U. IC50 (s.e.) μM
C33-A	176.13 (10.08)	33.43 (14.56)	9.76 (2.75)	63.13 (17.07)	35.86 (13.32)
WI-38	153.62 (48.53)	178.09 (68.05)	164.72 (55.21)	148.6 (54.76)	174.12 (74.09)
A2780	28.5 (13.92)	10.69 (3.66)	3.85 (1.45)	17.73 (4.39)	10.86 (0.64)
5637	102.86 (14.25)	93.55 (16.06)	73.36 (14.59)	112.61 (19.96)	103.17 (22.4)
HMEC	31.67 (11.21)	45.38 (20.54)	36.02 (11.27)	27.76 (6.27)	29.65 (4.5)

Table 5.1: IC50 values for CB1954 cytotoxicity in cell lines infected with Ad-hTERC-NTR and Ad-hTERT-NTR.

Relative cell densities were calculated from optical density measurements at 570nm to measure the quantity of MTT-formazan in individual wells. For an independent experiment, each IC50 value was calculated from the mean value of the 50% y-intercept determined from triplicate plates. IC50 values in the table are the means and standard errors, given in brackets, for each virus and multiplicity of infection calculated from 3 independent experiments.

CHAPTER 6
SUMMARY OF EXPERIMENTAL RESULTS AND FINAL DISCUSSION

- Validation of the use of hTERC and hTERT promoters for transcriptionally directed cancer gene therapy.
- Final discussion.

6 SUMMARY OF EXPERIMENTAL RESULTS AND FINAL DISCUSSION

6.1 Summary of experimental results

A major aim of this thesis was to examine the transcriptional regulation of hTERC and hTERT promoters and to determine whether the sequences are useful in the context of an anticancer gene therapy approach. The hTERC and hTERT sub-units are differentially regulated at a transcriptional level between normal and cancer cells and the expression of the sub-units is a major factor that determines whether telomerase is active in the cell. The data presented in chapter 3 show clear differentials in the activity of hTERC and hTERT promoter fragments in normal and cancer cells that validate the promoters for use in cytotoxic gene therapy.

It would be expected that the expression of a pro-drug activating enzyme under the control of these sequences would selectively activate the pro-drug to significant levels in cancer cells with high promoter activity. This indeed appears to be the case in stable cell lines harbouring hTERC-NTR and hTERT-NTR expression constructs. In this model, the response of cells to CB1954 challenge fell into two groups, apparently dependent upon promoter activity: those cell lines that had high promoter activities and were sensitised to CB1954, and those cells that had low promoter activities and were not. The sensitivity of two cell lines to telomerase-nitroreductase gene therapy was retained in xenograft models, resulting in significant reductions in tumour volume upon challenge with CB1954. In both xenograft models, the reduction in tumour volume mirrored the shape of the cytotoxicity curves determined by *in vitro* cytotoxicity assay, confirming that the promoter dependent sensitisation to CB1954 is retained *in vivo*. These data, presented in chapter 4, were encouraging for the further development of the telomerase-nitroreductase gene therapy model and the model was extended by cloning the expression constructs into an adenovirus backbone.

Infectivity assays showed that adenovirus was a highly efficient gene delivery vehicle in most of the cell lines used in this model and gene expression analysis indicated that NTR RNA and protein was expressed in a promoter dependent manner in cell lines infected with adenovirus gene therapy vectors. Northern analysis and sequencing of cDNA confirmed

that the major expression product was a splice variant with a 187bp deletion from the expected sequence. The variant encodes a 22 amino acid deletion with a further 5 amino acid mutation. The mutation is not expected to abrogate the function of NTR, as it is distal from the site of catalysis, but two deleted residues contact the internal electron donor molecule to stabilise binding suggesting that the efficiency of catalysis could theoretically be reduced. However, functional studies of CB1954 induced cytotoxicity in Ad-NTR infected cell lines indicated that functional NTR was expressed.

A cervical carcinoma cell line and an ovarian adenocarcinoma cell line could be efficiently targeted by infection with Ad-hTERC-NTR and Ad-hTERT-NTR followed by CB1954 challenge, while a further three mortal and immortal cell strains with low promoter activities were not sensitised to CB1954. Thus, Ad-hTERC-NTR and Ad-hTERT-NTR sensitise human cancer cells to CB1954 to an extent that is partly dependent on promoter activity and partly dependent on infection efficiency.

In order to make a more detailed analysis of the possible functional effects of the mutation, it would be necessary to perform a comparative enzyme activity assay using a defined proportion of adenovirus infected cells compared with a similar proportion of stable cells mixed with the necessary proportion of NTR negative cells. An assay for NTR enzyme activity based on menadione and cytochrome P450 reduction has previously been described (Plumb et al. 1994). However, the degree of sensitisation observed in the cervical cell line was comparable with that observed in stable cell lines harbouring the plasmid DNA NTR expression constructs despite the infection of less than 100% of cells and, taking into account the low infectivity of the ovarian cell line, the degree of sensitisation observed was also comparable to the stable cell line model. This indicated that the catalytic function of NTR was not adversely affected by the deletion, in terms of its ability to bioactivate CB1954 resulting in cell death. Additionally, C33-A xenografts infected with telomerase-nitroreductase adenovirus gene therapy vectors showed decreases in tumour volume on challenge with CB1954 that were comparable with those seen in xenografts established from stable hTERC-NTR and hTERT-NTR expressing cell lines. Thus, the functional evidence suggests that NTR activity was not significantly affected by the mutation.

The data presented in this study confirm that a therapeutic window for exploitation by cytotoxic gene therapy exists in the differential telomerase promoter activities between

normal and cancer cells and support the further development of telomerase-nitroreductase gene therapy vectors.

6.2 Final discussion.

Cancer is a major worldwide health problem. In the UK, cancer accounts for approximately 24% of all fatalities annually. Conventional systemic anticancer chemotherapeutics are limited in their efficacy by a low therapeutic ratio resulting in dose limiting toxicity to normal tissue. Often, this is because the targets and mechanisms of action of conventional chemotherapeutic agents are not directed against malignant cells, but rather cells that are in cycle. For this reason, the identification and exploitation of novel mechanism based targets for the treatment of cancer is a major aim of cancer research. Cytotoxic gene therapy represents an attractive approach for management of malignant disease as it promises to allow tumour specific expression of therapeutic constructs, thereby enhancing the therapeutic ratio.

The association of telomerase activity and telomerase sub-unit expression with human cancer is unique in its prevalence and specificity (Kim et al. 1994; Hiyama et al. 1995; Shay et al. 1997; Soder et al. 1998; Sarvesvaran et al. 1999; Hiyama et al. 2001). Telomerase promoter constructs have now been used to direct the cancer cell specific expression of a number of therapeutic transgenes including HSTK (Majumdar et al. 2001), diptheria toxin (Abdul-Ghani et al. 2000), Bax (Gu et al. 2000), caspase 8 (Koga et al. 2000), caspase 6 (Komata et al. 2001), FADD (Koga et al. 2001), and the noradrenaline transporter gene that facilitates the uptake of ^{131}I-MIBG (Boyd et al. 2001). Because of the many attractive features of the nitroreductase/CB1954 system, the addition of hTERC-NTR and hTERT-NTR expression systems to this potential anticancer armoury is an exciting development (Plumb et al. 2001).

Current gene therapy systems are limited by a requirement for increased efficiency and selectivity of delivery to the target *in situ*. However, several key advances of the last decade in adenovirus-based gene transfer technology may be expected to help overcome the current limitations to efficacious anti-cancer gene therapy when appropriately combined with cytotoxic gene therapy approaches.

The development of high-capacity vectors allows for longer transgene persistence at the site of infection coupled with the possible development of vectors encoding multiple

tumour targeting expression cassettes (reviewed in Morsy et al. 1999). The enhanced efficacy of a double suicide gene therapy approach has previously been demonstrated using a combination of cytosine deaminase and viral thymidine kinase genes (Uckert et al. 1998).

The identification of the primary receptor, hCAR (Bergelson et al. 1997), and the integrin mediated internalisation pathway of adenoviruses (Wickham et al. 1993) has allowed the development of novel technologies designed to retarget adenoviruses to other cellular receptors. Wickham and colleagues defined the hCAR/knob contact residues (Kirby et al. 1999; Roelvink et al. 1999; Kirby et al. 2000), and recent mutational experiments have seen the development of vectors with both hCAR and integrin specificity completely ablated (Einfeld et al. 2001). Such vectors effectively represent a "blank page" on which completely novel targeting specificities may be engineered. Interestingly, the exposed HI loop of the adenovirus knob domain is not involved either in the primary receptor interaction, or in the interactions required for the essential trimerisation of fiber (Krasnykh et al. 1998) and contains a unique restriction site that facilitates easy cloning of sequences of interest (Mizuguchi et al. 2001). Thus, this region of the molecule is an attractive site for incorporation of novel binding specificities. The validity of this approach for tumour targeted delivery of cancer therapeutics will therefore rely mainly upon the identification of tumour associated cell surface markers that may enable the more effective systemic administration of adenoviral vectors. Adenoviruses have been targeted to a number of alternative receptors using genetic approaches, or with the use of bispecific antibodies (Douglas et al. 1996; Gu et al. 1999; Haisma et al. 1999; Dmitriev et al. 2000).

Recent studies have seen the development of chimeric viral vectors, a method for combining attractive features of two viruses in completely novel constructs (reviewed in Reynolds et al. 1999). This approach has at its heart an extension of the pseudo-typing approach that has been used to confer new virus tropism, in the case of adenoviral vectors, by generating capsid combinations derived from two different adenovirus serotypes (Zabner et al. 1999). In a recent study, two adenoviral constructs were generated incorporating retroviral packaging and integration functions in separate vectors. Infection of an ovarian carcinoma cell line with both adenoviruses resulted in the transient production of a retrovirus producer cell line. Xenografts generated from a mixture of producer cells and virgin tumour cells showed stable incorporation of a GFP transgene encoded in the retroviral sequence (Feng et al. 1997). Alternative approaches to the development of chimeric vectors include mixing viral and non-viral gene delivery

technologies. For example, the enhancement of efficiency of transfection of naked plasmid DNA by co-internalisation with adenovirus or adenovirus capsid components is well documented (Seth et al. 1994).

A final development in adenovirus gene delivery technology that may be expected to allow for local enhancement of infectivity at the target site is the development of selectively replicating adenoviral vectors. Interestingly, a patent application has recently been filed by the Geron Corporation of California for the development of an adenoviral vector in which expression of the E1 region is restricted by the use of the hTERT promoter (Morin et al, World Intellectual Property Organisation publication number WO 00/46355).

Although the development and refinement of advanced generation gene delivery systems will be central to the realisation of cytotoxic gene therapy systems that are of genuine therapeutic value, their necessity does not outweigh the requirement for efficient tumour specific expression systems. Rather, it is the combination of these technologies that may herald the dawn of a new era in anticancer therapeutics. From this point of view, telomerase is an excellent candidate for the development of tumour specific therapeutic gene expression systems.

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