Characterisation of Transcriptional Regulation of the Human Telomerase RNA Gene

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To my family

Abstract

The human telomerase core enzyme consists of an essential structural RNA (h*TERC*) with a template domain for telomeric DNA synthesis and of a catalytic protein (hTERT) with reverse transcriptase activity. Expression of the h*TERC* and hTERT are essential for telomerase activity. Variation in telomerase activity is correlated with cellular senescence and tumour progression. Recent studies indicate that the regulation of telomerase activity is multifactorial in mammalian cells. The primary mode of control of hTERT appears to be transcriptional regulation but very little is known about the molecular mechanisms involved in the regulation of h*TERC* transcription. In this study, I have cloned and characterised the genomic sequences and promoter of the h*TERC* gene to provide evidence that transcriptional mechanisms are involved in h*TERC* gene regulation. Transient transfection with a series of 5´-deletion mutants demonstrated that between -5.0 kb and -51 bp of the h*TERC* gene is responsible for high promoter activity, the minimal promoter region was defined as 176 bp (-107 to +69 bp). With the aid of *in vitro* DNase I footprinting, electrophoretic mobility shift assays (EMSAs) and mutagenesis analysis, four Sp1 binding sites and one CCAAT-box bound by the transcription factor NF-Y were identified to be involved in regulation of h*TERC* transcription. Co-transfection experiments showed that Sp1 and the retinoblastoma protein (pRb) are activators of the h*TERC* promoter and Sp3 is a potent repressor. Mutation of the CCAAT-box or the coexpression of a dominant negative nuclear factor-Y (NF-Y) significantly attenuated the transactivation by pRb and Sp1, suggesting that NF-Y binding is a prerequisite for pRb and Sp1 to activate the h*TERC* promoter. The different transcriptional regulators appear to act in a species-specific manner. Whilst Sp1 and Sp3 act on the human, bovine and mouse *TERC* promoters, pRb activates only the human and bovine promoter and NF-Y is important for the human *TERC* gene. The h*TERC* gene is expressed during embryogenesis and then down-regulated during normal development but is re-expressed in tumour cells, the h*TERC* promoter activity was therefore further investigated and a higher promoter activity in immortal cells than in two mortal cell strains (WI38 and IMR90) was shown. In conclusion, h*TERC* promoter contains sequence elements that allow interactions with several different transcription factors. The interplay between NF-Y, pRb, Sp1 and Sp3 within the architecture of the h*TERC* promoter may combine to enable a wide variety of cell types from mortal to immortal to regulate h*TERC* expression through transcriptional control.

List of Tables

List of Figures

Table of Contents

Abbreviations

5'-FTR	5'-flanking transcriptional regulatory region
ALT	alternative lengthening of telomeres
AP1, AP2, AP4	activator Protein 1, 2, 4
ATF	activating transcription factor
b	bovine
BLAST	basic local alignment search tool
b*TERC*	bovine telomerase RNA gene
c/EBP	CCAAT/enhancer binding protein
CBF	CCAAT-binding factor
CBP	CREB-binding protein
CMV	cytomegalovirus
CP1	CCAAT protein 1
CR	conserved region
CREB	cAMP-responsive element binding protein
CTF	CCAAT-binding transcription factor
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
EST	essential for telomerase activity
FISH	fluorescence in situ hybridisation
FITC	fluorescein isothiocyanate
GC	GC box element
GFP	green fluorescent protein
h	human
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEK	human embryonic kidney cell
HFM	histone fold motifs
HMEC	human mammary epithelial cell
HPV	human pappillomavirus
h*TERC*	human telomerase RNA gene
hTERT	human telomerase reverse transcriptase
Luc	luciferase
m	mouse
Mam	mammalian
Max	Myc-associated factor x
MHC	major histocompatibility complex
m*Terc*	mouse telomerase RNA gene
MYOD	myoblast determining factor
MZF-2	myeloid-specific zinc finger protein 2
NCBI	national centre for biotechnology information
NF-Y	nuclear factor Y
P/CAF	p300/CBP binding protein-associated factor
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PNA	peptide nucleic acid

Pol I, II, III	RNA polymerase I, II and III
pRb	retinoblastoma protein
PSE	proximal sequence element
Rap1	repressor/activator protein
RCC23	renal cell carcinoma cell
RCE	retinoblastoma control element
RCP	retinoblastoma control element protein
Rif1p, Rif2p	Rap1p interacting factors
RNP	ribonucleoprotein
RT	reverse transcriptase
SCC	human squamous cell carcinoma lines
SEAP	secreted form of human placental alkaline phosphates
Sp1, Sp3	stimulating protein 1 and 3
TAF	TBP associated factor
TBP	TATA-binding protein
TER1	*Tetrahymena* thermophila
TERC	telomerase RNA gene
TERT	telomerase reverse transcriptase
TESS	transcription element search software
TGF	transforming growth factor
TK	thymidine kinase
TLC1	the RNA component of yeast telomerase
TP1	telomerase associated protein 1
TRAP	telomeric repeats amplification protocol
TRF1, 2	telomere repeat-binding protein 1, 2
TSA	trichostatin A
TSS	transcriptional start site
UWGCG	university of wisconsin genetics computer group
WT1	Wilms' tumor 1 suppressor gene
YY-1	Yin Yang-1

Reagents

APS	ammonium persulphate
AZT	azidothymidine
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dH_2O	de-ionised water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dNTP	3' deoxyribonucleoside 5'-triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetra-acetic acid

ET-743	*Ecteinascidia turbinata,*
EtBr	ethidium bromide
FCS	foetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HMN-154	(E)-4-[2-[2-(p-methoxy-benzene-sulfonamide)phenyl]ethenyl] pyridine
mRNA	messenger ribonucleic acid
PBS	sterile phosphate-buffered saline
PDL	population double level
PE	PBS+EDTA
PEG	polyethylene glycol
PMSF	phenymethylsuephonyl fluoride
RNA	ribonucleic acid
RNA'ase	ribonuclease
SDS	sodium dodecyl sulphate
TAE	tris, acetic acid, ethylenediaminetetra-acetic acid
TBE	tris, boric acid, ethylenediaminetetra-acetic acid
TE	tris, ethylenediaminetetra-acetic acid
TEMED	tetramethylenediamine
Tris	2-amino-2-(hydroxymethyl)propane-1, 3-diol

Units

bp	base pair(s)
Ci	curie
Da	dalton
g	gram
k	kilo
kb	kilo base pair
l	litre
m	milli
n	nano
nt	nucleotides
°C	degree celsius
rpm	revolutions per minute
sec	second
UV	ultra violet
v/v	volume for volume
w/v	weight for volume
wt	wild type
μ	micro

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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

- The end-replication problem
- Telomere
- Telomerase
- Patterns of telomerase expression
- Regulation of telomerase
- Aims of the thesis

Chapter 1 Introduction

1.1 The End-Replication Problem

The genetic material of eukaryotic cells is dispersed on multiple linear chromosomes, with a total of 46 chromosomes in each human somatic cell (for example Figure 1.1). One could imagine that the presence of these multiple independent chromosomes offers great selective advantages during evolution due to the potential for variation. However, linear chromosomes pose two problems for the cell: how to protect the free DNA ends from degradation and fusion, and how to replicate the extreme termini of the DNA. Exposed 5' and 3' termini of DNA are highly susceptible to nuclease degradation. In addition, such exposed DNA ends are highly recombinogenic and can ligate with other free DNA ends or can integrate into the heart of chromosomal DNA (Haber & Thorburn; 1984).

The mechanisms of DNA replication in linear chromosomes is different for each of the two strands (called leading and lagging strands, see Figure 1.2, Morin, 1997). The lagging strand is made as a series of discrete fragments, each requiring a new RNA primer to initiate synthesis. The DNA between the last RNA priming event and the end of the chromosome cannot be replicated because there is no DNA beyond the end to which the next RNA primer can anneal, thus this gap cannot be filled in (this is referred to as the "end replication problem"). Since one strand cannot copy its end, telomere shortening occurs during progressive cell divisions. The shortened chromosomes are inherited by daughter cells and the process repeats itself in subsequent divisions (Olovnikov, 1971; Watson, 1972).

1.2 The telomere

1.2.1 Telomeres cap the chromosome end

In the early 1970s, the end replication problem was put forth as a dilemma for maintaining the integrity of linear chromosomes. Given the limitations of conventional DNA

polymerases, genetic information was predicted to be lost with each round of replication, causing the chromosomes to shorten each cell cycle (Watson, 1972 ; Olovnikov, 1973). In 1985, Greider and Blackburn identified telomerase activity in the ciliate protozoa, *Tetrahymena* (see Table 1.1 for history). In 1987, Greider and Blackburn demonstrated a new and unexpected mechanism of DNA replication-telomere extension by reverse transcription. Eukaryotic telomeres generally consist of simple arrays of tandem G rich repeats that run 5' to 3' at chromosome ends, with the C rich complementary strand sometimes recessed, forming a G rich 3' overhang. Single stranded G rich telomeric sequences can form intra- and interstrand structures, but their biological importance is unclear (Fang & Cech, 1993).

Telomere sequences can vary from species to species, but a given organism has a characteristic repeat at all telomeres, and their function is the same: (1) they form specific complexes with telomere-binding proteins; (2) they protect chromosome ends from exonuclease digestion; (3) they prevent aberrant recombination; and (4) telomeres prevent the chromosome ends from activating checkpoint controls that sense DNA damage. Telomeres have been implicated in a large number of cellular processes: chromosome length maintenance, chromosome stability, transcriptional silencing, and chromosome positioning in the nucleus, chromosome healing, recombination, chromosome fragmentation and cellular senescence.

Therefore, telomeres are specialised heterochromatin structures that act as protective caps at the ends of the chromosomes. One critical difference between normal and tumour cells lies at the ends of their chromosomes. Normal somatic cells progressively shorten their telomeres due to the end-replication problem with increasing cell divisions until a growth arrest (senescence/age) occurs. Tumour cells have elongated telomeres due to expression of telomerase. An enzyme called telomerase is the key to maintaining the length of the telomeres and overcome the end-replication problem.

1.2.2 Telomeres in cellular aging and molecular biologic clock

In human and all vertebrates studied, a single telomere sequence (TTAGGG) is found at chromosome ends (Figure 1.1) (Moyzis et al., 1998). In human, telomeres are made up of an average of 5000-15000 base pairs of $(TTAGGG)_n$ repeats and telomere-binding proteins. Every time a cell divides it loses 25-200 DNA base pairs off the telomere ends. Once this pruning has occurred about 100 times, a cell senesces (or ages) and does not continue dividing. Eventually, shortening of the telomeric DNA at chromosome ends is postulated to limit the lifespan of human cells, such DNA deletion would lead to the loss of cell viability. In mouse, telomeres are approximately 40 kb long (Kipling & Cooke et al., 1990; Starling et al., 1990; Prowse & Greider, 1995), mouse cells lacking telomerase would require many more doublings to exhaust telomeric sequences compare to human cells (de Lange & Jacks, 1999).

Telomeres are shorter in all somatic tissues from older than from younger individuals (Hastie et al., 1990; Lindsey et al., 1991) and in somatic tissues than in germline cells (Cooke & Smith, 1986; Allshire et al., 1988). Telomeres in normal cells from young individuals progressively shorten when grown in cell culture (Harley et al., 1990), and children born with progeria have shortened telomeres compared to age-matched controls (Allsopp et al., 1992). Finally, experimental elongation of telomeres extends the proliferative capacity of cultured cells (Wright et al., 1996b; Bodnar et al., 1998).

It has been proposed that entry into senescence is initiated when a critically shortened telomere length is reached (Harley, 1991; Allsopp et al., 1995) perhaps by the induction of a DNA damage checkpoint pathway. Thus mounting evidence suggests that the sequenctial shortening of telomere DNA may be an important intrinsic molecular timing mechanism, or replicometer. Telomeres may both protect chromosome termini from degradation and recombination (Blackburn, 1991; Zakian, 1989) and promote correct mitotic segregation of sister chromatids (Kirk et al., 1997), as well as provide a buffer of expendable non-coding DNA to accommodate the end-replication problem.

Figure 1. 1 The Telomeres

Fluorescence in situ hybridisation of human telomeres in a metaphase chromosome spread from human cells. The metaphase chromosomes were hybridised with a FITC-labeled PNA $(TTAGGG)_3$ probe. The chromosomes were counterstained with propidium iodide. Telomeric TTAGGG repeats show as yellow dots at ends of the human chromosomes. The 92 telomeres in cells are considered essential for maintaining chromosome structure and function. They are composed of the hexameric nucleotide repetitive sequence TTAGGG (thymidine, thymidine, adenosine, guanosine, guanosine, and guanosine). Telomeres shorten each time a cell divides and may be the timing mechanism that limits the growth of normal cells.

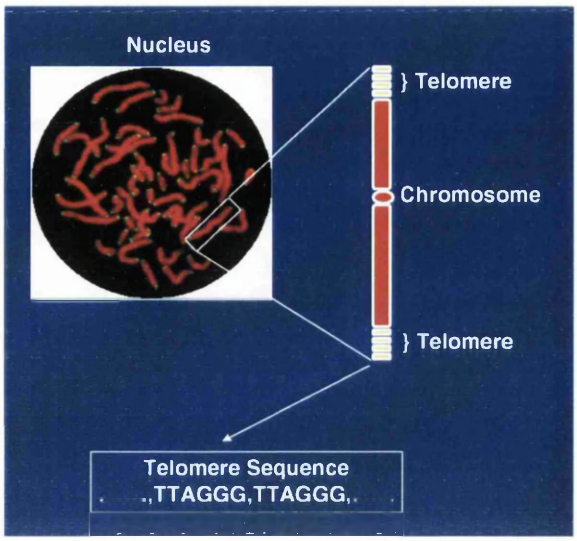


Table 1. 1 A brief history of telomerase in relation to cell senescence and cancer

Date	Events
1961	L. Hayflick describes cellular aging or senescence
1971	Soviet scientist, A.M. Olovnikov proposes loss of telomeres limits cell division
1985	C.W. Greider & E.H. Blackburn identify telomerase activity in the ciliate protozoa, *Tetrahymena*.
1986	H. Cook at the MRC in Edinburgh showed telomere shortening in somatic tissues relative to germline cells
1987	Greider & Blackburn demonstrated a mechanism of DNA replication-telomere extension by reverse transcription
1989	G.B Morin identified human telomerase activity in HeLa cell lines
1990	N. Hastie et al. At the MRC in Edinburgh show telomere reduction in human colorectal cancer
1990	C.B. Harley, A.B. Futcher and C.W. Greider, propose telomerase necessary for immortalisation
1992	Geron Corporation formed
1994	Kim et al. Publish the TRAP assay for the detection of telomerase activity
1995	Telomerase RNA gene, h*TERC*, cloned by Feng et al. (Science, 277: 1236)
1995	First demonstration that inhibition of telomerase can kill a cancer cell (Science, 277: 1236)
1997	Telomerase knockout mouse (Cell, 91: 25)
1997	Telomerase protein component gene cloned (Science, 277: 955, Cell 90: 785)
1997	Keith et al. at the CRC Dept of Medical Oncology, Glasgow, identify amplification of the telomerase RNA gene in human cancers (Oncogene, 14: 1013)
1998	Extension of proliferative life span of normal cells by forced expression of telomerase (Science 279: 349)
1998	Keith et al. at the CRC Dept of Medical Oncology, Glasgow, identify the promoter sequences for the telomerase RNA gene (Oncogene, 16: 1345)
1999	Identification of promoter sequences for the telomerase protein component gene
1999	Most recent examples of inhibition of telomerase leading to cell death (Nature Medicine 5:1164)

Figure 1.2 Model for telomerase addition of repeats at chromosome ends

The two components of human telomerase h*TERC* and hTERT are indicated. The putative template domain of the RNA component of human telomerase is shown aligned against an arbitrary 3' end of a human telomere. This primer template configuration allows extension of the telomere in the first round of elongation until the extended product reaches the 5' end of template domain. Translocation then moves the extended DNA back one repeat relative to the template domain, positioning it for another round of elongation in which a full repeat (ggttag) is added to the 3' end of the chromosome (adapted from Greider & Blackburn 1985). DNA replication doesn't reach the end of the chromosome on one of the strands of DNA when a human cell divides, One strand is left with a gap at its end. That end gets shorter after each division until the cell can on longer divide. Adding the enzyme telomerase lengthens the strand opposite the gap. Another enzyme then fills in the gap, keeping the strand at youthful length and allowing the cell to keep dividing.

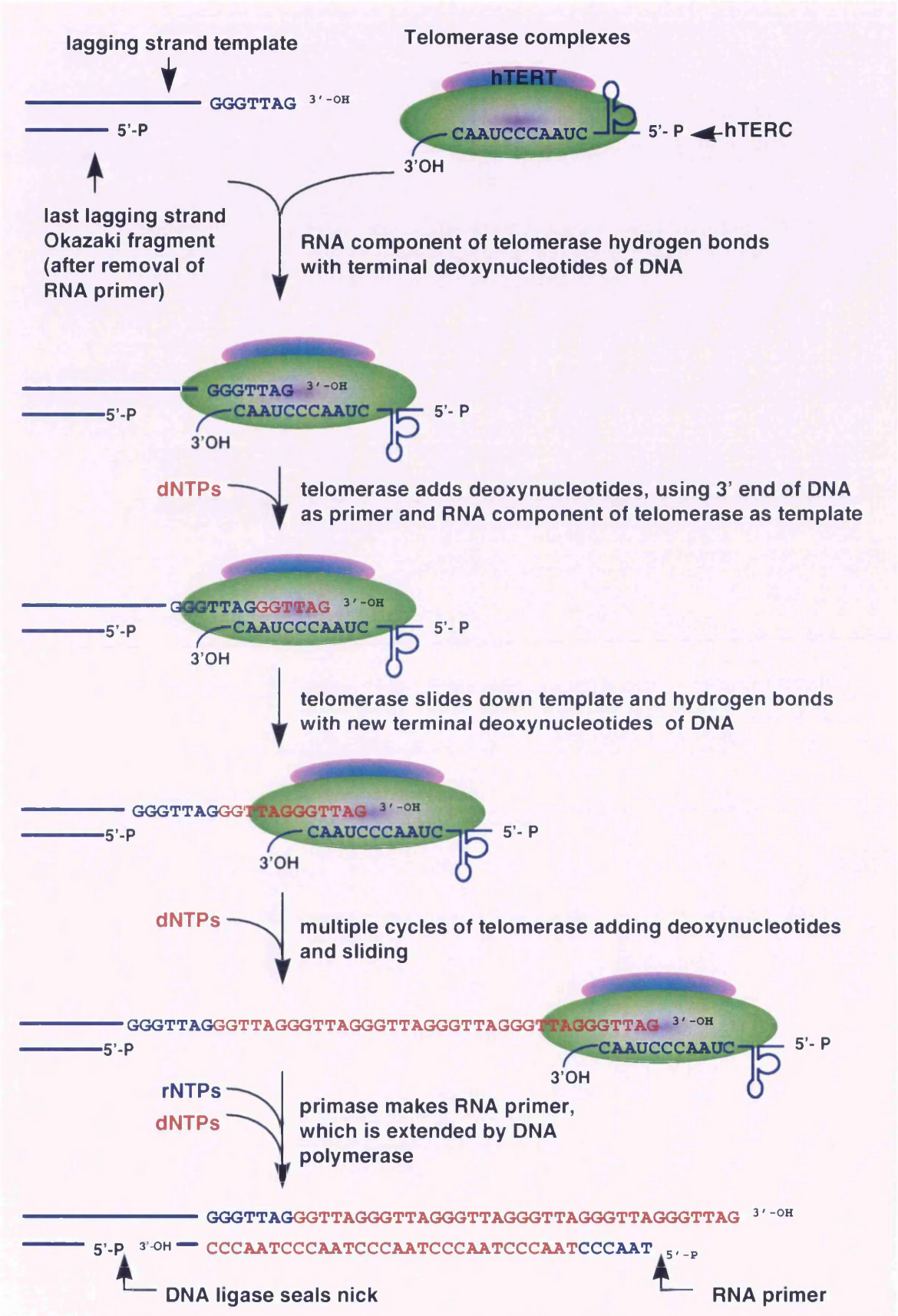

lagging strand template
Telomerase complexes
hTERT
GGGTTAG 3'-OH
5'-P
CAAUCCCAAUC
5'- P
hTERC
3'OH
last lagging strand Okazaki fragment (after removal of RNA primer)
RNA component of telomerase hydrogen bonds with terminal deoxynucleotides of DNA
dNTPs
telomerase adds deoxynucleotides, using 3' end of DNA as primer and RNA component of telomerase as template
GGGTTAGGGTTAG 3'-OH
telomerase slides down template and hydrogen bonds with new terminal deoxynucleotides of DNA
GGGTTAGGGTTAGGGTTAG 3'-OH
multiple cycles of telomerase adding deoxynucleotides and sliding
GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG 3'-OH
rNTPs
primase makes RNA primer, which is extended by DNA polymerase
CCCAATCCCAATCCCAATCCCAATCCCAATCCCAAT 5'-P
DNA ligase seals nick
RNA primer

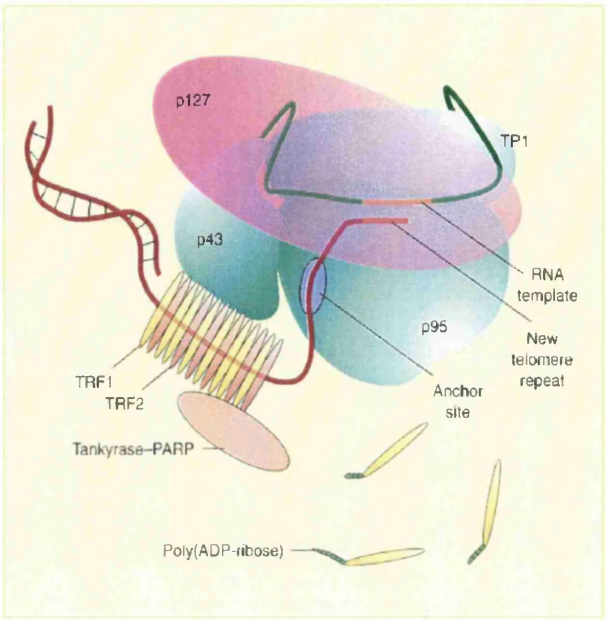
Figure 1. 2

The proliferative capacity of normal cells is thought to be controlled by an endogenous generation clock (Ouellette &Smith, 1999; Allshire et al., 1988; Allsopp et al., 1995; Greider, 1990; Harley et al., 1990; Hastie et al., 1990; DeLange et al., 1990). The shortening of telomeres appears to be the cellular clock that determines the number of cell divisions, and this in turn, appears to control the ageing of the cells. Extension of replicative lifespan and perhaps immortalization of human cells will have many important applications for the treatment of age-related diseases. Significantly, the expression of telomerase in normal mortal cells extends their life-span without transforming them into malignant cancer cells, demonstrating that telomerase makes tumour cells immortal, but that other genetic alterations are responsible for the malignant characteristics of cancer cells. These results suggest that telomerase is both an important target for cancer and for the treatment of age-related disease.

1.3 Telomerase

Telomerase is a ribonucleoprotein complex that contains several protein subunits, as well as an RNA component (Figure 1.3). The RNA component (h*TERC*, in human) contains a domain that is complementary to one hexameric unit of the DNA telomeric repeat sequence, TTAGGG. The protein moiety contains the recently cloned catalytic subunit (hTERT, in human) that is homologous to reverse transcriptase. Thus, this enzyme complex comprises both template and polymerase activity. Telomerase binds to the 3' ends of DNA strands and extends them by copying its own RNA template in multiples of the hexamer repeat sequence and thus stabilising telomeres (Greider & Blackburn, 1985; Blackburn, 1992), telomerase enzyme function results from a collaboration of both protein and RNA functional groups contributed by TERT and TERC (Blackburn, 2000).

Figure 1.3 A speculative model of human telomerase complexes



A speculative model of human telomerase ribonucleoprotein complex is shown. The p127 catalytic subunit (human telomerase reverse transcriptase, hTERT) is drawn overlaying the RNA-binding subunit, the telomerase associated protein (TP1, or TEP1, shown in green), and the substrate DNA-binding subunit, p95, with its anchor site. Also shown are the small p43 subunit, which has no known function to date, and the telomere repeat-binding factors 1 and 2 (TRF1 and TRF2), which are involved in regulating telomerase activity. Tankyrase, a poly adenosine diphosphate ribose polymerase, (PARP), adds poly (ADP) ribosyl complexes to TRF1, which can then dissociate from the telomere. The human counterparts of the ciliate p95 and p43 subunits have yet to be cloned. The catalytic subunit is shown in the process of copying the RNA template (yellow) using its reverse transcriptase function (from McKenzie et al., 1999).

*1.3.1 Telomerase RNA component (*TERC*)*

The telomerase RNA component, TERC, was first identified in *Tetrahymena* (Greider & Blackburn, 1987) and subsequently cloned from a number of other organisms. The full-length RNA can serve as template for target-primed reverse transcription. Because of this specialisation, the recent studies in the *T. thermophila* telomerase suggested that telomerase RNA gene must have a minimum of three functional motifs: (1), the template, (2), the TERT binding site, and (3), additional sequences required to form a catalytically competent polymerase active site.

The template region itself is an unlikely candidate for a high-affinity protein binding site because it is accessible to chemical modification in the ribonucleoprotein (RNP) (Zaug & Cech, 1995) and can be altered without preventing RNP assembly *in vitro* and *in vivo* (Collins et al., 1999). Expressing mutant telomerase RNA genes *in vivo* have suggested that non-template regions of telomerase RNA are important for telomerase activity. Thus, the RNA sequences required for activity independent of templating and TERT binding could serve a variety of roles, including involvement in substrate binding, active site conformation, or catalysis (Licht & Colline, 1999).

1.3.1.1 h*TERC transcribed region*

Telomerase RNAs have been identified from 24 ciliate species (148 to 209 nucleotides [nt] in length), two yeasts (~1,300 nt), and 35 vertebrate species [382 to 559 nt including mouse (397 nt), bovine (~443 nt), and human (451 nt)] (Singer and Gottschling, 1994 ; Blasco et al., 1995 ; Feng et al., 1995 ; McEachern and Blackburn, 1995 ; Tsao et al., 1998 ; Chen et al., 2000; Collins, 1999). The human telomerase RNA gene, h*TERC,* has been well studied by several groups from different angles. The 5' and 3' boundaries of the transcribed region of h*TERC* have been determined using *in vitro* assembly systems derived from rabbit reticulocyte lysates and human cell extracts (Tesmer et al., 1999). The region spanning nucleotides +33 to +325 of h*TERC* is the minimal sequence required to produce levels of telomerase activity that are comparable with that made with full-length h*TERC* (Figure 1.4). In addition, two regions of h*TERC* (nucleotides +33 to +147 and +164 to +325) have been shown to be unable to produce telomerase activity when combined separately with hTERT but can function together to assemble active telomerase (Tesmer et al., 1999). Sequences upstream of the template of h*TERC* are not essential for producing active telomerase *in vitro*, although they do contribute to the overall level of activity. Consistently, Autexier et al. (1996) have shown that the residues 1-44, 5' to the template region (residues 46-56) are not essential for telomerase activity. However, removal of the first 43 nt of h*TERC*, but not the first 32 nt, reduces the level of active telomerase, suggesting that nt +33 to +43 may aid in assembly or in catalysis,

perhaps by anchoring the template region of h*TERC* on hTERT and facilitating translocation during processive elongation. The significance of sequence in this region of h*TERC* is unknown. Telomerase activity was abolished when the template region of h*TERC* (nt +46 to +56) was deleted. All together, the h*TERC* functional region for reconstitution of active telomerase can be defined as +44 to +325 nt (Figure 1.4) with a requirement for a functional template region (Tesmer et al., 1999).

Telomerase RNA templates from several organisms have been mutated and studied, including those from *Tetrahymena. thermophila* (Autexier and Greider, 1994; Gilley et al., 1995; Gilley and Blackburn, 1996), *Kluyveromyces lactis* (McEachern and Blackburn, 1995), human (Marusic et al., 1997; Tesmer et al., 1999) and *Saccharomyces cerevisiae* (Prescott and Blackburn, 1997). Biochemical studies of such mutated telomerase (assembled *in vivo* and extracted from cells) have shown that base changes in the template RNA are usually copied into DNA products. Template mutations can also cause specific changes in enzymatic properties *in vitro*. Expression of mutant h*TERC* in immortal human cells resulted in the appearance of mutant telomerase activity and in the synthesis of mutant telomeres. These results suggest that the presence of mutant telomerase, even if coexpressed with the wild-type enzyme, can be deleterious to cells, possibly as a result of the impaired function of hybrid telomeres (Marusaic et al., 1997).

1.3.1.2 Conserved structure

These TERC RNAs vary remarkably in their size and sequence, with comparison of sequences from vertebrate, yeast, and ciliates revealing no obvious similarity among these three distantly related groups. However, a conserved secondary structure has been established using phylogenetic comparative analysis (Figure 1.5) (Romero and Blackburn, 1991 ; ten Dam et al., 1991 ; Lingner et al., 1994 ; McCormick-Graham and Romero, 1995).

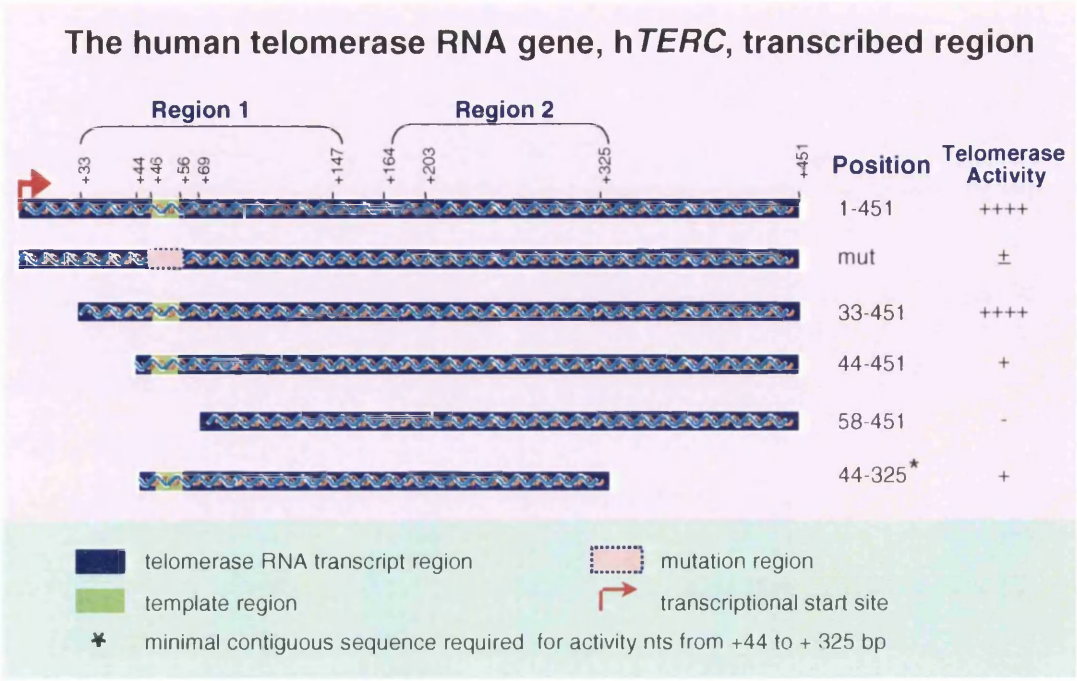
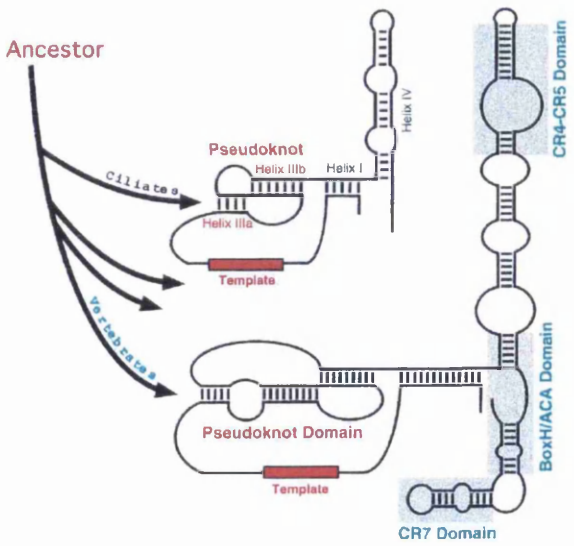


Figure 1.4 Schematic model for the h*TERC* transcribed region

The 451 nucleotides (nt) h*TERC* sequence is represented schematically with respect to the template region (nt +46 to +56). Truncations are denoted by the transcribed nucleotides within h*TERC*. The sizes of the transcribed RNAs are also indicated. The two fragments of h*TERC* (nt +33 to +147 and +164 to +325) that cannot produce telomerase activity when combined separately with hTERT but can function together to assemble active telomerase are indicated as Region 1 and Region 2. Relative activity was determined by defining the level of activity with full-length h*TERC* as 100% (++++). (Adapted from Tesmer et al., 1999 and Autexier et al., 1996)

Figure 1.5 Consensus structure of ciliate and vertebrate telomerase RNA

The outline of the minimum-consensus structures of ciliate and vertebrate telomerase RNAs are shown. Template regions are indicated with red filled boxes. Vertebrate-specific structural elements are shaded. The diagram on the left illustrates a possible evolutionary course from the ancestral telomerase RNA to ciliate and vertebrate RNAs (Adapted from Chen et al., 2000).



A study of 35 TERC genes has proposed a secondary structure model for vertebrate telomerase RNA based on phylogenetic comparative analysis (Chen et al., 2000). The most remarkable feature of this structure is the evolutionary conservation of four structural domains: the pseudoknot domain, the CR4-CR5 domain, the Box H/ACA domain and the CR7 domain (Figure 1.5). Each conserved structural domain in the consensus structure might play a distinctive role in either the function, stability, processing, or localisation of telomerase RNA. These regions are also good candidates as binding sites for TERT or other telomerase accessory proteins. This proposed structure of vertebrate telomerase RNA displays striking similarities in overall architecture to the structure of ciliate RNAs (Chen et al., 2000). Experiments with h*TERC* have also shown that the pseudoknot structure is required for human telomerase function *in vivo*. A mutant human telomerase RNA with a 17-base insertion at position 176, which changes the natural structure, failed to reconstitute telomerase activity (Feng et al., 1995 ; Autexier et al., 1996 ; Weinrich et al., 1997). Secondary structure predictions reveal a putative double-stranded element adjacent to the template of telomerase RNA in several other species, including *S. cerevisiae* and human (Tzfati et al., 2000). A study of yeast TERC has demonstrated that the phylogenetically conserved, long-range base-pairing interaction adjacent to the template specifies one boundary of the telomerase template, thus determining the end of the telomeric repeat synthesised. Thus, it would appear that the RNA structure, rather than its sequence, specifies the template boundary, and entire structure is important for active telomerase function (Tzfati et al., 2000).

1.3.1.3 Transcription of TERC

The 5' ends of the human and mouse telomerase RNA transcripts were previously mapped by both RNase-protection and primer-extension analyses (Hinkley et al., 1998). The 5' end of human RNA is 45 nt upstream of the template sequence, the mouse 5' end is only 2 nt upstream from the template sequence. Biochemical evidence suggests that both the human and mouse telomerase RNA genes are transcribed by RNA polymerase II (Feng et al., 1995; Zhao et al., 1998; Hinkley et al., 1998; Chen et al., 2000). The

upstream region of each vertebrate telomerase RNA gene contains sequence elements typical of RNA polymerase II promoter (Chen et al., 2000).

A comparison of the proximal pomoter regions of several ciliate telomerase RNA genes detected two highly conserved sequence elements, a proximal sequence element (PSE) at -55, and an A/T-rich element at -25 position, which constitute the promoter of the *T. Thermophila* TER1 gene. The metazoan U6 small nuclear RNA which is transcribed by RNA polymerase III appears to have a similar promoter architecture to the ciliate telomerase RNA gene suggesting that ciliate telomerase RNA gene are likely to regulated by RNA polymerase III (Hargrove et al., 1999). In contrast to the ciliate TER1 and vertebrate snRNA gene promoters, the 5' regions of the human and mouse telomerase RNA genes lack a PSE. The putative difference in transcription may reflect an increased complexity of regulation of telomerase in vertebrate species compared to that in the ciliates, however, the transcriptional regulatory mechanisms of the telomerase gene are not well defined to the date.

1.3.2 Telomerase Reverse Transcriptase (TERT)

1.3.2.1 hTERT protein

The human telomerase reverse transcriptase (hTERT) previously referred to as hTRT (Nakamura et al., 1997), hEST2 (Meyerson et al., 1997), and hTCS1 (Kilian et al., 1997), is the catalytic protein subunit that is responsible for the enzymatic activity of telomerase (Harrington et al., 1997b; Feng et al., 1995; Nakamura et al., 1997; Nakayama et al., 1997; Meyerson et al., 1997). The hTERT gene encompasses more than 37 kb and is subdivided into 16 exons and 15 introns varying fundamentally in size. The hTERT gene encodes a 1,132-amino acid polypeptide with a molecular weight 127 kD (Meyerson et al., 1997; GenBank AF015950). Like other reverse transcriptase family members, hTERT contains seven highly conserved reverse transcriptase (RT) motifs (Wick et al., 1999; Lingner et al. 1997). These RT motifs, as well as the telomerase-specific T motifs, are encoded either alone or in pairs on distinct exons. In contrast, the less conserved N-

terminal and C-terminal regions of hTERT are encoded by two exons or one large exon, respectively. However, the specific exon-RT motif-assignment differs fundamentally between the human and yeast *S. pombe* TERT genes (Nakamura et al., 1997), suggesting that TERT genes of evolutionary distant organisms have a highly conserved function but show a phylogenetic diversity in their genomic structure (Wick et al., 1999). Recent analysis of all of the cloned TERTs revealed several salient features of their structural organisation: (i) all of the RT motifs are located in the C-terminal half of the protein; (ii) a telomerase-specific motif (T motif), located just N terminal to the RT motifs, can be discerned in all TERTs; and (iii) a motif positioned further toward the N terminus appears to be much more highly conserved among all the ciliate telomerases and may perform a function specific to ciliate telomere formation (Bryan et al., 1998a; Xia et al., 2000). hTERT has been demonstrated to be an important factor for telomerase activity both biologically and enzymatically (Hahn et al., 1999; Nakayama et al., 1998; Counter et al., 1998a, b). This issue will be discussed further in section 1.5.2.

1.3.2.2 hTERT promoter

Genomic sequences that encompassed the complete hTERT transcription unit have been cloned by different groups to investigate the regulation mechanisms of hTERT gene expression. The essential promoter region controlling hTERT expression has been identified (Kyo et al., 2000; Takakura et al., 1999; Cong et al., 1999; Horikawa et al., 1999; Oh et al., 1999; Wick et al., 1999; Devereux et al., 1999; Fujimoto et al., 2000). Transient transfections of immortal human cells with potential regulatory 5'-sequences linked to a reporter, combined with deletion analysis of these sequences, indicated that elements responsible for promoter activity are contained within a region extending from 330 bp upstream of the ATG. Assays in different cell types showed that the hTERT promoter is inactive in normal and in transformed pre-immortal cells, but, like telomerase, it is activated with cell immortalization.

The hTERT promoter is GC-rich, lacks TATA and CCAAT boxes, and contains at least five Sp1-consensus sites and two c-Myc consensus binding sites in the core promoter region (Figure 1.6) (Kyo et, 2000; Wick et al., 1999; Cong et al; 1999). Additional luciferase assays using a series of constructs containing unidirectionally deleted fragments revealed that a 59-bp region (-208 to -150) is required for the maximal promoter activity (Horikawa et al., 1999). Multiple binding motifs for myeloid-specific zinc finger protein 2 (MZF-2) are present within a 400 bp region of the hTERT promoter between -776 and -378 bp upstream of the proximal core promoter (Fujimoto et al., 2000). The Wilms' Tumour 1 (WT1) regulatory sequence, GCGCGGGCG, is located between -307 and -423 bp (Oh et al., 1999). A palindromic estrogen receptor element (ERE) and an Sp1/ER element, targeted by estrogen, are present at -2677 and -873 bp respectively (Kyo et al., 1999). All these elements, and possibly others, combine to regulate hTERT gene expression. A large CpG island with a GC content 70% was identified in the 5'-flanking hTERT gene sequence spanning from -900 bp into exon 2. One additional smaller CpG island with a GC content >60% is located further upstream at position -4300 to -4600 bp (Wick et al, 1999). Because of this, methylation status of the hTERT promoter has been investigated to determine if this plays a part in gene regulation, however no correlation with expression levels was found (Devereux et al., 1999; Dessain et al., 2000). It would therefore appear that hTERT expression may be subject to multiple levels of control and may be regulated by different factors in different cellular contexts. Specific transcription factors involved in the regulation of hTERT promoter will be further discussed in section 1.5.2.

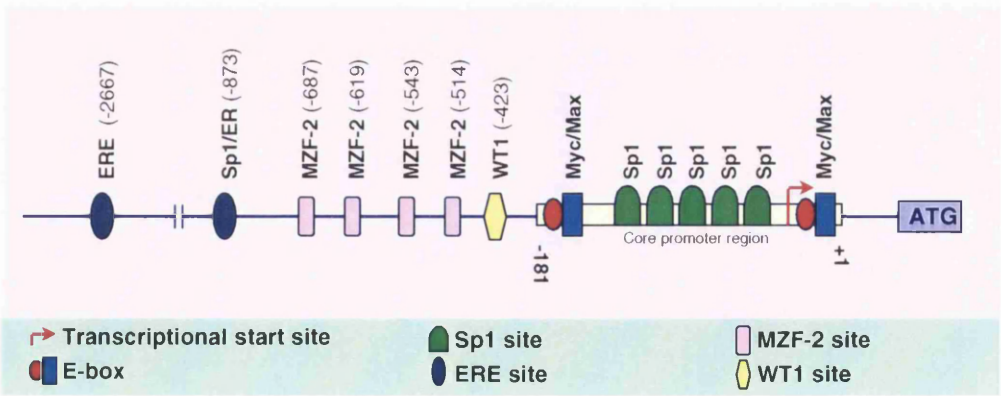


Figure 1.6 Schematic diagram of hTERT promoter region

The identified transcriptional elements between -3.3 kb and +1 bp in the hTERT promoter region are shown. Binding sites for c-Myc and Sp1 in the core promoter of hTERT (-181 bp; yellow box) are shown. The transcriptional start site (TSS) upstream of the ATG initiation codon is marked as "+1". The numbers indicate the position of DNA-binding sites relative to the TSS.

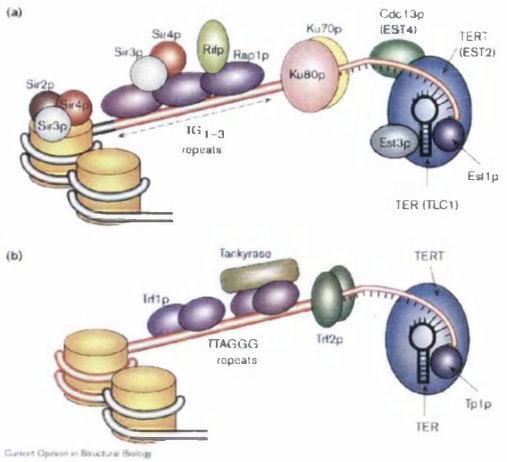


Figure 1.7 Telomerase-Telomeric complexes

Schematic representations of the composition of telomeric complexes of (a) the yeast *S. cerevisiae* telomere and (b) the human telomere (from O'Reilly et al., 1999). Telomeric and nontelomeric DNA are represented as red and grey tubes, respectively. Histone octamers are depicted as orange cylinders. Other components of the telomere complex are labelled.

Table 1. 2 Proteins in the telomeric complexes

Genes	Mammalian Homologue	Function	Effect telomere length
Est2	TERT	Telomerase enzyme component	+
TLC1	TERC	Telomerase enzyme component	+
Cdc13p		Recruitment of telomerase to the chromosome end	+
Est1p			+
Cdc13p	TRF2?	Protect telomere ends from degradation	+
Stn1p			+
Hdf1p			+
Hdf2p			+
Rad50p	Rad51	Telomerase independent mechanisms of telomere maintenance	+
Rag51p			+
Rad52			+
Mre11P	Mre11p		+
Xrs2p		ALT in mammals?	+
Rap1p	TRF1	Inhibit telomerase access to the chromosome end	-
Rif1	TIN2?		-
Rif2p			-
Tel1p	ATM	Positively regulate telomerase	+
Tel2p			+
Pif1p	DNA polymerase	Affect telomerase indirectly	-
Cdc17p	G4-DNA-dependent nuclease		+
Kem1p			+
Cdc44p	DNA replication factor C		-
Dna2p			+
Rad27p			+

1.3.3 Proteins in the Telomeric Complexes

During the past few years, much of the emphasis in the study of telomeres has been on telomeric DNA, but it is becoming clear that proteins are major players and will be a key to understanding what actually goes on at the chromosome end. Human/vertebrate telomeres consist of many kilobases of (TTAGGG)n together with various associated proteins (Kipling, 1995). Studies of telomerase-positive immortalised cells (Bryan et al., 1998b; Jones et al., 1998) and of telomerase-negative normal cells (Martens et al., 1998) reveal evidence for the existence of factors other than telomerase that regulate telomere length.

The list of known proteins with effects on the structure and function of telomeres continues to grow (Figure 1.7, Table 1.2). In conjunction with the RNA template these proteins act as a committee to maintain the telomere. One group includes protein components of telomerase (TERT/Est2), proteins essential for the *in vivo* activity of telomerase, and proteins isolated by virture of physical association with the enzyme. The other group includes proteins that constitute the telomeric complex (e.g., Rap1, TP1, TRF1 and TRF2, some of which will be discussed below). This group contains proteins that affect the sizes of the telomere but do not necessarily appear to be essential for telomerase activity (this group may include the proteins preventing chromosome end-to-end-fusion, for which the DNA repeats may act primarily as anchor points). These two groups may overlap since several of the telomeric proteins are thought to affect the activity of telomerase.

To the date, over 25 genes affect telomere length in addition to Est2(TERT) and TLC1 (telomerase RNA component in yeast, Figure 1.7a). Some of these gene products may help recruit telomerase to the end of the chromosome (e.g., Cdc13p and Est1p) (Nugent et al., 1996; Virta-Pearlman et al., 1996) or protect the ends from degradation (e.g., Cdc13p, Stn1p, Hdf1p, and Hdf2p) (Garvik et al., 1995; Boulton & Jackson, 1996; Porter et al., 1996; Grandin et al., 1997; Gravel et al., 1998; Laroche et al., 1998; Nugent et al., 1998; Polotnianka et al., 1998). Some may be important in alternative pathways of telomere length maintenance that use DNA recombination (Rad52p, Rad50p, Rad51p,

Mre11p, and Xrs2p) (Lundblad & Blackburn, 1993; Lendvay et al., 1996; Le et al., 1999), some have been suggested to negatively (e.g., Rap1p, Rif2p, and Rif1p) (Hardy et al., 1992; Kyrion et al., 1992; Marcand et al., 1997b; Wotton and Shore, 1997) or positively regulate telomerase activity (e.g., Tel1p and Tel2p) (Lustig & Petes, 1986; Greenwell et al., 1995; Morrow et al., 1995; Runge & Zakian, 1996), while others may affect telomerase activity indirectly (Cdc17p, Pif1p, Kem1p, Cdc44p, Dna2p, and Rad27p) (Carson & Hartwell, 1985; Schulz & Zakian, 1994; Liu et al., 1995; Adams & Holm, 1996; Formosa & Nittis, 1999; Parenteau & Wellinger, 1999). The mechanisms by which most of these gene products actually regulate telomere length are unclear. There is a rapidly increasing list of proteins reported to bind to the telomere and/or affect telomere length (Table 1.2), a few of which are discussed here, hTERT having been discussed previously (section 1.3.2.1).

1.3.3.1 Telomerase-associated proteins

In *T. Thermophila* two proteins, p80 and p95 have been identified which interact with telomerase RNA and TERT, and p95 has also been shown to bind single-stranded substrate DNAs (Collins et al., 1995; Collins & Gandhi, 1998; Gandhi & Collins, 1998). The mammalian TP1 protein has sequence homology to p80 and also interacts with telomerase RNA and TERT (Licht & Colline, 1999; Harrington et al., 1997a, b; Nakayama et al., 1997). The mouse/rat TP1 (2629 aa) and human TP1 (2627 aa) proteins (~230-240 kDa) lack any RNA-dependent DNA polymerase domain indicating that TP1 is not the catalytic subunit of telomerase but it may be associated with a catalytic subunit in a multiple component telomerase complex.

1.3.3.2 Telomere repeat-binding proteins

TRF1 was the first mammalian telomere binding protein to be identified (Zhong et al., 1992). Homodimers of TRF1 bind double-stranded telomeric repeats but not single-

stranded DNA, through a Myb-type DNA-binding domain (reviewed in Griffith & de Lange, 1998; Smith & de Lange, 1997). TRF1 belongs to a large family of proteins conserved during evolution which contain one or two related myb repeats, such as for example the Rap1p yeast protein (Brun et al., 1997). Rap1p controls telomere length, telomeric silencing and telomere stability in yeast. Recent studies have demonstrated that Rap1p, in concert with Rifs (Rap1 interacting factors), serves as a sensor of the telomere length, leading either to onset or to repression of the telomerase activity.

TRF1 is located at the telomeric end (Hanish et al., 1994) and is involved in the regulation of telomere length. It behaves as a negative regulator of telomere maintenance since TRF1 deletion results in a faster telomere elongation (van Steense & de Lange, 1997). Over-expression of TRF1 in the tetracycline-responsive human fibrosarcoma cell line HTC75 results in a gradual decline in telomere length at a rate of ~10 bp/population double level (PDL). Conversely, the expression of a dominant negative allele of TRF1, which removes endogenous TRF1 from telomere, leads to telomere elongation. In this system, TRF1 did not affect the activity of telomerase detectable in cell extracts, suggesting that TRF1 does not affect telomerase activity globally in the cell. Instead, TRF1 acts in *cis* as a negative length regulator at each individual telomere (Hanish et al., 1994; van Steense & de Lange, 1997).

During the search for proteins interacting with TRF1, tankyrase that is a new protein localised to telomere, has been identified (Smith et al., 1998). Tankyrase presents ankyrin repeats (involved in the interaction with TRF1) and a catalytically active region homologous to poly(ADP)-ribose polymerase (PARP). Tankyrase is catalytically active towards itself and TRF1 and modifies TRF1 such that it is unable to bind DNA. This suggests that negative regulation of telomerase by TRF1 might occur through a poly(ADP)-ribosylation reaction, performed by this telomere-specialised enzyme.

A second telomere binding protein, TRF2, is a distantly related homologue of TRF1 which also binds to double-stranded telomere repeats and is ubiquitously expressed (Broccoli et al., 1997a, b). TRF2 has the same general protein architecture as TRF1 except that its

amino terminus carries mostly basic residues instead of the acidic residues seen in TRF1. Overexpression of dominant negative alleles of TRF2 in human cells caused loss of the terminal single-stranded 3' overhang, an increase in chromosome end-to-end fusion, and irreversible growth arrest in a senescence-like state (van Steesel et al., 1998). It was also reported that cells forced to express a dominant negative allele of TRF2 rapidly initiate an apoptotic pathway (Karlseder et al., 1999). It is not known whether TRF2 serves to protect the G-rich overhang directly (which would seem unlikely, as TRF2 cannot bind single-stranded DNA *in vitro*) whether it stimulates the formation of G tails by recruiting an exonuclease or other factor, or whether TRF2 recruits other telomere binding proteins that protect G tails.

In human cells, both TRF1 and TRF2 are predominantly located at chromosome ends where they contribute to the protection and maintenance of telomeric DNA. Based on biochemical studies, the formation of t-loops, which engage the 3' single-stranded DNA overhang in an interaction with double-stranded telomeric repeats, was proposed to involve both TRF1 and TRF2. TRF1 has the ability to induce bending, looping, and pairing of duplex telomeric DNA (Bianchi et al., 1997, 1999; Griffith et al., 1998, 1999), activities that could facilitate the folding back of the telomere. TRF2 was found to induce the invasion of the 3' single-stranded TTAGGG repeat tail into duplex telomeric DNA, forming t-loops *in vitro* (Griffith et al., 1999; Smogorzewska et al., 2000). Thus, a t-loop-based mechanism for telomere length regulation would predict that both TRF1 and TRF2 are required for the length homeostasis of human telomeres (Smogorzewska et al., 2000). The complete functions of TRF1 and TRF2 proteins in regulating telomerase and telomeres require further studies.

1.3.3.3 Mapping h*TERC and hTERT genes*

Both h*TERC* and hTERT are required for telomerase activity and expression of both is upregulated in cancer cells (Meyerson et al., 1997; Yi et al., 1999; Soder et al., 1998). h*TERC* and hTERT genes have been mapped in our own and other Labs to 3q26 and

5p15 respectively (Bryce et al., 2000; Soder et al., 1997a; Heselmeyer et al., 1997; Bjorkqvist et al., 1998). Interestingly, some tumour types show increases in copy number of h*TERC* and hTERT. 5p15, and more specifically 5p15.3, has been found at increased numbers in such tumours as uterine cervix cancer (Heselmeyer et al., 1997), non-small cell lung carcinoma (Bjorkqvist et al., 1998), squamous cell carcinoma of the head and neck (Bockmuhl et al., 1996; Liehr et al., 1998; Wolff et al., 1998; Pack et al., 1999). It seems increasing the copy number of hTERT was common, with 5p isochromosoimes seen in 2 cell lines (HeLa and SiHa). No Copy number alterations were observed in the WI38 normal mortal cell line or in normal lymphocytes. Increasing the copy number of hTERT in cancer cells may titrate out repressors, or compensate for low levels of transcriptional activators, thus allowing a tumour to increase the levels of the catalytic subunit of telomerase (Bryce et al., 2000). Over-representation of 3q26 has been demonstrated for uterine cervix cancer, squamous cell carcinoma of the head and neck, small cell and non-small cell lung carcinoma (Heselmeyer et al., 1997; Bjorkqvist et al., 1998; Wolff et al., 1998; Pack et al., 1999; Ried et al., 1994). It has been demonstrated that h*TERC* was involved in this region of amplification (Soder et al., 1997a), and recent study has narrowed down the region of lung and cervix tumours to 1 to 2Mb centred around h*TERC,* further supporting it as a potential target (Sugita et al., 2000). These gains in both 3q26 and 5p15 are potentially interesting given the presence of the h*TERC* and hTERT genes.

1.4 Patterns of Telomerase Expression

In recent years, telomerase has attracted much attention because of its potential utility as both a diagnostic marker and a therapeutic target in cancer. This enzyme is present in almost all cancer, but not in normal somatic cells. Understanding telomerase structure, expression, and activity regulation is pivotal for the prospects of using it as a target in cancer therapy, with respect to both therapeutic and undesirable side effects.

1.4.1 Telomerase in somatic cells and germline cells

All normal human somatic tissues and cells show progressive telomere shortening. In contrast, germline and certain stem cell populations must have a mechanism to compensate for the continuous telomere erosion as they manage to divide indefinitely without telomere shortening. This difference has been shown to be due to the absence or presence of telomerase.

During normal embryogenesis, the germline (Wright et al., 1996a; Mantell & Greider, 1994) and somatic cells express telomerase activity (Wright et al., 1996b), which persists in the male germline, fully maintaining telomere length. However, repression of telomerase occurs in most somatic tissues during development, with the exception of a small number of cells in renewal tissues (Hiyama et al., 1996; Taylor et al., 1996; Harle-Bachor et al., 1996; Ramirez et al., 1997; Hiyama et al., 1995; Iwama et al., 1998; Kyo et al., 1997; Saito et al., 1997; Brien et al., 1997). Thus, low levels of telomerase are detected in activated lymphocytes (Counter et al., 1995; Hiyama et al., 1995; Iwama et al., 1998), intestinal crypt cells (Hiyama et al., 1996), premenopausal endometrium (but only in the proliferative phase) (Kyo et al., 1997; Saito et al., 1997; Brien et al., 1997), and the basal layer of the skin (Taylor et al., 1996; Harle-Bachor et al., 1996; Ramirez et al., 1997). Although telomerase is "on" in the proliferating cells of these renewal tissues, its level is insufficient to fully maintain telomere length and the telomeres of these tissues progressively shorten (Hastie et et, 1990; Lindsey et al., 1991; Hiyama et al., 1995; Iwama et al., 1998). Proliferating normal cells without detectable telomerase have the greatest rate of telomere attrition and eventually undergo senescence or proliferative growth arrest. The stem cells of renewal tissues maintain a low or intermittent level of telomerase that may provide the increased proliferative capacity required for these tissues, which have a high rate of turnover.

Telomerase RNA and hTERT are necessary and sufficient for the production of human telomerase, as demonstrated by the recent *in vitro* reconstitution of telomerase using only these two components (Weinrich et al., 1997). Transient telomerase expression is sufficient to elongate critically short telomeres, resulting in an increase of the life span of

normal human fibroblasts (Steinert et al., 2000). Transfection of only the hTERT into normal human cells without detectable telomerase results in restoration of telomerase activity. Normal human cells with stable expression of introduced telomerase demonstrate extension of life span, providing direct evidence that telomere shortening controls cellular aging (Bodnar et al., 1998; Vaziri et al., 1998). In the presence of limited expression of hTERT, long telomeres shorten while short telomeres are maintained by the expression of telomerase, suggesting that cellular factors preferentially recruit hTERT to critically short telomeres (Ouellette et al., 2000).

Table 1. 3 Telomerase activity detection in human tissue

Pathology	No. Positive/no. tested	Telomerase positive(%)
Normal*	1/196	0.5
Preinvasive(benign)	125/410	30
Malignant	1734/2031	85
Adjacent to malignant	77/660	11

*Cells from renewal tissues (intestinal crypts, basal layer of skin) as well as inflammatory cells have low but detectable telomerase activity (review see Holt & Shay, 1999a).

Table 1. 4 Telomerase activity detection in human cancer

Cancer Type	Positive/Tested(%)	Cancer Type	Positive/Tested (%)
Lung	113 / 140 (81%)	Stomach	72 / 85 (85%)
Colon	123 / 138 (89%)	Liver	149 / 173 (86%)
Breast	300 / 339 (88%)	Leukemias	142 / 194 (73%)
Prostate	52 / 58 (90%)	Brain	90 / 131 (69%)
Pancreatic	41 / 43 (95%)	Kidney	95 / 115 (83%)
Ovarian	21 / 23 (91%)	Skin	94 / 102 (92%)
Cervical	16 / 16 (100%)	Head/Neck	112 / 130 (86%)

(for review see Shay & Wright, 1996)

1.4.2 Telomerase in tumour development

The advent of a highly sensitive telomere repeat amplification protocol (heretofore referred to as the TRAP assay) allowed screening of a variety of human tumours, utilising less than 5 ug of protein for each sample (Kim et al., 1994; Wright et al., 1995b). A recent literature search indicated more than five hundred manuscripts published on telomerase since 1994, and most of them published in the past four years. Telomerase is specifically associated with all of the 20 broadly defined cancer types investigated, including lung, colorectal, breast, prostate, pancreatic, ovarian, cervical, stomach, liver, leukaemia, brain, kidney, skin, head/neck, and other cancers. Across all cancer types, telomerase is detectable in an average 85% of specimen samples (see Table 1.3 and 1.4). No other cancer marker offers this level of universality and specificity.

While some highly proliferative normal cells have detectable telomerase activity, most exhibit levels of activity significantly lower than those found in cells extracted from high-grade malignant tumours. The use of telomerase as a screen for the early detection of human cancer, prior to progression to high-grade malignant status is of potential interest. For instance, many precarcinoma lesions have detectable telomerase activity (on the order of 15%; Shay & Wright, 1996), yet are classified as premalignant by standard cytopathology and histopathology. Detection of telomerase activity in these samples may indicate that these seemingly benign lesions contain a small subset of malignant cells and may assist in patient risk stratification. The hope is that telomerase measurements may have a place in early cancer diagnostics and prognostics. However, additional studies on the level and distribution of telomerase activity in the early stages of cancer are needed to determine the utility of telomerase detection in early diagnosis, especially as it relates to clinical outcome. Several studies have proposed detection of telomerase activity as a predictor of poor clinical outcome (Hiyama *et al.,* 1995a,b 1996). One example of this is the childhood cancer, neuroblastoma, where certain subclasses (stages III and IV) have high levels of detectable telomerase activity and poor prognosis (Hiyama *et al.,* 1995a). Interestingly, one subclass (stages IV-S) has been shown to spontaneously regress and coincidentally is telomerase negative. Therefore, without telomerase, the tumour

undergoes telomere shortening, the cells senesce, and the cancer is eventually cleared from the body. This study also demonstrates that while telomerase or another mechanism is likely required for the sustained growth of advanced tumours, it is not required for the initiation of cancer.

Another area of interest is monitoring patients for residual or recurrence of disease using telomerase as a marker. As can be seen in Table 1.3 and 1.4, telomerase activity is detected in adjacent, presumably cleared regions in more than 10% of tumour specimens obtained during biopsy. In these instances, occult micrometastases of the primary tumour can invade the surrounding normal tissue and small amounts of tumour may remain undetected by pathologists. Recurrence of cancer could be monitored by fine needle aspiration of cancer patient tissues to assess the regression of the tumour due to this micrometastatic event using telomerase detection.

1.5 Regulation of Telomerase

1.5.1 Regulation requires both h*TERC* and hTERT

Several telomerase-associated proteins have been identified in various organisms, however, h*TERC* and hTERT are both necessary and sufficient for telomerase activity *in vitro* (Weinrich et al., 1997; Beattie et al., 1998). Telomerase genes have been reported to have different expression profiles in normal somatic cells, and also two molecular mechanisms have been described that may contribute to the regulation of telomerase activity in human: transcriptional regulation of h*TERC* and hTERT and post-transcriptional alternative splicing of hTERT (Feng et al., 1995; Nakamura et al., 1997; Meyerson et al., 1997; Harrington et al., 1997b; Kilian et al., 1997; Ulaner et al., 1998).

1.5.1.1 Reconstitution require both h*TERC and* hTERT

Recently, some groups reported *in vitro* reconstitution of telomerase and demonstrated

the essential role of hTERT and h*TERC* (Beattie et al., 1998; Weinrich et al., 1997; Holt et al., 1999; Riou, 1998; Masutomi et al., 2000). Using the dominant-negative mutation of hTERT abrogated this *in vitro* reconstitution assays (Hahn et al., 1999) suggesting the hTERT and h*TERC* are the minimal essential components for telomerase activity (Masutomi et al., 2000). It has been reported that the production of active recombinant telomerase in *Tetrahymena* requires a factor in rabbit reticulocyte extract that promotes ribonucleoprotein assembly (Licht & Collins, 1999; Bachand & Autexier, 1999). Other factors such as Hsp90 and p23 may participate in reconstitution for telomerase activity with recombinant hTERT synthesised *de novo* in rabbit reticulocyte extract *in vitro* (Holt et al., 1999b).

1.5.1.2 Interaction between h*TERC and hTERT*

Normal human diploid cells contain the integral RNA component of telomerase (h*TERC*) but generally lack the mRNA for the catalytic subunit (hTERT) (Meyerson et al., 1997; Nakamura et al., 19997), although there are some cases in which alternative splicing forms of hTERT are present in telomerase-negative cells (Kilian et al., 1997; Ulaner et al., 1998). The catalytic subunit is thought to be the only missing component necessary for at least a minimally functional enzyme. This is based on two lines of evidence: transfection of plasmids directing the expression of hTERT into telomerase-negative cell types results in the appearance of telomerase activity, and *in vitro*-transcribed h*TERC* mixed with *in vitro*-translated hTERT in a rabbit reticulocyte lysate results in detectable telomerase (Yi et al., 1999; Beattie et al., 1998; Bodnar et al., 1998; Counter et al., 1998; Vaziri et al., 1998; Weinrich et al., 1997). A 3- to 10-fold increase in steady-state levels of h*TERC* has been observed in a variety of immortal cells (Avilion et al., 1996), indicating that some regulation of h*TERC* is associated with the acquisition of telomerase activity. In situ hybridisation of human biopsy specimens indicates that the relative levels of h*TERC* in tumour cells are sufficiently different from those in adjacent tissues to be clinically useful in the diagnosis of cancer (Soder et al., 1997a; Morales et al., 1998a,b; Yashima et al., 1997a,b).

It is known that the hTERT binds the structure h*TERC* RNA and this complex interacts with the telomeres. A physical complex formation between hTERT protein and h*TERC* was detected by EMSA *in vitro* when equimolar amounts of two molecules were mixed, these complexes were specifically eliminated by excess amounts of nonlabeled h*TERC* (Masutomi et al., 2000). These studies provide direct evidence that hTERT as a RNA-binding protein interacts with RNA component of telomerase.

1.5.2 Transcriptional regulation of telomerase

Myc Up to date, several gene products have been demonstrated directly or indirectly targeting the promoter to regulate hTERT transcription or telomerase activity (Figure 1.6 and Table 1.5). Wang et al (1998) has firstly reported that Myc induces telomerase in both normal human mammary epithelial cells (HMECs) and normal human diploid fibroblasts. Myc increases expression of hEST2 (hTRT/TP2) and can extend the life span of HMECs. Furthermore, this activation has been proven in transcription level. Over-expression of c-Myc/Max resulted in a significant increase in transcriptional activity of the core promoter (Kyo et, 2000; Oh et al., 1999a; Horikawa et al., 1999; Takakura et al., 1999) and telomerase activity (Wu et al., 1999). Levels of activation depended upon the Myc/Max ratio used.

Mad1 Other studies by Gunes et al. (2000) showed that over-expression of Mad1 in transient transfection assays in U937 cells resulted in a consistent and significant decrease of hTERT promoter activity, and this repressive effect of Mad1 was dose dependent and required for an intact proximal E-box. Thus, c-Myc and Mad1 exert their transcriptional effects via the same site in the hTERT promoter. It was discussed that Mad1 repression of the hTERT promoter may involve an active repression mechanism rather than simple competition with c-Myc.

Sp1 was shown to be a potent transactivator of hTERT, and Sp1 function was required for Myc-mediated activation of hTERT. Myc/Max and Mad/Max regulate hTERT transcription differently in a cell type-specific manner, and transactivation by Myc/Max is achieved with the cooperative action of Sp1 (Kyo et al., 2000). It was reported that levels of c-Myc and Sp1 expression were correlated with the transcriptional activity of hTERT in various cell types, and expression of c-Myc and Sp1 increased during malignant progression of fibroblast cells, in association with telomerase activity. Therefore, c-Myc and Sp1 cooperatively function to activate hTERT transcription and up-regulation of these expressions might be critical for telomerase activation during carcinogenesis (Kyo et al., 2000).

Estrogen Telomerase activity in estrogen receptor (ER)-positive MCF-7 cells was up-regulated by the treatment with 17 beta-estradiol. This activation accompanied up-regulation of the telomerase catalytic subunit, hTERT mRNA. Gel shift assays revealed that the imperfect palindromic estrogen-responsive element in the hTERT promoter specifically binds to ER. Transient expression assays using luciferase reporter plasmids containing various fragments of hTERT promoter showed that this imperfect palindromic estrogen-responsive element is responsible for transcriptional activation by ligand-activated ER. Furthermore estrogen has been shown to activate c-Myc expression in MCF-7 cells, and E-boxes in the hTERT promoter that bind c-Myc/Max play additional roles in estrogen-induced transactivation of hTERT. Estrogen thus activates telomerase via direct effects on the hTERT promoter (Kyo et al., 1999).

WT1 and MZF-2 The Wilms' tumour 1 suppressor gene (WT1) has been shown to repress hTERT promoter activity in 293 kidney cells but not in HeLa cells which express no endogenous WT1. WT1 represses the endogenous hTERT promoter and telomerase enzyme activities suggesting that WT1 may be a transcriptional repressor of the hTERT gene, at least in some specific cells (Oh et al., 1999). In addition, Fujimoto et al (2000) have recently identified a 400 bp silencer of the hTERT promoter between -776 and -378 upstream of the proximal core promoter. The myeloid-specific zinc finger protein 2 (MZF-2) has been demonstrated to recognise this region. Mutation introduced in these MZF-2

sites resulted in significant activation of hTERT transcription. Over-expression of MZF-2 in cells led to down-regulation of hTERT transcription as well as telomerase activity. MZF-2 may be an effector of negative regulation of hTERT (Fujimoto et al., 2000).

To the date, identified factors in regulating of hTERT promoter are listed in Table 1.5. Taken together, these studies demonstrate that expression of hTERT is regulated mainly at the transcriptional level and that the promoter of the hTERT gene is essential for transcriptional activation in cancer cells and immortalised cells. Up- or down-regulation of these factors during the course of carcinogenesis is therefore thought to be responsible of telomerase regulation.

Table 1. 5 Factors regulating the hTERT gene promoter

Proteins	Binding sites	EMSA	Regulation of hTERT promoter	Regulation of telomerase activity	Telomerase activity in the cell lines
c-MYC	E-box	C+S	activation	activation	IMR90, HMEC, C33A, SiHa
Sp1	Sp1	C+S	activation		
Estrogen	ER, ERE	C+S	activation	activation	MCF-7
WT1	WT1	C	repression	repression	293, COS, BHK
Mad1	E-box	S	repression		
MZF-2	MZF-2	C+S	repression	repression	C33A, SiHa

C=competition; S= antibody supershift

1.5.3 Regulation of *hTERT* expression

High levels of expression of hTERT have been observed in telomerase-positive tumour tissues and immortal cell lines, but not in normal tissues and telomerase-negative cells (Nakamura et al., 1997; Meyerson et al., 1997). There is significant correlation between level of hTERT expression and telomerase activity in a variety of types of cancers (Ito et al., 1998; Kanaya et al., 1998; Kyo et al., 1999b; Takakura et al., 1998). Introduction of hTERT cDNA into telomerase-negative normal cells leads to activation of telomerase in these cells (Nakayama et al., 1998; Weinrich et al., 1997) and normal cell clones with high levels of hTERT expression attain immortal capacity (Bodnar et al., 1998; Hahn et al., 1999a). The ectopic expression of the hTERT in combination with two oncogenes (the

simian virus 40 large-T oncoprotein and an oncogenic allele of H-ras) results in direct tumourigenic conversion of normal human epithelial and fibroblast cells (Hahn et al., 1999a). Therefore, regulation of hTERT expression levels is an important mechanism used in a variety of developmental contexts to determine the amount of telomerase activity present in specific cell lineages. Interestingly, studies in Kathleen Collins group have also shown that h*TERC* is necessary for telomerase or immortalisation in real cells (Mitchell et al., 1999b). Up-regulation of hTERT and h*TERC* message may be an important mechanism through which telomerase becomes activated during both cellular immortalization and the progression of malignant tumours (Meyerson et al., 1997; Mitchell et al., 1999b).

Expression of a mutant catalytic subunit of human telomerase results in complete inhibition of telomerase activity, reduction in telomere length and death of tumour cells. Expression of this mutant telomerase eliminated tumorigenicity *in vivo* (Hahn et al., 1999b). Alteration of the carboxyl terminus of hTERT does not affect telomerase enzymatic activity but impedes the ability of this enzyme to maintain telomeres. Telomerase-positive cells expressing this mutant enzyme fail to undergo immortalization, further tightening the connection between telomere maintenance and immortalization (Counter et al., 1998a). Inducible dominant-negative mutants of hTERT dramatically reduced the level of endogenous telomerase activity in tumour cell lines, it has been reported that telomerase inhibition in cells with short telomeres lead to chromosomal damage, which in turn trigger apoptotic cell death (Zhang et al., 1999). These results provide the first direct evidence that telomerase is required for the maintenance of human tumour and immortal cell viability, and suggest that tumours with short telomeres may be effectively and rapidly killed following telomerase inhibition.

1.5.4 Onco- and cellular- proteins regulate telomerase activity

Evidence accumulated over the past several years has shown that telomerase is activated in many different kinds of human cancer (Harley et al., 1994) and tumour-

suppressor genes have joined oncogenes on centre stage. One pathway by which telomerase can be directly activated has recently been identified. Human papillomavirus (HPV) E6 and MYC activate telomerase activity (Klingelhutz et al., 1996; Kiyono et al., 1998; Wang et al., 1998), and hTERT is the target for MYC activation and co-operates with E7 in immortalization. These findings are evidence for the complexity of telomerase control mechanisms and constitute a point of departure for piecing together an integrated picture of telomerase structure, function, and regulation in aging and tumour development (Liu, 1999a).

1.5.4.1 Cellular oncoprotein MYC activates telomerase

Expression of the MYC oncogene in primary human epithelial cells that lack telomerase activity leads to telomerase activation (Wang et al., 1998). By contrast, several other cellular and viral oncoproteins, including MDM2, RAS^{V12}, cyclinD1, CDC25C and CDC25A, do not activate telomerase. Expression of MYC activates telomerase by up-regulating the mRNA encoding the hTERT. The activation of telomerase by E6 might also be a MYC-mediated event. Expression of E6 leads to the activation of MYC (Auewarakul et al., 1994; Kinoshita et al., 1997b) and subsequent activation of telomerase in epithelial cells. This pathway appears to be cell type specific. In fibroblasts, expression of E6 does not activate MYC (Wang et al., 1998). In some cell types telomerase activation leads to an increase in telomere length, while in other cell types it does not, suggesting that different factors are required to facilitate telomere elongation by telomerase. As described in section 1.5.2., the c-Myc activates telomerase activity by acting on the hTERT gene promoter through the E-box. The up-regulation of telomerase by MYC might help to elucidate the pathway of telomerase activation in human cancer.

1.5.4.2 Viral oncoprotein HPV E6 activates telomerase

One pathway by which telomerase can be directly activated has recently been identified. HPV infections are linked to human cervical cancer; HPV type 16 and 18 predispose normal human cells to tumour formation (Zur Hausen, 1991; Howley, 1991). Two viral genes, E6 and E7, are primarily responsible for this tumourigenicity. In cell-culture models, the HPV16 E6 and E7 oncogenes cooperate to allow primary cells to become immortalised (Munger et al., 1989), and in transgenic mice both genes are necessary for tumour formation (Pan & Greip, 1994). Expression of E6 alone directly activated telomerase in pre-immortal human keratinocytes (Klingelhutz et al., 1996), however, this induction of telomerase activity did not lead to telomere elongation (Stoppler et al., 1997; Klingelhutz et al., 1996). In fact, experiments from a number of groups have shown that cells expressing HPV E6 and E7 do not show telomere elongation until after cells have passed through crisis (Shay et al., 1993; Klingelhutz et al., 1994). Recent evidence suggests that the activation of telomerase by E6 occurs, at least in part, through the activation of the MYC oncogene (Wang et al., 1998). This suggests that, although telomerase activity is present, either the levels are too low, or that in some cell types other factors might be needed to recruit telomerase to telomere.

1.5.4.3 Telomerase co-operates with HPV E7 in cellular immortalization

hTERT expression elongates telomeres and extends the lifespan of primary human cells (Bodnar et al., 1998) leading to the speculation that such ectopic expression of telomerase might allow cellular immortalization and, thus, promote tumour formation (de Lange, 1998). Expression of HPV E6 or E7 alone will extend the life span of primary human keratinocytes past the normal senescence checkpoint. When expressed together E6 and E7 cooperate to allow the generation of immortal variants, which survive after the culture undergoes crisis (Galloway & McDougall, 1996). hTERT expression from a retroviral vector induces telomerase activity but does not immortalise cells in keratinocytes and human mammary epithelial cells. When hTERT is expressed along with

HPV E7, established cell lines were selected after crisis (Kiyono et al., 1998). The ability of hTERT to cooperate with E7 in cellular immortalization suggests that telomerase fulfils one definition of cooperative oncogenes (Land et al., 1983).

1.5.4.4 Regulation of telomerase by the c-Abl tyrosine kinase

Recently Kharbanda et al. reported that hTERT associates directly with the c-Abl protein tyrosine kinase. c-Abl phosphorylates hTERT and inhibits hTERT activity. Exposure of cells to ionising radiation induces tyrosine phosphorylation of hTERT by a c-Abl-dependent mechanism, phosphorylation of hTERT by c-Abl is associated with inhibition of telomerase activity. The finding that c-Abl-mediated phosphorylation of hTERT inhibits telomerase activity supports a function in negatively regulating telomere length (Kharbanda et al., 2000). Ubiquitously expressed c-Abl tyrosine kinase is activated by DNA double-strand breaks (Kharbanda et al., 1995), and protein involved in the repair of these lesions function on telomere control (Laroche et al., 1998; Polotnianka et al., 1998; Nugent et al., 1998a). These findings of functional interactions between c-Abl and hTERT support a role for c-Abl in the regulation of telomerase function.

1.5.4.5 Chromosome 3 represses telomerase activity

It has been reported that introduction of chromosome 3 but not chromosomes 7 or 11 resulted in repression of telomerase activity, followed by progressive telomere shortening, and after 60-90 days, inhibition of cell proliferation. Direct inhibition of telomerase activity using micro-cell-mediated chromosome 3 transfer has no immediate effect on cellular proliferation but does result in gradual telomere shortening and the eventual renewal of the senescence program (Ohmura *et al.,* 1995). Experiments by Ohmura et al. (1995) utilised introduction of a normal human chromosome 3 to a renal cell carcinoma cell line (RCC23), which contains significant rearrangements and deletions of genes on the short arm of chromosome 3. While fusion using other chromosomes showed no changes in

telomeres or telomerase, RCC23 with introduced chromosome 3 exhibited a marked decline in telomere lengths coupled with repression of telomerase activity. These microcell hybrids continued to proliferate with similar growth rates as wild-type RCC23 cells and eventually senesced after 30 to 40 PDs (Ohmura *et al.,* 1995). Follow-up experiments have shown that introduction of chromosome 3 results in a substantial decrease in hTERT mRNA expression and after growth arrest, expression of a stress response and senescence-activated β-galactosidase (Horikawa *et al.,* 1998). These data suggest that there is a functional telomerase repressor on chromosome 3 and that repression of telomerase activity in tumour-derived cell lines results in progressive telomere shortening and reprogramming of cellular senescence. Microcell mediated transfer of chromosome 3 results in telomerase inhibition by repression of hTERT transcription (Horikawa et al., 1998). This telomerase repressor region on chromosome 3p has been narrowed to 3p12-p21.1 and 3p21.3-p22 (Cuthbert *et al.,* 1999), 3p13-p14.2 (Vieten *et al.,* 1998), 3p14.2-p21.1 (Tanaka *et al.,* 1998), and 3p14.2-p21.2 and 3p24.1-p24.3 (Mehle *et al.,* 1998).

1.6 Telomerase in Therapeutics

The finding that normal somatic cells lack telomerase expression may well lead to a highly specific treatment with fewer side effects than conventional chemotherapy (Morin, 1995; Shay, 1995; Harley & Villeponteau, 1995; deLange, 1994). The high frequency of telomerase expression in certain cancers reinforces the notion that the telomerase enzyme and the telomeric structure are exciting targets for potential anti-cancer strategies. Indeed, inhibition of telomerase in cancer cells in culture results in cell death, giving strong support to efforts to develop telomerase inhibitors for clinical use. Different strategies can be used to inhibit telomerase; (1) Small molecule or protein inhibitors can be directed against hTERT; (2) Antisense directed against h*TERC* results in the shortening of telomeres and cell death, after 20 cycles, of tumour cells; (3) Gene therapy can be conducted with a suicide gene under the control of the hTERT, h*TERC*, or/and

h*TERC*/hTERT promoter. Our knowledge of the basic biology of telomere length maintenance suggests that discovery of drugs that target telomerase would represent a new class of therapeutic agents that act through a novel mechanism. Telomerase is a complex enzyme that offers many targets for the design of inhibitors, and their development and testing will provide the only sure way to address the telomerase challenge to drug discovery.

Numerous potential therapeutic strategies have been proposed with the telomere or telomerase enzyme as the molecular target. Telomerase-interactive compounds currently being developed include peptide nucleic acids, antisense oligonucleotides, ribozymes and reverse transcriptase inhibitors, with both the catalytic and RNA components as the target. For example, recent studies suggest that targeting h*TERC* may prove particularly beneficial in the treatment of small cell lung cancer, a common cancer associated with very poor prognosis. h*TERC* gene expression was detectable by RNA in situ hybridisation in 98% of small cell lung cancer samples, making it the best available candidate for targeted therapy in this cancer type (Sarvesvaran et al., 1999).

A major unmet clinical need in the treatment of cancer is accurate determination of the aggressiveness, or stage, of cancer. Knowing the stage of a cancer guide the physician in treatment decisions is impacting the patient's chance of survival. For example, 70% of early stage breast cancer patients will be cured after primary surgery; no additional radio or chemotherapy is usually recommended. However, this practice does not benefit the 30% of patients who will experience recurrent cancer and perhaps could be helped by additional therapies. An accurate prognostic marker that could identify high risk early stage breast cancer patients has been one of the most sought after tests in breast cancer management. Telomerase has significant potential in predicting the aggressiveness of cancer in multiple cancer types, and assisting the oncologist to treat more effectively.

Another example of the importance of developing additional diagnostic screens is the use of telomerase in detecting bladder cancer as an adjuvant to normal cytology. Almost 50% of early-stage bladder tumour cases are initially missed due to the lack of effective

cytological markers for detection. Telomerase can be detected in the majority (approaching 90%) of bladder cancers using less invasive biopsy techniques (bladder washings or voided urine; Lin *et al.,* 1996; Dalbagni *et al.,* 1997; Kinoshita *et al.,* 1997a; Yoshida *et al.,* 1997). Detection of telomerase activity relative to human cancer development is likely to be an important and novel method, in combination with traditional cytology, for early cancer diagnosis.

It has been shown that inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death (Herbert et al., 1999). Telomerase inhibitors could target any one of several features of human telomerase. These include: the hTERT active site; the 11-base RNA template; the 'anchor site' where hTERT interacts with telomeric DNA; the extended telomere, possibly a G-quadruplex structure; additional, as yet unknown interacting proteins; or antisense targeting of the mRNA for either h*TERC* or hTERT. The hTERT would be a preferred target in this antisense-targeting strategy because hTERT is not expressed in telomerase negative cells. In a variation in this theme, the gene for an inactive hTERT mutant could be delivered to telomerase positive cells and suppress telomerase activity though a dominant-negative approach. The progress of investigations of described inhibitors is discussed in following section and outlined in Table 1.6.

1.6.1 Nucleotide inhibitors

Telomerase shares substantial identity with reverse transcriptase, so it is not surprising that nucleotide analogs known to inhibit polymerase, such as AZT (azidothymidine) (Strahl & Blackburn, 1996; Gomez et al., 1998), 7-deaza-ATP (Fletcher et al., 1996), ddG (Ku et al., 1997; Pai et al., 1998) and carbovir (Yegorov et al., 1996), also inhibit telomerase. Unfortunately, these molecules also inhibit other polymerases, making it difficult to relate changes in cell proliferation to inhibition of telomerase. Identification of nucleotide inhibitors that are selective for telomerase may be able to take advantage of reverse transcriptase inhibitors synthesised during programs to investigate inhibition of

other polymerases, some of which may have been poor inhibitors of their original targets. The recent cloning of h*TERC* and hTERT and possession of reconstituted enzyme will facilitate development of protocols for mass screening of these derivatives. Identification of hTERT and h*TERC* should lead to high-resolution structural information, and this should also facilitate development of nucleotide analogs.

Table 1. 6 Reported inhibitors of human telomerase activity

Inhibitor	Proposed target
Expression of antisense RNA	h*TERC*
Peptide nucleic acid	h*TERC* template
Phosphorothioate DNA	h*TERC* template/hTERT
2'-O-methyl RNA	h*TERC* template
Retroviral antisense	h*TERC* template
Phosphodiester DNA oligos	h*TERC* template
Anthraquinone derivatives	G-quadruplex/telomere
Cationic porphyrin	G-quadruplex/telomere
Perylenetetracarboxylic dimide	G-quadruplex/telomere
Nucleoside derivatives	hTERT active site
Tea catechins	Unknown
Oligonucleotide with 2'-5' A linkage	h*TERC*/RNase L-mediated
Small molecules	Different protein
Ribozyme	h*TERC*
Phosphorothioate DNA	Anchor site
4-(hydroxyphenyl)retinamide (4HPR)	Unknown
Trichostatin A (TSA)	hTERT in HAC1 cell

(For review see Pitts & Corey, 1999)

1.6.2 G-quadruplex interactive agents

An approach to telomerase inhibition that has been the focus of much attention is the design of small molecules that bind G-quartet structures. Two classes of them, anthraquinone derivatives (Sun et al., 1997) and cationic porphyrins (Whealhouse et al., 1998), have been demonstrated by NMR spectroscopy to interact with G-quadruplex structures, and both molecules also inhibit telomerase *in vitro*.

1.6.3 Oligonucleotides as inhibitors

The 11-base template within h*TERC* is intrinsically accessible to binding by telomere ends and this recognition is essential for maintaining telomere length, suggesting that telomerase should be an ideal target for inhibition by oligonucleotides that target this sequence. It has been found that peptide nucleic acids (PNAs) complementary to the template inhibit telomerase with IC50 values of 1-10 nM (Hamilton et al., 1997). There have been two reports of anti-telomerase oligonucleotide causing cell death soon after addition, results that are contrary to the dogma that telomerase inhibitors require a lag phase before their effects become apparent. One involves a hexamer oligonucleotide directed to the template region of h*TERC* (Mata et al., 1997), while a second contains a 2'-5' adenosine linkage that is meant to direct RNase L-mediated cleavage to a non-template region predicted to be accessible (Kondo et al., 1998). Phosphodiester-linked 2'-0-methyl-RNA, however, proved to be a highly selective inhibitor and displayed a potency comparable with the best PNA inhibitors (Pitts & Corey, 1998). Introduction of phosphorothioate linkages at the 3'- and 5'- termini of the 2'-*0*-methyl-RNA increased stability towards nuclease digestion without sacrificing potency or specificity. This modified 2'-*0*-methyl-RNA inhibited telomerase within cells upon delivery with cationic lipid. These intriguing reports could indicate that telomerase inhibitors can function by a mechanism other than gradual telomere erosion.

1.7 Aims of This Thesis

Recent studies indicate that the regulation of telomerase activity in mammalian cells is multifactorial. It is known that expression of the h*TERC* and hTERT are essential for activity, and the primary mode of control of hTERT appears to be transcriptional regulation. However, very little is known about the molecular mechanisms involved in the regulation of h*TERC*. The main target to be studied of this project is to detect the telomerase RNA gene functional promoter region and to investigate whether transcription is involved in regulation of the RNA component (see Figure 1.8 for strategy). The h*TERC* and m*Terc* 5'-flanking region cDNA will be cloned and sequenced (Chapter 3). Secondly, the sequence elements bound by transcription factors will be investigated to clarify the molecular basis involved in regulation of telomerase RNA gene expression (Chapter 4). To elucidate the regulatory mechanism of telomerase RNA gene transcription, the major regulators of the h*TERC* promoter will be identified. The h*TERC* promoter could allow targeting by several regulators including viral oncoprotein or tumour suppresser gene products to effect the h*TERC* gene expression phenotype in certain cell situation, the candidate gene products such as E1A and pRb will be further tested. The regulation of mammalian TERC genes from different species will be also studied by comparison of 5' flanking transcriptional region (Chapter 5). This study may offer a way to identify molecular mechanisms participating in the modulation of telomerase activity during tumourgenesis and also open up possibilities for the gene therapeutics (Chapter 6).

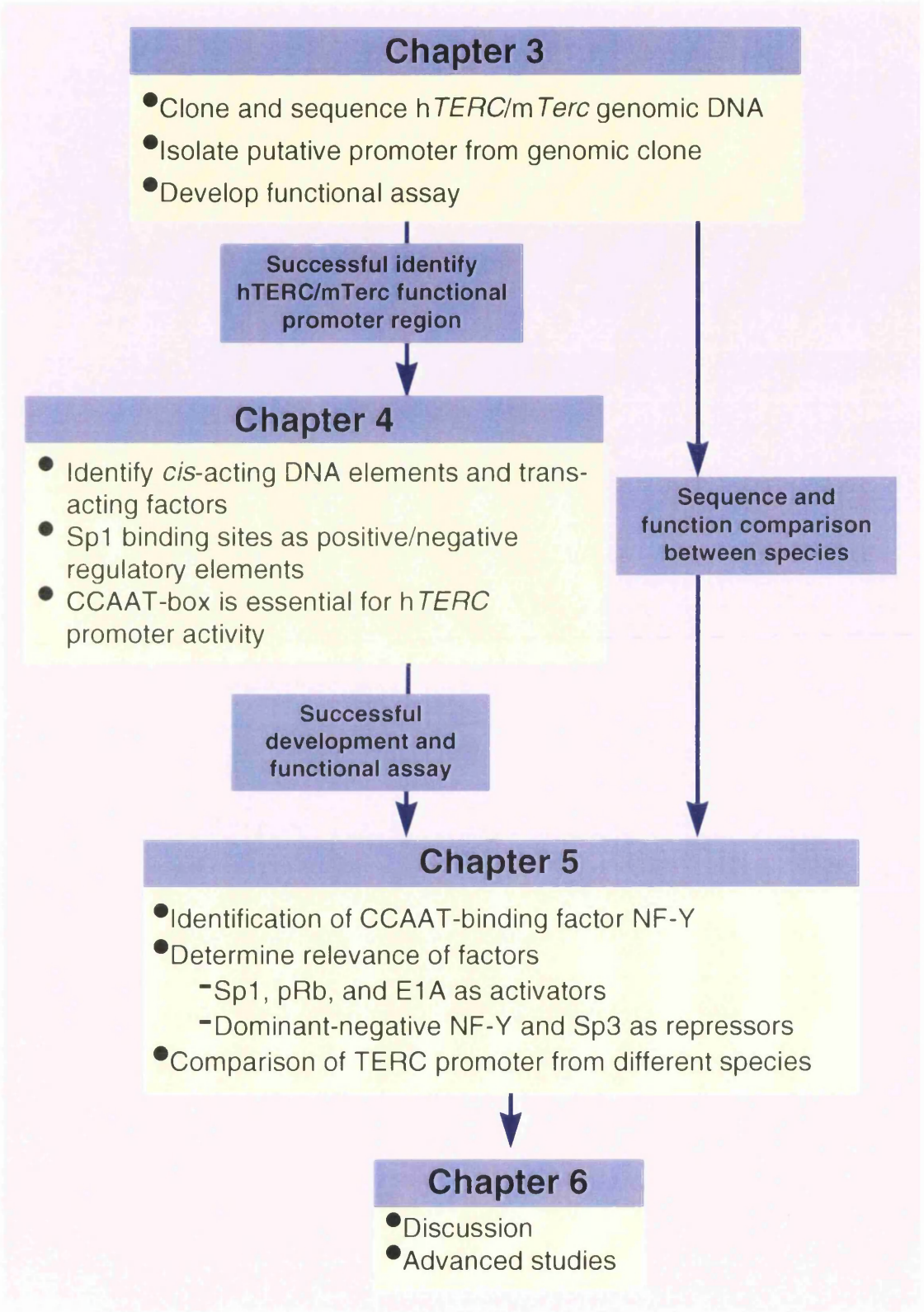


Figure 1.8 Structure of this thesis for analysis of the h*TERC* transcriptional regulation mechanism

Chapter

2

Materials and Methodology

- Materials
- DNA Recombination Techniques
- DNA Analysis
- Cell Culture and Reporter Gene Assay
- DNA-protein Interaction
- Computer Program Analysis

Chapter 2 Materials and Methodology

2.1 Materials

2.1.1 Plasmids

Human P1 clone, 9913, is derived from a human foreskin fibroblast P1 library. Mouse P1 clone, 11792, is derived from a mouse C127 fibroblast P1 library.

pGL3 Basic plasmid (Promega) contains the firefly luciferase gene with no promoter or enhancer. pGL3 Control plasmid contains the firefly luciferase gene driven by the SV40 promoter and enhancer.

pEGFP-1 vector (Clontech) contains a green fluorescent protein (GFP) reporter gene without the promoter. pEGFP-N1 protein fusion vector contains a GFP reporter driven by the CMV promoter.

pPCR-Script Amp phagemid (Stratagene) was used as a cloning and sequencing vector.

pSEAP2 Basic Vector (Clontech) is used for expression of the reporter gene secreted alkaline phosphateas (SEAP) with no promoter. pSEAP2 Control Vector is a positive control vector under control of the SV40 early promoter and SV 40 enhancer.

The Sp1 expression vectors, pCMVSp1, were generously provided by Dr. Robert Tjian, University of California, Berkeley. pCMVSp3 was a gift from Dr Guntram Suske, Institut fur Molekularbiologie und Tumorforschung, Germany. pCMV control plasmid was created by deletion of the Sp3 Bgl II/BamH I fragment from pCMVSp3.

pCMV-Rb, the wild-type human Rb expression construct driven by CMV promoter, was a gift from Dr. Lee F. Johnson, University of Ohio State. pSV40-Rb, the wild-type human Rb expression construct driven by SV40 promoter, was a gift from Dr. Lee F. Johnson, University of Ohio State.

Dominant-negative mutants pNF-YA13m29 and wild-type pNF-YA13 were generously provided by Dr. Roberto Mantovani (Universita Degli Studi Di Milano) and were constructed from NF-YAm29 and NF-YA cDNA cloned in down-stream of SV40 promoter (Mantovani et al., 1994).

pE1A wt, expression vector with full-length E1A gene, was a gift from Dr David Gillespie (Beatson Laboratories).

2.1.2 Kits and Enzymes

Products	Supplies
Protein Assay Dye Reagent Concentrate	BioRad Labs: 500 0006
Luciferase Assay System	Promega: E1500
Gel Shift Assay System	Promega: E3300
T4 Polynucleotide Kinase	New England BioLabs: 201S
Restriction enzyme	GIBCO
T4 DNA Ligase, ver. 2.0	MBI:EL0332
Qiaquick Gel Extraction Kit	Qiagen: 28704
Superfect Transfection Reagent(1.2ml)	Qiagen: 301305
Qiaquick Nucleotide Removal Kit	Qiagen: 28304
Calf Intestine Alkaline Phospatase	Cambridge Bioscience: 8410 1
SureTrack Footprinting Kit	Pharmacia Biotech: XY-029-00-05
pCR Bluescript II Cloning kits SK+	Stratgaene: 212206XXX
DNA Ligation Kits	Stratagene: 203003
Taq DNA Polymerase PCR Kit	Boehringer Mannheim: 1435094
Quick Change Site-Directed Mutagenesis Kit	Stratagene: #200518
Lyse-N-Go PCR Reagent	PIERCE: #78882)
Sentinel Molecular Beacon GAPDH Detection kit	Stratagene: #200571
[γ^{32}-P] ATP, >5000Ci/mmol, 10mCi/ml	Amersham: AA0018
[α^{32}-P] dCTP, >3000Ci/mmol, 10mCi/ml	Amersham: AA0005

2.1.3 Antisera

Table 2. 1 Antisera

Antisera for EMSA	Supplier
Anti-NF-YA rabbit monoclonal antibodies	A gift from Dr Roberto Mantovani
Anti-NF-YB rabbit polyclonal antibodies	A gift from Dr Roberto Mantovani
CBF-A (C-19): goat polyclonal IgG,	Santa Cruz : sc-7711X
CBF-C (N-19): goat polyclonal IgG,	Santa Cruz : sc-7715X
CBF-B (C-18): goat polyclonal IgG,	Santa Cruz : sc-7712X
CBF-B (N-19): goat polyclonal IgG,	Santa Cruz : sc-7713X
Sp1 (1C6): mouse monoclonal IgG,	Santa Cruz : sc-420X
Sp3 (D-20)-G: rabbit polyclonal IgG,	Santa Cruz : sc-644-GX
Ap2 (C-18): rabbit polyclonal IgG,	Santa Cruz : sc-184X
Ap1 (D): rabbit polyclonal IgG,	Santa Cruz : sc-44X
Ets-2 (C-20): rabbit polyclonal IgG,	Santa Cruz : sc-351X
NF-1 (N-20): rabbit polyclonal IgG,	Santa Cruz : sc-870X
c/EBPα (14AA): rabbit polyclonal IgG,	Santa Cruz : sc-61X
Rb (C-25-G): goat polyclonal IgG,	Santa Cruz : sc-50-GX
NF-YA: purified mouse IgG,	Pharmingen:65131A

2.1.4 Consensus oligonucleotides and sequence primers

Table 2. 2 Commercial consensus oligonucleotides

Oligos	Sequence*	Source
Sp1	ATTCGATCGGGGCGGGGCGAGC	Promega E323A
TFIID	GCAGAGCATATAAGGTGAGGTAGGA	Promega E322B
AP2	GATCGAACTGACCGCCCGCGGCCCGT	Santa Cruz: sc-2513
AP1	CGCTTGATGAGTCAGCCGGAA	Promega E320B
NF-kB	AGTTGAGGGGACTTTCCCAGGC	Promega E329B
c/EBP	TGCAGATTGCGCAATCTGCA	Santa Cruz: sc-2525
NF-1	TTTTGGATTGAAGCCAATATGATAA	Santa Cruz: sc-2553
NF-Y	ATTTTTCTGATTGGTTAAAAGT	Becton Dickinson UK Ltd
Ets-2	GATCTGGAGCAGGAAGTTCGA	Santa Cruz: sc-2555
CBF	AGACCGTAGGTGATTGGTTAATCTCTT	Santa Cruz: sc-2591
T7	TAATACGACTCACTATAGGG	Promega: Q5021
T3	ATTAACCCTCACTAAAGGGA	Promega: Q5741
RVprimer3	CTAGCAAAATAGGCTGTCCC	Promega: E4481
GLprimer2	CTTTATGTTTTTGGCGTCTTCC	Promega: E1661
LucR1	TTTGAATCTTGTAATCCTGAA	
LucF1	CTACCGTGGTGTTCGTTTC	

* Nucleotide sequences are given in uppercase letters from 5' to 3'. T7 and T3 are the sequencing primers for pPCR-Script Amp or pCR Bluescript SK (+) cloning vector (Stratagene). Rvprimer3 and Glprimer2 are the sequencing primers for the pGL3 luciferase reporter plasmid (Promega). LucR1 and LucF1 primer are for PCR semi-quantitation.

Table 2. 3 Oligonucleotides for h*TERC* promoter

Oligos	Position	Sequence*	Purpose
hTR14R	+46 to +69	ggatccTACGCCCTTCTCAGTTAGGGTTAG	For deletion mutation (with BamH I site)
hTR17F	-436 to -417	ggatccACTGAGCCGAGACAAGATTC	For deletion mutation (with BamH I site)
hTR20F	-798 to -779	gcgctcgagAGCTACTCAGGAGGCTGAGA	For deletion mutation (with Xho I site)
hTR21F	-627 to -609	gcgctcgagcATCAAGACACAGCACTACT	For deletion mutation (with Xho I site)
hTR22F	-272 to -253	gcgctcgagGTCTGGTCTGCAGAGGATAG	For deletion mutation (with Xho I site)
hTR23R	+46 to +69	cgcaagcttTACGCCCTTCTCAGTTAGGGTTAG	For deletion mutation (with HindIII site)
hTR5mF	-473 to -452	gcgctcgAGGAAAAAGGTTCAGGGTTGGA	ΔSp1.5 mutation (with Xho I site)
hTR5F	-473 to -452	gcgctcgAGGAAAAAGGGGCAGGGTTGGA	For deletion mutation (with Xho I site)
hTR26n	-51 to -28	gcgctcgaGTGCGGTCGGCGGCCGCTCCCTTT	For deletion mutation (with Xho I site)
hTR29F	-107 to -88	gcgctcgAGCCCGCCCGAGAGAGTGAC	For deletion mutation (with Xho I site)
h4 [a]	-110 to -91	ACCAGCCCGCCCGAGAGAGT	h4 DNA footprinting region
h41m [a]	-110 to -91	ACCAGCCCG**AA**CGAGAGAGT	ΔSp1.2, from cc to aa, (-101/-100)
h5 [a]	-471 to –452	GAAAAAGGGGCAGGGTTGGA	h5 DNA footprinting region
h5m [a]	-471 to -452	GAAAAAGG**TT**CAGGGTTGGA	ΔSp1.3, from gg to tt, (-463/-462)
h9 [a]	-44 to -17	CGGCGGCCGCTCCCTTTATAAGCCGACT	h9 DNA footprinting region
h91 [a]	-44 to -21	CTTACGCCGCTCCCTTTATAAGCC	h9 mutation from ggcg to ttac, (-43/-40)
h910 [b]	-53 to -29	CCGTGCGGTC**TTAC**GCCGCTCCC	h9 mutation from ggcg to ttac, (-43/-40)
h911 [a]	-44 to -21	CGGCG**TAAA**CTCCCTTTATAAGCC	h9 mutation from gccg to taaa, (-39/-36)
h92 [a]	-44 to -21	CGGCGGCC**ATAG**CCTTTATAAGCC	h9 mutation from gctc to atag, (-36/-33)
h921 [a]	-44 to -21	CGGCGGCCGCTC**ATGC**TATAAGCC	h9 mutation from cctt to atgc, (-32/-29)
h93 [a]	-44 to -21	CGGCGGCCGCTCCCTT**CGAC**AGCC	h9 mutation from tata to cgac, (-28/-25)
h930 [b]	-38 to -14	CCGCTCCCTT**CGAC**AGCCGACTCGC	h9 mutation from tata to cgac, (-28/-25)
h9con [b]	-49 to -20	GCGGTCGGCCCCCGCCCCGTTTATAAGCCG	Point mutation from gg to cc(-39/-40), t to c(-34), c to g(-31)
h10 [a]	-63 to -42	CTTGGCCAATCCGTGCGGTCGG	h10 footprinting region containing CCAAT binding site
h101 [a]	-63 to -42	CTTGG**AGTC**TCCGTGCGGTCGG	ΔCCAAT, from ccaa to agtc, (-58/-55)
h10m11 [b]	-74 to-45	GCGAGAGTCAGCTTGG**AGTC**TCCGTGCGG	ΔCCAAT, from ccaa to agtc, (-58/-55)
h10m2 [a,b]	-63 to -42	CTTGGCCAATCC**TGAT**GGTCGG	h10 mutation from gtgc to tgat, (-51/-47)
h11 [a]	-2 to +36	CCGGGTTGCGGAGGGTGGGCCTGGGAGGGGTGGT GGCC	Sp1.3 (+11,+16) and Sp1.4 (+26, +31) binding site
h111 [a]	"	CCGGGTTGCGGA**AAAA**TGGGCCTGGGAGGGGTGGT GGCC	ΔSp1.3, from ggg to aaa (+11/+13,)
h112 [a]	"	CCGGGTTGCGGAGGGTGGGCCTGGG**TAA**GGTGGT GGCC	ΔSp1.4, from agg to taa (+24/+26)
h113 [a]	"	CCGGGTTGCGGA**AAAA**TGGGCCTGGG**TAA**GGTGGT GGCC	ΔSp1.3 and ΔSp1.4 binding site(+11/+13, +24/+26)
h11c [a]	-2 to +23	CCGGGTTGCGGAGGGTGGGCCTGGG	Sp1.3 binding site
h11d [a]	+14 to +36	GCCTGGGAGGGGTGGTGGCC	Sp1.4 binding site
h112b [a,b]	+15 to +36	GGGCCTGGG**TAA**GGTGGTGGCC	ΔSp1.4, from agg to taa, +24/+26
h112C [a,b]	"	GGGCCTGGG**TAA**GGT**AA**TGGCC	ΔSp1.4, from aggggtgg to taaggtaa(+24/+26, +30/+31)
h11e [a]	"	GGGCCTGGGAGGGGT**AA**TGGCC	Mutation from gg to aa (+30/+31)

* Nucleotides corresponding to promoter sequences are given in uppercase letters from 5' end to 3' end; The core sequences mentioned in the Purpose column are underlined. Bold characters indicate mutated nucleotides and lowercase letters indicate flanking nucleotides that introduce restriction enzyme sites.

[a] Complementary sequence for EMSA not shown.

[b] For PCR-directed *in vitro* mutagenesis, complementary sequence not shown.

2.1.5 Oligonucleotides for PCR and EMSA

All oligodeoxynucleotides used were obtained from GENOSYS (Genosys Biotechnologies, Europe, Ltd). Each oligonucleotide was resuspended in deionised H_2O according to the manufacturers product information; the concentration of 250 pmolar was calculated by: 250pM = Oligo Weight (mg/ml) X 4 / Molecular Weight. Oligonucleotide solutions were stored at - 20 ^{o}C.

For PCR cloning, primers from hTR14R to hTR29F in Table 2.3 (F, sense; R, antisense) including a restriction enzyme recognition sites (lowercase), respectively, were used for construction of the plasmids. DNA fragments with mutation of specific DNA element(s) were generated by introducing a mutated nucleotide into PCR primers when the mutation was directed into the middle region of the DNA fragment. For EMSA experiments, oligonucleotides h4, h5, h9 and h10 were used for construction of each DNase I footprinting protection region.

Table 2. 4 Oligonucleotides for m*Terc* promoter

Oligos	Position	Sequence*	Purpose
mTR25F	-190 to -99	gcgctcgagGTGTCTCACAGCAAGAAACA	For deletion mutation (with Xho I site)
mTR26 F	-129 to –110	gcgctcgagGTGACTGGCTAGGAAGAGTG	For deletion mutation (with Xho I site)
mTR27 F	-549 to –530	gcgctcgagTGTGACCTTGAACTACAGAC	For deletion mutation (with Xho I site)
mTR28 F	-339 to –320	gcgctcgagGGACTGGGTTGAAGGTGGAA	For deletion mutation (with Xho I site)
mTR29 F	-379 to –360	gcgctcgagTGCGCCACTTTTCCCCACTT	For deletion mutation (with Xho I site)
mTR30 R	-180 to +59	cgcaagctTCCGCTGGAAGTCAGCGAGAA	For deletion mutation (with Hind III site)
mTR31 R		cgcaagctTTGTAGGCGGGTACGGGACTG	For deletion mutation (with Hind III site)
mTR32 R		cgcaagctTTGACAGAGGCGGAGCTCTTC	For deletion mutation (with Hind III site)
mTR33 F		gcgctcgaGGCTCTTAGAAGGCACCGTGG	For deletion mutation (with Xho I site)
mTR34 R	-8 to +27	cgcaagcttCTAGCCGCCCTCGACCTTAA	For deletion mutation (with Hind III site)
mTR35 R		cgcaagctTCTAGAGTTGAAAGTAGGGAT	For deletion mutation (with Hind III site)
mTR36 F	-55 to -34	gcgctcgagTCGACCAATCAGCGCGCGCCAT	For deletion mutation (with Xho I site)
mTR37R	-34 to -55	CgcaagcttATGGCGCGCGCTGATTGGTCGA	For deletion mutation (with Hind III site)

* Nucleotides corresponding to promoter sequences are given in uppercase letters from 5' end to 3' end. Lowercase letters indicate flanking nucleotides that introduce restriction enzyme sites.

[a] Complementary sequence for EMSA not shown.

[b]For PCR-directed *in vitro* mutagenesis, complementary sequence not shown.

2.1.6 General laboratory supplies and miscellaneous

Beatson Institute Central Services

LB-Medium (Luria-Bertani Medium)

Sterile distilled water

Sterile phosphate-buffered saline (PBS)

Sterile phosphate-buffered saline (PBS)+EDTA(PE)

Sterile glassware and pipettes

Penicillin (7.5 mg/ml)

Streptomycin (10gm/ml)

Amphotericin B (250ug/ml)

Tissue culture

Gibco Europe Life Technologies Ltd.

DMEM, RPMI 1640,

200mM glutamine

100mM sodium pyruvate

7.5%(w/v) sodium bicarbonate

2.5%(w/v) trypsin

2.2 DNA Recombination Techniques

The human P1 clone, 9913, is derived from a human foreskin fibroblast P1 library and the mouse P1 clone, 11792, is derived from a mouse C127 fibroblast P1 library. Briefly, in order to subclone the promoter regions, the P1 clones were digested with EcoRI/HindIII and ligated into pCR Bluescript to create phTR (1765 bp) and pmTR (4040 bp). Colonies containing telomerase RNA gene sequences were identified by hybridization with PCR generated probes as previously described (Soder et al., 1997a, b). Plasmid DNA was prepared from positively hybridizing colonies, and inserts sequenced on both strands using the ABI PRISM dye terminator cycle sequencing kit (PE Applied Biosystems,

Warrington, UK). DNA fragments with various lengths of 5'-flanking regions were generated by PCR amplification.

2.2.1 Polymerase Chain Reaction

The amplification of DNA fragments using the polymerase chain reaction is performed in either the Perkin-Elmer Cetus DNA Thermal Cycler or the Perkin-Elmer Cetus Cycler 9600, by adding the following reagents to a 0.5 ml PCR tube in a total volume of 50 µl: 50 ng plasmid DNA or 1 µg genomic P1 clone as template, 25 pM sense (F) and antisense (R) primers (Table 2.3 and 2.4), 10 mM dNTP's, 10 X PCR buffer and 5 units Taq DNA polymerase (Boehringer). The cycling protocol consisted of 30 cycles of three temperatures: strand denaturation at 95 °C for 30 seconds; primer annealing at 55 °C for 30 seconds; primer extension at 72 °C for 1 or 2 minutes. The reaction mixtures were overlaid with two drops of mineral oil. After PCR, 5 µl aliquots of the mixture, typically, were analysed by agarose gel electrophoresis to detect amplified product.

2.2.2 Cloning PCR products

Isolation and purification To clone the PCR products, 45 µl of PCR products were incubated with 2 µl of Xho I and Hind III (10 U/µl) enzyme at 37 °C for 60 minutes. DNA fragments to be used for cloning were separated from unwanted products of the restriction digest reaction by electrophoresis on 1 % non-denaturing agarose gels. The fragment band was cut out of the gel using a clean scalpel blade and the gel slice placed in a sterile microcentrifuge tube. Extraction of the DNA fragment from the agarose was achieved using a QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions. Each of the digested PCR fragments were ligated into Xho I/Hind III linearized pGL3 Basic vector (Promega), pEGFP-1 vector (Clontech) or pPCR-Script™ SK (+) vector (Stratagene).

Ligation and transformation Standard DNA ligations were performed in 10 µl by incubating DNA inserts with 1 µl of linearized vector in the presence of buffer, 50% PEG rATP, and T4 DNA ligase (MBI, Lithuania), reactions were incubated at room-temperature for 1 hour. Then the ligation reaction was mixed with 50 µl competent DH_{α}-5 E.coli (Gibco, Life Tech) and incubated on ice for 45 minutes. The cells were placed in a 42 °C water-bath for 45 seconds and transferred to ice for 5 minutes. After this step 200 µl SOC medium (2% Bacto-tryptone, 0.5% bacto-yeast extract, 20 mM Glucose, 10 mM NaCl, 10 mM $MgCl_2$) was added and incubated for 1 hour in an orbital shaker at 37 °C. After completion of this step, 50 to 100 µl cells were plated to bacteriological grade dishes (Bibby Sterilin) coated with 1.5% Agar containing L-Broth and with the appropriate selection antibiotic. Plates were incubated in an inverted position in a 37 °C incubator for 18 to 24 hours.

2.2.3 Cloning of site-directed mutagenesis constructs

The basic strategy for site-directed mutagenesis of the proximal h*TERC* promoter consisted of the following steps, illustrated in Figure 2.1. The PCR mutageneses were performed separately by using a site-directed mutagenesis kit (Stratagene, QuikChange™) following the manufacturer's instructions. An oligonuleotide, complementary to the single-strand template except for the mutated nucleotide of interest, was synthesised and annealed with the single-strand template DNA. The mutant strand was then synthesised by extending the oligonucleotide with DNA polymerase followed by annealing, to create double-stranded DNA. The mutant forms of h*TERC* (-107/+69) were constructed using oligonucleotides designed with the mismatches shown in Table 2.3. The mutant sequences did not create any known binding site for transcription factors as predicted by TFSEARCH and TESS data base searchs.

The PCR reactions were performed using the following standard conditions. All PCRs were performed using 2 µl of pC2923 (-107/+69) plasmid DNA (50 ng/µl, 3.1 Kb) as template, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 25 nM of appropriate mutagenic primers in the

sense orientation and 1.0 µl (2.5 U/µl) Pfu DNA polymerase in 50 µl reaction volume. The same oligonucleotides mentioned above were used as antisense primer. A total of 16 cycles (16 cycles at 95 °C for 30 sec, 55 °C for 30 sec, and 68 °C for 6 or 10 min, 2 minutes/Kb plasmid length) were used after originally denaturing at 95 °C for 30 seconds in either the Perkin-Elmer Cetus DNA Thermal Cycler or the Perkin-Elmer Cetus Cycler 9600.

5 µl aliquots of the PCR products were then analysed on a 1% agarose gel and the remainder of the reaction was digested by adding 1 µl of Dpn I (10 U/µl) at 37 °C for 60 minutes to remove all pure parental DNA template. Then, 2 µl of Dpn I treated PCR products were transformed into competent XL-2 Blue cells (Stratagene). After amplification of DNA in E.coli., the mutations were verified by DNA sequencing.

Then, mutants of luciferase reporter construct were constructed by subcloning Xho I/Hind III digested fragment into linearized pGL3 Basic vector. 5 µg of mutant construct which cloned in pPCR-Script™ AMP(+) vector (Stratagene) were digested by Xho I/Hind III at 37 °C for 60 minutes. Then, DNA was applied for 1.5% agarose gel or 6% PAGE for electrophoresis to purify 0.2 Kb insert.

The mutant sequences are shown in Table 2.3. For example, the Sp1.1 binding site-replaced mutant, pLh92(ΔSp1.1), was constructed by subcloning Xho I/Hind III digested pC92 Xho I/Hind III fragment amplified with the primer set h92 [a] . The Sp1.1 and Sp1.2 double site-replaced mutant, pLh29m292(ΔSp1-1.2), was constructed by using pC92 as template for PCR amplification with the primer set h41m [a]. The multiple site-replaced mutant, pLh114(ΔSp1.1, ΔSp1.2, ΔSp1.3, ΔSp1.4), was created by using pC29m292 as template for three separate PCR amplifications with the primer sets h111a[a,b], h112b[a,b] and h112c[a,b], respectively. All mutation constructs were sequenced from both 5' and 3' ends to confirm orientation and sequence.

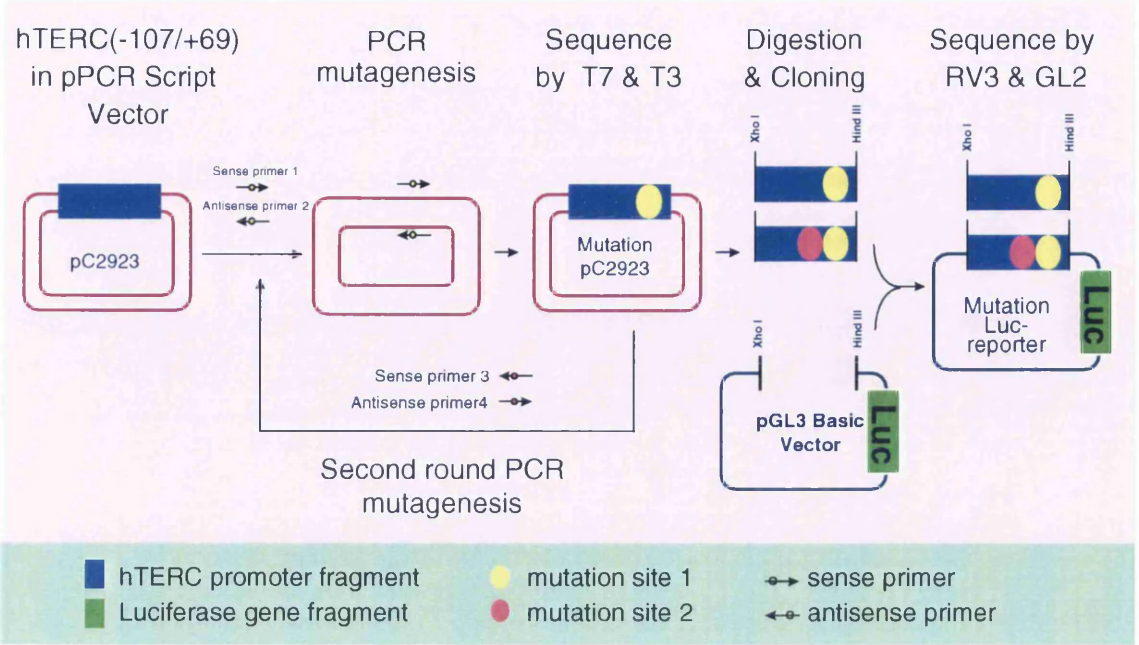


Figure 2. 1 The strategy of proximal promoter mutagenesis

Table 2. 5 The h*TERC* site-directed mutation constructs

Name	Mutation Sites	Mutation and position
pLh2923	none	wild type
pLh29m223	ΔSp1.2	cc to aa, -101 to -100
pLh1011	ΔNF-Y	ccaa to agtc, -58 to -55
pLh102		gtgc to tgat, -51 to -47
pLh910		ggcg to ttac, -43 to -40
pLh911		gccg to taaa, -39 to -36
pLh92	ΔSp1.1	gctc to atag, -36 to -33
pLh921	ΔSp1.1	cctt to atgc, -32 to -29
pLh930	ΔTATA	tata to cgac, -28 to -25
pLh29111	ΔSp1.3	ggg to aaa, +11 to +13
pLh29112	ΔSp1.4	agg to taa, +24 to +26
pLh29m292	ΔSp1,2; ΔSp1.1	-101/-100; -36/-33
pLh29m921	ΔSp1,2; ΔSp1.1	-101/-100; -32/-29
pLh111	ΔSp1.2; ΔSp1.1; ΔSp1.3	-101/-100; -36/-33; +11 /+13
pLh112	ΔSP1.2; ΔSp1.1; ΔSp1.4	-101/-100; -36/-33; +24/+26
pLh113	ΔSp1.2; ΔSp1.1; ΔSp1.3; ΔSp1.4;	-101/-100; -36/-33; +11 /+13; +24/+26
pLh114	ΔSp1.2; ΔSp1.1; ΔSp1.3; ΔSp1.4;	-101/-100; -36/-33; +11 /+13; +24/+26; +30/+31
pLh471	ΔNF-Y	ccaa to agtc, -58 to -55 in pLh2223

Δ Indication of the site-directed mutation.

As shown in Table 2.3, the Sp1.1 core binding sequence 5'-GGCCGCTCCC-3' has been replaced by 5'-GGCC*ATAG*CC-3', NF-Y core binding sequence 5'-CCAAT-3' has been replaced by 5'-*AGTC*T-3', two motifs of Sp1 site core sequence 5'-GGGTGGGCCTGGGAGGGGTGGTGG-3' have been replaced by 5'-AAATGGGCCTGGG*TAA*GGT*AA*TGG-3'. The resultant plasmid DNAs were prepared and sequenced to confirm specific nucleotide changes. The site-directed mutation constructs that have been made in this study are shown in Table 2.5.

2.2.4 Glycerol Stocks

750 µl of the overnight culture, which contained exponentially growing cells, was added to 750 µl 50% sterile glycerol in a 1.5 ml Nunc cryotube. The mixture was vortexed to ensure that the glycerol was mixed throughout the bacterial cell suspension and frozen rapidly by placing the tube in dry-ice for 5 minutes. Glycerol stocks were stored at -70 ^{o}C.

2.3 DNA Analysis

2.3.1 Minipreparation of plasmid DNA

Bacterial pellets were recovered in a 0.5 ml PCR tube by centrifugation at top speed in a microcentrifuge. The pellet was resuspended in 270 µl STET solution (0.1 M NaCl, 10mM Tris.HCl, pH8.0, 1mM EDTA, pH8.0, 5% Triton X-100) containing 0.667 µg/µl lysozyme, by vortexing briefly. After 5 minutes incubation on ice, the reaction was treated at 97 °C for 60 seconds in either the Perkin-Elmer Cetus DNA Thermal Cycler or the Perkin-Elmer Cetus Cycler 9600. Next, the reaction was chilled on ice for 5 minutes and the insoluble matter spun down at room temperature for 10 minutes, then removed with the assistance of tooth picks. DNA was precipitated by adding 140 µl isopropanol and centrifugation. The pellet was washed by 100% (v/v) ethanol, and air dried for 5 min. The pellet was resuspended in 50 µl TE (pH 8.0) containing heat-treated RNase (10 µg/ml), vortexed for 20 seconds and incubated at 55 °C for 5-10 minutes. Diagnostic digestion was performed

by using 2 µl miniprep DNA, 2 µl reaction buffer, 0.1-0.2 units of restriction enzyme in a 20 µl reaction which was incubated at 37 °C for 1 hour. 10 µl aliquots of the reaction mixture were loaded onto an agarose gel and electrophoresed to detect restriction fragments.

2.3.2 Maxipreparation and purification of plasmid DNA

0.5 ml fresh cultures from bacterial stocks were added into 100 ml of L-Broth containing appropriate antibiotic [20 µg/ml ampicillin + 80 µg/ml methicillin (Sigma M-6535)] and incubated overnight in an orbital shaker at 37 °C. The cultures were divided into two 50 ml falcon tubes and centrifuged at 4,000 rpm for 10 minutes at 4 °C in a Beckman GS-6R centrifuge.

The supernatant was discarded and the cell pellet was resuspended in 5 ml solution I(50 mM Tris-HCl pH 8.0, 10 mM EDTA pH8.0) by vigorous vortexing. The tubes were placed on ice, 10 ml solution II(0.2 M NaOH, 1% SDS) was added, the lysate was mixed by careful inversion and incubated at room temperature for 10 minutes. After this step, 15 ml solution III (5 M Potassium Acetate, 11.5% v/v Glacial Acetic Acid) was added and the lysate was mixed thoroughly. Samples were incubated in ice for 10 minutes then centrifuged at 4,000 rpm for 10 minutes at 4 °C in a Beckman GS-6R centrifuge. The supernatant was filtered through gauze and 0.6 volumes of isopropanol (about 12 ml) was added, mixed, and the solution was left for 10 minutes at room temperature. Then the solution was centrifuged at 4,000 rpm for 10 minutes at 4 °C in a Beckman GS-6R centrifuge.

After centrifugation the supernatant was removed carefully, the pellet was resuspended in 1.0 ml TE (pH 8.0) containing heat-treated RNase (10 µg/ml) and the plasmid DNA incubated at 55 °C for 30 minutes. Then plasmid DNA was precipatated by adding 1.0 ml solution IV (13.5% Polyethylene Glycol 6,000, 1.6 M NaCl) and centrifugation at 4,000

rpm for 10 minutes at 4 °C in a Beckman GS-6R centrifuge. Plasmid DNA also was purified using a plasmid maxi kit (QIAGEN).

The pellet was resuspended in 0.4 ml TE by repetitive pipetting. Sample was transferred to 1.5 ml centrifuge tube and extracted twice with phenol/chloroform, precipitated with 1 ml 100% (v/v) ethanol by centrifugation at 14,000 rpm for 10 minutes, then the pellet was washed with 70% and 100% (v/v) ethanol. After drying the pellet at room temperature, the pellet was dissolved with 0.4 ml TE to read concentration at OD260/280. Finally, plasmid DNA concentration was adjusted to 0.5 or 1.0 µg/µl.

2.3.3 Taq Terminator sequencing on ABI 373A

All of the constructs in this study were sequenced on both strands by dideoxy chain termination using the ABI PRISM dye teminator cycle sequencing kit (PE Applied Biosystems, Warrington, UK) and 25 ng oligonucleotide primers (Table 2.2). The sequence reaction was performed by mixing 1 µg of plasmid DNA, 3.2 pmoles of primer, 8 µl premix from dye terminator cycle sequencing kit up to 20 µl. PCR was performed with the following conditions in Perkin Elmer 9600: Preheat to 96 °C, 96 °C 15 seconds, 50 °C 1 seconds, 60 °C 4 minutes, and 25 cycles hold at 4 °C

After amplification, the PCR product was transfered to a 0.5 ml eppendorf tube and precipitated by adding 2 µl of 3M Sodium Acetate (pH4.5) and 50 µl 100% ethanol. Dye labelled products were resolved and detected using the Applied Biosystems DNA sequencer ABI373 that was performed by Beatson Institute technical service staff. The sequence data was collected and analysed by using the Sequencing Analysis program v3.0 and ABI software.

2.3.4 Quantitation of plasmid DNA for transfection

The concentration of nucleic acid in a solution was determined spectrophotometrically in a Beckman DU 650 spectrophotometer. Samples were diluted in TE and transferred to a quartz cuvette with a pathway of 1 cm. The spectrophotometer was initially calibrated using TE buffer only as a blank. The opticlal density reading were obtained a t 260 nm and 280 nm; an O.D. reading of 1 at 260 nm (A_{260}=1) corresponds approximately to a concentration of 50 µg/ml of double stranded DNA, for oligonucleotides an A_{260} of 1 was taken to correspond to 35 µg/ml, and for RNA an A_{260} of 1 was taken to correspond to 40 µg/ml. The ratio between reading at 260 nm and 280 nm (OD_{260}:OD_{280}) provided an estimate of the sample purity: samples with ratio of between 1.8 and 2.0 were taken as being sufficiently pure for all the techniques undertaken in this study.

To use equal amount of mutant constructs for transfection assays, plasmid DNA was carefully diluted and quantitated via UV spectrophotometer first, then 1.5 µg of mutation plasmid DNA was used for Xho I/Hind III restriction enzyme digest at 37 °C for 1 hours. The samples were loaded onto an 6% PAGE gel and electrophoresed to detect 176 bp products. After EtBr (10 mg/ml) staining, the gel was visualised in UV light and insert amount of each DNA sample was analysed by a computing PhosphorImager with ImageQuant analysis software (BioRad).

2.4 Cell Culture and Reporter Gene Assays

2.4.1 Mammalian cell lines

5637 cell line, originally established from the primary bladder carcinoma of a 68-years-old man in 1974, purchased from DSMZ (No: ACC 35). Swiss 3T3 feeders (ATCC CCL92), originally established in 1962 by G. Todaro and H. Green (Todaro and Green, 1963), are an immortalised mouse fibroblast cell line which were a kind gift from Rosewlyn McCaffery, Beatson Institute for Cancer Research, Glasgow. All cell lines used in this study are listed in Table 2.6.

Table 2. 6 Cell lines in this study

Cell type	Origin	Rb status[1]	Telomerase activity	
HeLa	Cervical carcinoma	HPV 18 E7-pRB	Positive	Immortal cell
5637	Bladder carcinoma	Gross rearrangement	Positive	Immortal cell
C33A	Cervical carcinoma	Partly deleted exon 20	Positive	Immortal cell
GM847	Established fibroblast	SV40	Negative	Immortal cell
WI38	Diploid fibroblast	Normal	Negative	Mortal cell
IMR90	Diploid fibroblast	Normal	Negative	Mortal cell
A9[2]	Areolar Adipose		Positive	Immortal cell
Swiss 3T3[2]	Established fibroblast		Positive	Immortal cell

[1]Original references on pRb gene and protein status can be found in Bartek et al., (1992) and Lukas et al., (1994). In the cell lines expressing DNA virus oncogenes, pRB is inactivated through protein-protein sequestration (For review see Aagaard, 1995)

[2]All cell types are of human origin, except for Swiss 3T3 and A9 (mouse).

2.4.2 Cell maintenance and storage

All cell lines were grown in medium supplemented with 10% FCS (450 ml suitable medium, 5 ml 200mM Glutamine, 2.5 ml Streptomycin/ Penicillin and 50 ml 100% FCS). Cell lines were trypsinized in 1.0 ml of PE/trypsin and resuspended in growth medium with 10% FCS and divided to an appropriate number of dishes.

Parental cell lines and transfected stable cells were kept in liquid Nitrogen for long term storage. For such a purpose cell lines were washed once with PBS and 1 ml of PE/trypsin solution was added to the plate. After trypsinisation cells were centrifuged for 5 minutes at 1,000 rpm at room temperature and the trypsin-containing medium aspirated. For one 10 cm dish 1 ml freezing solution (90% FCS, 10% DMSO) was added, mixed and the aliquot placed into a 2 ml Nunc tube and directly were transferred to -70 °C. After 2-4 days the aliquots were placed in liquid nitrogen barrels. To recover cells from nitrogen, vials were thawed in 37 °C dH_2O and resuspended in growth medium with 10% FCS, centrifuged for 5 minutes at 1,000 rpm at room temperature and the DMSO-containing medium aspirated. Immediately after this step, the cells were resuspended in growth medium with 10% FCS and plated to 10 cm dishes.

2.4.3 SuperFect Transfection

The day before transfection, 2-3 X 10^5 cells were seeded in each well of six-well plates (~ 9.5 cm ^{2}) in 2 ml of appropriate growth medium, and incubated at 37°C and 5% CO_2 overnight to give 40% to 80% confluence. 1.5 µg of construct was dissolved in TE pH 8.0 with cell growth medium containing no serum to a total volume of 75 µl. For co-transfection of each individual site-mutation Luc-reporter construct, 3 µg plasmid DNA was mixed with 3 µg of Sp1, Sp3, pCMVb or 0.5 µg of NF-YAm29 expression vector, or 0.25 µg pRb vector. 7.5 µl Superfect™ Transfection Reagent (QIAGEN) was added to the DNA solution and mixed by pipetting up and down 5 times. The mixtures were incubated for 10-15 min at room temperature to allow complex formation. Then, 0.4 ml cell growth medium (containing 10% FCS and antibiotics) was added to the tube containing transfection complexes and mixed by pipetting up and down twice. Growth medium was gently aspirated from the plate and the cells were washed once with 2.0 ml PBS. The complexes were immediately transfered to the cells and incubated with cells for 2-3 hours at 37 °C with 5% CO_2 in an incubator. Then, the medium containing the remaining complexes was removed from cells, and cells were washed once with 2 ml PBS, replaced with fresh 10% FCS growth medium, and incubated for a further 48 hours.

2.4.4 Luciferase activity assay

Cells were harvested for analysis after 48 hours transfection. Equivalent amounts of cellular protein as determined by Bio-Rad assay (BioRad), were used in the luciferase assay. Luciferase assays were performed according to the manufacturers protocols. 10 µl of cell extract was mixed with 50 µl of room temperature Luciferase Assay Reagent (Promega E1500). The reaction was placed in a luminometer or scintillation counter to measure the light produced for 10 seconds to 5 minutes. To ensure reproducibility in the assays, particular care was taken over the following: DNA was quantified by spectrophotometry and direct visualisation by gel electrophoresis. All transfections were carried out in duplicate wells and we found this to be a good measure of the

reproducibility of transfection. In each experiment, all deletion constructs were analysed together with both the basic cloning vector, pGL3-Basic, and the positive control vector, pGL3-Control, which contains SV40 promoter and enhancer sequences. Each extract was measured for luciferase activity at least twice. All transfections were carried out at least three times. Initial transfection conditions were determined by using promoter fragments linked to a green fluorescent protein (GFP) reporter gene (Clontech), as this allowed direct visualisation of promoter activity in live cells. The means and standard deviations of duplicate samples from representative transfections are shown and all experiments were carried out at least three times in duplicate.

2.4.5 SEAP reporter gene assay

A useful reporter, which is the secreted form of human placental alkaline phosphatase (SEAP), was used in transient transfections as an internal control against which the efficiency of transfection can be adjusted for (Clontech, cat. # 6049-1). An important advantage of this reporter for some purposes is that the reporter protein is secreted into the culture medium, allowing reporter detection without cell lysis. 1.5 μg SEAP plasmid DNA was co-transfected with the construct of interest using Superfect™ Transfection Reagent (QIAGEN). 48 post-transfection, SEAP protein maintained in culture was quantified by chemiluminescent detection, using Clontech's kits. The transfected cells can be harvested for luciferase assay at a later time. SEAP activity assays were performed according to the manufacturers protocols.

2.4.6 PCR quantitation of plasmid DNA for transfection

The Great EscApe™ SEAP System (Clontech) was used to control for transfection efficiency in experiments with the mutant promoter constructs. In keeping with others the Sp1 and Sp3 expression vectors modulated the activity of the SV40 promoter used in the pSEAP2-Control vector (Conn et al., 1996; Chen et al., 1998). For these experiments

semi-quantitative PCR of transfected plasmid was carried out using luciferase specific primers (for primer sequence see Table 2.2) and products adjusted relative to input genomic DNA.

The nuclear pellet after luciferase assay was directly resuspended in 50 µl of Lyse-N-Go™ PCR Reagent (PIERCE, #78882) and homogenised by pipetting the sample up and down. Then, a thermal cycling program was performed for up to three cycles as follows: 65 °C for 30 seconds; 8 °C for 30 seconds; 65 °C for 90 seconds; 97 °C for 180 seconds; 8 °C for 60 seconds; 65 °C for 180 seconds; 97 °C for 60 seconds; 65 °C for 60 seconds; then hold on 80 °C for about 20 minutes. I.0 µl of lysis sample was used as template for 25 µl total volume of PCR amplification by adding luciferase specific primers or GAPDH primers (suppled in Sentinel™ Molecular Beacon GAPDH Detection kit, Cat~200571; Stratagene). The cycling protocol consisted of : (i) 25 cycles of three temperatures for amplification of luciferase DNA, strand denaturation at 95°C for 30 seconds, primer annealing at 60 °C for 50 seconds and primer extension at 72 °C for 40 seconds. (ii) one cycle of 95°C at 3 minutes for denaturation of genomic GAPDH DNA, followed by 30 cycles of three temperatures for amplification of genomic GAPDH DNA, 95°C for 15 seconds, primer annealing at 60 °C for 30 seconds and primer extension at 72 °C for 60 seconds. After PCR, 10 µl aliquots of the mixture, typically, were analysed by agarose gel electrophoresis and by a computing PhosphorImager with ImageQuant software (BioRad).

2.5 DNA-Protein Interaction

2.5.1 Preparation of nuclear protein extracts

Nuclear protein preparation: Approximately 10^{10} cells were washed in PBS and then twice with TMS (5 mM Tris-HCl pH 7.5, 2.5 mM $MgCl_2$, 125 mM sucrose). Cells were lysed in 200 ml of TMS plus 0.25% Triton X-100, and nuclei were harvested by centrifugation (1,600 x *g*, 20 minutes). Nuclei were washed three times in 200 ml of TMS and

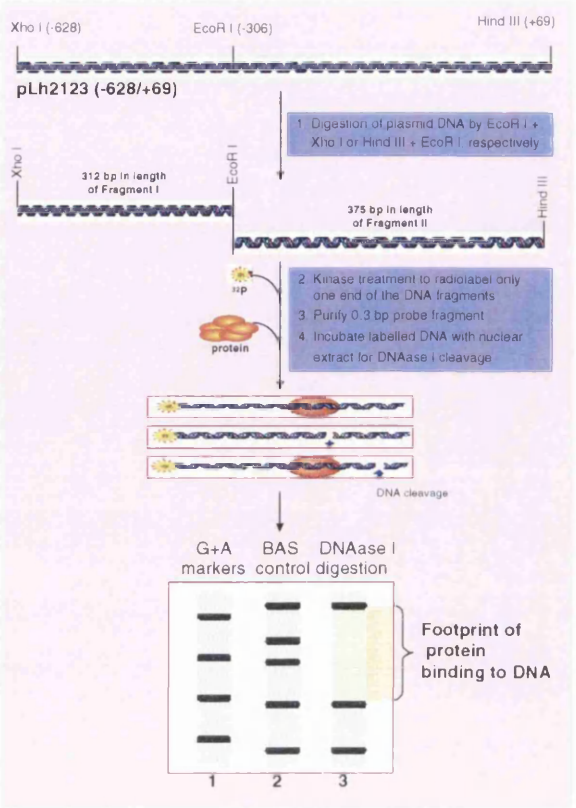
resuspended in approximately 5 ml of TMS (5 to 10 mg of DNA per ml), and 0.1 volumes of 4 M NaCl was added dropwise with stirring. The solution was centrifuged at 10,000 x *g* for 20 minutes, and the supernatant was spun at 10,000 x *g* for 60 minutes. Solid ammonium sulfate was added to 0.35 g/ml and left on ice for 30 minutes. The precipitate was pelleted at 10,000 x *g* for 30 minutes and re-dissolved in 5 ml of E50 buffer (50 mM ammonium sulfate, 20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1%[v/v] Brij 35, 20%[v/v] glycerol, 1 mM DTT) and dialysed for 16 hours against 1 litre of storage buffer (50 mM NaCl, 20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 20%[v/v] glycerol, 1 mM DTT). The crude protein extract was cleared by centrifugation at 10,000 x *g* for 60 minutes, and aliquots were stored at –70°C.

A microscale technique for the preparation of DNA-binding protein was also employed for electrophoretic mobility shift assays (EMSAs). Where possible, microscale and large-scale nuclear protein preparation were compared in EMSAs and found to produce identical results. All manipulations were carried out on ice, and all solutions contained 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 10 mM sodium butyrate, 10 mM ß-glycerophosphate, 2 mM levamisole, 50 mM sodium orthovanadate, pH 8.0, and 1 ug (each) of leupeptin, aprotinin, bestatin, and pepstatin per ml.

2.5.2 *In vitro* DNase I footprinting

As shown in Figure 2.1, plasmid pLh2223(–272/+69) or pLh2123(-628/+69) were used for the h*TERC* promoter DNA footprint analysis and pL2530 (–188/+80) was used for the m*Terc* promoter DNase I footprinting analysis. Digestion of pLh2223 and pL2530 plasmid with Xho I/Hind III, pLh2123 with Xho I/EcoR I or EcoR I/Hind III released the promoter fragment to be investigated. The sense strand was labelled by filling in the Xho I (or EcoR I) site by using Klenow fragment in the presence of [α-^{32}P]dATP (Amersham), and the antisense strand was labelled by filling in the Hind III (or EcoR I) site.

Figure 2.2 The strategy for DNase I footprinting analysis of h*TERC* promoter



The footprinting protocol was performed according to the SureTrack Footprinting Kit instructions (Pharmacia Biotech, XY-029-00-05). Labelled DNA (~20,000 cpm) was incubated for 30 minutes with 2 µg of poly(dI-dC) and 80 µg of nuclear protein extract in a final volume of 50 µl (8% glycerol, 20 mM Tris-HCl pH7.5, 100 mM NaCl, 5 mM $MgCl_2$ and DTT). For competition experiments oligonucleotide competitors (100-fold molar excess) were included in the binding reaction. To determine the optimal conditions, a titration was performed for each probe using increasing concentrations of DNase I for the same amount of nuclear extract. 5-10 milliunits of DNase I in DNase I buffer (2 mM $MgCl_2$, and 1 mM $CaCl_2$) were added and, following a 2-minutes incubation at room temperature, the reaction was terminated by the addition of 140 µl of DNase I stop solution (192 mM sodium acetate, 32 mM EDTA, 0.14% SDS and 64 µg/ml yeast RNA). After a 10 minute incubation at room temperature reactions were extracted twice with phenol/chloroform/isoamylalcohol and ethanol-precipitated before analysis in a 6% polyacrylamide, 7 M urea sequencing gel. DNA-protein complexes were autoradiographed on Kodak X-Omat films. For G+A sequence markers, the radiolabeled DNA was added to 4 µl of a standard sequencing gel loading buffer consisting of demonised formamide (98%), 0.05% each of Bromophenol blue and xylene cyanol FF and 20 mM Tris acetate adjusted to the desired pH 8.0. The samples were heated at 95°C for 20 minutes, cooled on ice and then loaded onto a sequencing gel (Song et al, 1997).

2.5.3 Oligonucleotide and EMSA

2.5.3.1 Oligonucleotide Design

Depending on the DNase I footprinting results, new oligonucleotide has been designed. Synthesise the oligonucleotide on either the ABI 392 or the Beckman Oligo 1000 automated DNA synthesizers according to manufacturers' instructions (Genosys Biotechnologies, Europe, Ltd). Single-stranded oligomers and their complementary sequences corresponding to the wt or mutants were synthesised as shown in Table 2.3 and 2.4. Computer program (described in section 2.6) analysed to prevent new DNA-binding site when mutagenesis wt DNA-binding site. The human Sp1 double-stranded oligodeoxynucleotide corresponding to the Sp1 consensus sequence ATTCGATCGGGGCGGGGCGACC was purchased from Promega. The some consensus oligonucleotides are shown in the Table 2.2.

2.5.3.2 Annealing and gel purification of oligonucleotides

To anneal the two complementary oligonucleotides, equal molar quantities of oligonucleotides were mixed in dH_2O or TE buffer, the oligomers were treated at 95 °C for 5 minutes in either the Perkin-Elmer Cetus DNA Thermal Cycler or the Perkin-Elmer Cetus Cycler 9600, then the Cycler was switched off to cool down slowly (about 4.5 hours). Annealing oligonucleotides were loaded in the 12% PAGE gel and run for 1 hour, DNA band was excised and placed into a 10 ml tube with 1 ml TE and allowed to elute overnight with agitation. The eluate was filter-treated through a pre-wetted acrodisk (0.2 μ m) (GelmanSciences), the eluate was collected and then 5 ml butanol was added. The mixture was vortexed for 30 seconds and spun at 3,500 rpm for 5 minutes. Organic (top) phase was removed and aqueous phase was placed into an eppendorf tube and the volume was adjusted to 500 ul with TE (pH8.0), followed by phenol/chloroform extraction and ethanol precipitation with 10 mM NaACO. After incubation at -20 °C overnight, the sample was spun 13,000 rpm for one hour, pellet was washed in 100 μl 70% (v/v) ethanol. Oligonucleotides were resuspended in TE (pH 8.0), the concentration of the

double-stranded oligonucleotide solution was measured spectrophotometrically and diluted with TE buffer to give a final concentration of 0.1 mg/ml. Oligonucleotide solutions were stored at -20 ^{o}C. Annealing oligonucleotide concentration was determined as described in section 2.3.4.

2.5.3.3 End labelling of double-stranded oligonucleotides

A typical 5'-kinase labelling reaction included the DNA to be labelled, [γ^{32}-P] dATP (Amersham), T4 polynucleotide kinase, and buffer (Promega). The components of the labelling reaction were added in the order listed:

Sterile d H_2O	5 µl
10X kinase buffer	1 µl
DNA or oligonucleotide (50 pm)	2 µl
[γ^{32}-P] dATP (10 mCi/ml)	1 µl
T4 polynucleotide kinase (3 U/ul)	1 µl
Total volume	10 µl

After incubation at 37 °C, reactions were heat inactivated by incubation at 80 °C for 15 minutes. Portions of the reactions were mixed with gel loading dye and loaded into a well of 6% non-denaturing polyacrylamide gel [15%(v/v) 40%(w/v) acrylamide: 2.1%(w/v) bisacrylamide, 0.5 X TBE (89 mM Tris base, 89 mM orthoboric acid, 2 mM EDTA, pH 8.0), 0.07% APS, 0.08% TEMED] and electrophoresed for 60 minutes at 150 volts.

The position of each radiolabelled oligonucleotide was determined by exposing the gel, covered with cling film, to a sheet of Kodak XAR-5 film for 1-2 minutes. Using the X-ray film as a guide, the band containing the labelled probe was excised from the gel using a clean scalpel blade, The gel slice was transferred to a clean eppendorf tube and TE buffer was added to the tube sufficient to cover the gel slice. The tube was placed in a lead pot, sealed and left at room temperature overnight during which time the oligonucleotide eluted from the gel slice into the TE buffer. The polyacrylamide gel slice

was removed from the solution using QIAquick spin columns (QIAGEN). The radioactive oligonucleotide solution was quantified and stored at -20 ^{o}C.

2.5.3.4 EMSA, competition and supershift assay

5.5 µg HeLa nuclear extract protein was incubated in 15 µl of reaction containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA, 50 mM NaCl, 10 mM Tris-HCl, pH7.5 and 2.0 µg poly(dI-dC) with or without excess moles of unlabelled DNA competitors on ice for 10 min, followed by addition of 5,000-10,000 cpm of the probe.

For competition experiments, non-radioactive double-stranded oligodeoxynucleotides were added in 100-fold molar excess prior to addition of the probe. For supershift assays, 1 or 2 µl of antibodies specific against Sp1, Sp3, Rb, Ap-2, c/EBP, c-Ets-2, NF-1, CBF (Santa Cruz Biotechnology), or NF-YA and NF-YB (PharMingen and Dr Mantoviani) were added to the reaction mixtures 25 minutes prior to the addition of the probe, then reaction mixture were incubated at room temperature for 1 hour or 4 °C overnight. All DNA-protein complexes were resolved on 5% native polyacrylamide gels running in 22.3 mM Tris, 22.3 mM boric acid, and 0.5 mM EDTA. Samples were electrophoresed at 150 volts at 4^{o}C until the blue dye front in the tracking wells was about 3cm from the end of the gel. The gel was transferred onto Whatman 3MM filter paper and dried down on a Biorad 583 gel drier at 80 °C for 45 minutes using a slow rise in temperature. Visualisation of the dried gel was performed using both a computing PhosphorImager with ImageQuant software analysis (BioRad) and autoradiography on Kodak XAR-5 films.

2.6 Computer Program Analysis

Homology searches were carried out using BLAST (Basic Local Alignment Search Tool), National Centre for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/ (see appendix V). Nucleotide sequences were analysed by using programs devised by the

University of Wisconsin Genetics Computer Group (UWGCG). Sequence comparisons were used the COMPARE program and were graphically displayed using the DOTPLOT program in the UWGCG package. Sequences were analysed for potential transcription factor binding sites by TESS: Transcription Element Search Software on the WWW, Jonathan Schug and G Christian Overton, Technical Report CBIL-TR-1997-1001-v0.0, of the Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, 1997. Identification of CpG islands was carried out using GRAIL: Gene Recognition and Assembly Internet Link, http://compbio.ornl.gov/ Grail-1.3/.

Sequence alignment and phylogenetic comparative analysis of 5' flanking transcription region of the 21 mammalian TERC genes were manually aligned using Vector NTI Suite program (InforMax). The sequences of closest relatives were aligned first on the basis of primary sequence similarity, The comparison of h*TERC* with other TERC gene promoter sequence were also used Gap or Fasta program in GCG package (UWGCG) to help align divergent regions. Each of aligned sequences was then aligned against each of the other groups. Sets of highly conserved nucleotides were identified and used as markers for aligning the more variable regions. The full sequences identified in present study have been submitted to GenBank, accession numbers AF047386, AF047387 and AF176663 (see appendixes I II and VI).

Chapter

3

Cloning Promoter Regions of the h*TERC* and m*Terc* Gene

Key issues

- Cloning the human and mouse TERC genomic DNA sequence
- Analysis of nucleotide sequence
- Prediction of potential promoter regions and transcription elements
- Functional identification of promoter regions by deletion analysis

Chapter 3 Cloning of the h*TERC* and m*Terc* promoter regions

The regulation of telomerase is a complex issue and has been reviewed in previous chapter. The results presented in this chapter begin to address the biological question as to whether telomerase RNA gene expression involves transcriptional control. In order to examine the transcriptional regulation of the TERC genes, genomic clones encompassing the TERC genes were identified and characterised (strategy shown in Figure 3.1).

3.1 Introduction

Telomeres are found at the end of linear chromosomes and consist of short repetitive sequences essential for the maintenance of normal chromosome structure and function (Wellinger and Sen, 1997). With each cell division, telomeres shorten due to the inability of DNA polymerases to replicate the ends of linear DNA molecules. However, telomere erosion is counteracted by the activity of the enzyme telomerase, a ribonuclear protein with reverse transcriptase activity, which adds telomeric repeats to the chromosomal termini (Morin, 1997; Nakamura et al., 1997). The genes for the human, (h*TERC*), and mouse, (m*Terc*), RNA components have been cloned, as has the human protein component; (hTERT) (Blasco et al., 1995; Feng et al., 1995; Nakamura et al., 1997; Soder et al., 1997a,b). Whilst telomerase expression is detectable in normal embryonic tissues and germ line stem cells, telomerase expression is repressed in most normal postnatal somatic cells (Blasco et al., 1995; Feng et al., 1995; Prowse and Greider, 1995; Soder et al., 1998; Wright et al., 1996a). The lack of telomerase expression may be the major reason for the progressive loss of telomeric sequences in somatic cells, which is considered to be one regulatory mechanism which monitors the number of times a cell divides before entering replicative senescence (Campisi, 1997). However, although telomerase appears to be stringently repressed in normal somatic tissues, there is substantial evidence to suggest that telomerase is expressed in the majority of human

cancers and contributes to the immortal phenotype through the maintenance of telomere integrity (Holt et al., 1997; Kim, 1997; Shay and Bacchetti, 1997).

The regulation of telomerase activity is likely to be a complex issue including control of the transcriptional activity of the telomerase RNA and protein component genes, interaction of telomerase with other telomere associated proteins and post-translational modification of the enzyme complex. Identification of the regulatory regions of the h*TERC* promoter sequence will be essential in understanding the molecular mechanisms of positive and negative regulation of telomerase RNA gene expression. There are few studies that directly address the mechanisms regulating telomerase expression in normal and cancer cells (Bodnar et al., 1996; Broccoli et al., 1997a; Li et al., 1997; Mandal and Kumar, 1997; Morin, 1997; Nakamura et al., 1997; Soder et al., 1998). In this chapter, genomic DNA sequences encompassing both the human and mouse telomerase RNA genes have been cloned in order to identify the functional promoter regions.

3.2 Cloning genomic sequences of the h*TERC* and m*Terc* genes

Our group have previously reported the identification of P1 genomic clones for the human, (h*TERC*), and mouse, (m*Terc*), telomerase RNA genes (Soder et al., 1997a,b). In order to obtain sequences flanking the genes, the P1 genomic clones were digested with EcoR I and Hind III, subcloned into the plasmid vector, pPCR script (+SK), and colonies containing h*TERC* or m*Terc* sequences identified by hybridisation to PCR generated probes specific for the genes. A 1.3 kb genomic clone encompassing h*TERC* was isolated as was a 4 kb genomic clone encompassing m*Terc* (Figure 3.2).

A BLAST search using the 1.3 kb human sequence identified three high-scoring segment pairs: HSU85256, HSU86046 and MMU33831. HSU85256 (598 bp of sequence) and HSU86046, (545 bp of sequence), are published sequences for the transcribed region of the human telomerase RNA gene and confirmed that we had cloned genomic sequences encompassing h*TERC* (Bryan et al., 1997; Feng et al., 1995). MMU33831 is the

sequence of the transcribed region of the mouse telomerase RNA gene that has previously been shown to have homology to the human gene (Blasco et al., 1995). In addition to the BLAST search, a text search for telomerase sequences carried out on the NCBI database revealed a 2.4 kb sequence, (I31750), from US patent number 5583016, (Inventors: Ville-ponteau B, Feng J, Funk W and Andrews WH, assigned to the Geron Corporation), which aligned with the Hind III site at the 3'-end of our genomic clone and extended 5' to the EcoR I site in our clone and thus completely overlapped the sequence of our 1.3 kb clone with only minor base differences. The sequence of P1 clone 5' to the EcoR I site was extended by designing a PCR primer, hProm867 from the patent sequence and cloning and sequencing the PCR product using the P1 clone as a template. In total 1765 bp of sequence information encompassing the human telomerase RNA gene has been generated and a schematic representation of this is shown in Figure 3.2a. Thus the sequences in both the 3' and 5' flanking regions of h*TERC* have been extended and have confirmed the unpublished patent sequence.

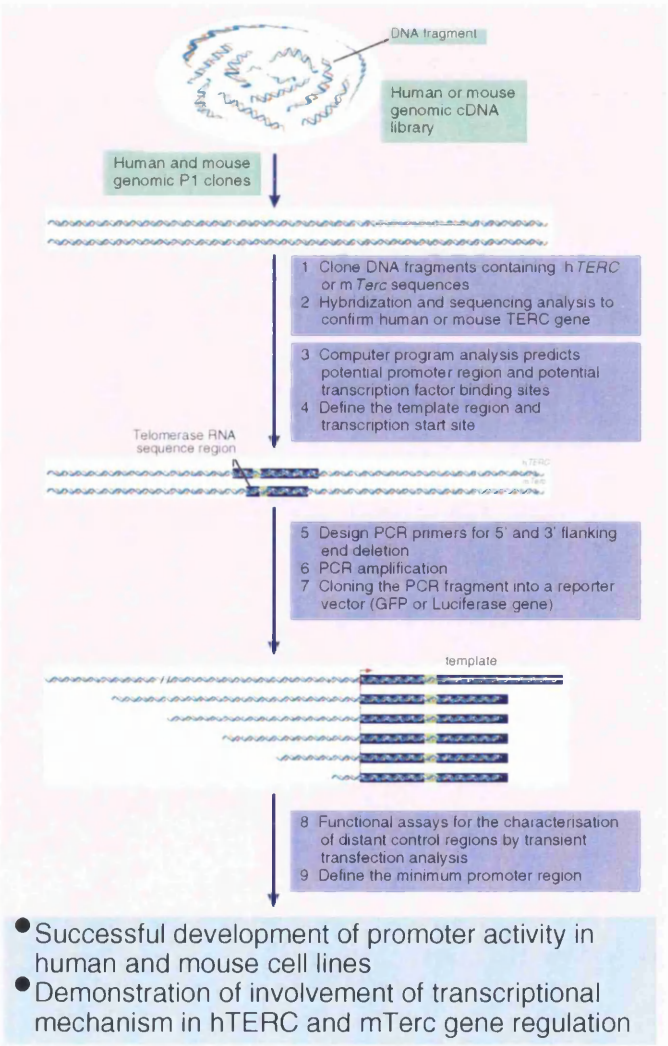


Figure 3.1 Strategy for characterising the functional promoter region

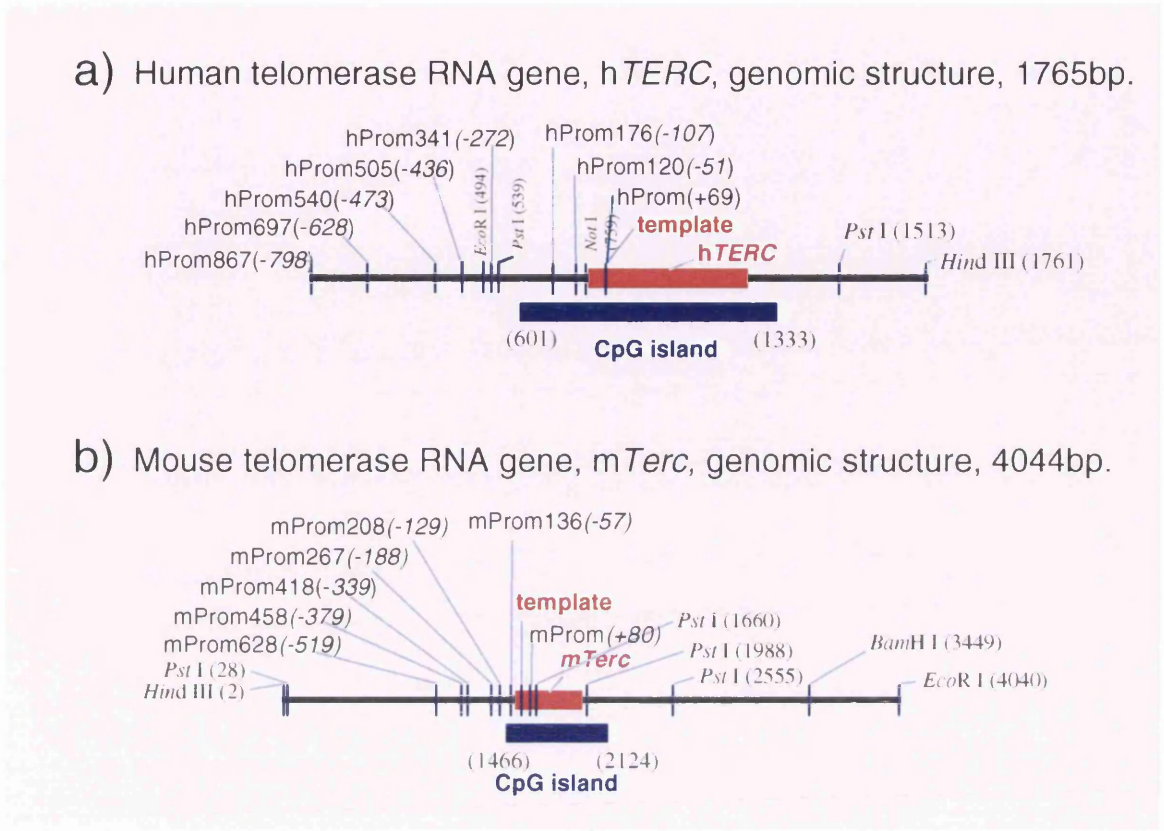


Figure 3. 2 Restriction enzyme map of the h*TERC* and m*Terc* genomic clones

Restriction enzyme map of the genomic clones encompassing the human, (a), and mouse, (b), telomerase RNA genes. The transcribed regions of h*TERC* and m*Terc* are depicted as red boxes within the central regions of the genomic sequences. The site of the template sequence within the telomerase RNA genes is indicated. The positions of the CpG islands are shown as a blue box beneath the genomic sequence. Numbers in brackets refer to the nucleotide position within the sequence. All the human promoter fragments are shown as hProm at the 3'-ends and fragments extend 5-prime to; hProm867, hProm697, hProm540, hProm505, hProm341, hProm176 and hProm120. All the mouse promoter fragments are shown as mProm at the 3'-end and fragments extend 5-prime to; mProm628, mProm458, mProm418, mProm267, mProm208 and mProm136. The numbers after the prefix hProm or mProm refer to the number of nucleotides of genomic sequence contained in the promoter fragment, and numbers in brackets refer to distance from TSS. The 5'-end deletion constructs are listed in Table 3.1.

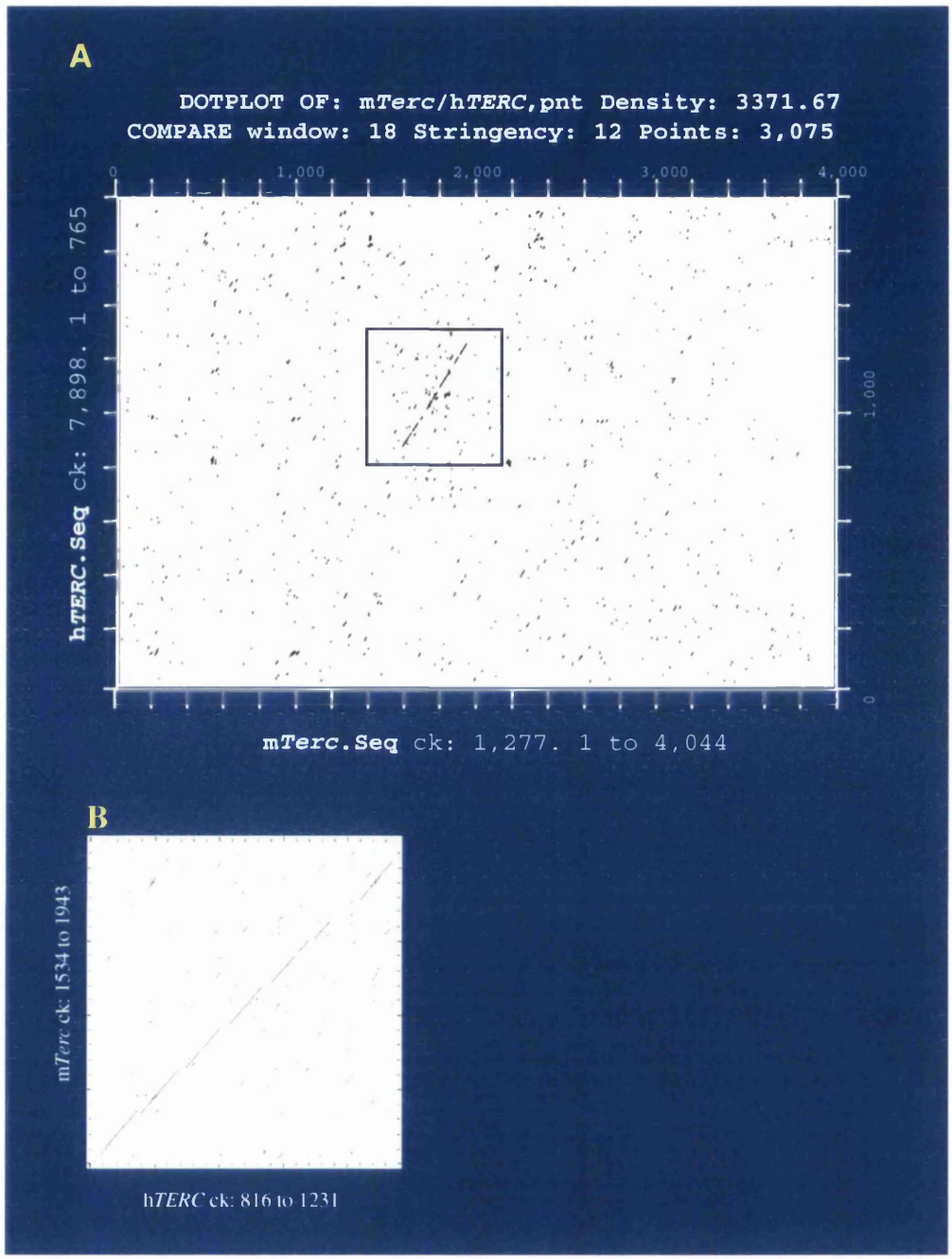


Figure 3.3 Genomic sequences comparison between h*TERC* and m*Terc*

(**A**) An overview comparing h*TERC* (vertical) and m*Terc* (horizontal) 5' and 3' flanking region sequence is shown where each dot represents significant nucleic acid sequence similarity of two genes. The genomic sequence was compared using the COMPARE program (window 18, stringency 14) and graphically displayed using the DOTPLOT program in the UWGCG package. The zoom-up window for the boxed area in (**B**) is shown with comparison between nucleotides 816 to 1231 bp of h*TERC* and 1534 to 1943 bp of m*Terc* in the transcribed region: this region indicates 70.4% sequence identity.

Figure 3.4 Nucleotide Sequence of the h*TERC* and m*Terc* gene 5'-flanking regions

Sequence of the human, (a), and mouse, (b), telomerase RNA gene 5'-flanking regions. Putative regulatory motifs are underlined. Red arrows indicate the transcriptional start sites (Feng et al., 1995; Hinkley et al., 1998) and numbers to the left of each figure refer to the number of bases upstream of the transcriptional start site. The template region is indicated. Sequences contained in promoter constructs are shown by vertical lines and labelled, hProm or mProm (see Figures 3.1 and Table 3.1). The regions containing elements responsible for minimum promoter activity are highlighted in bold (see text for details). The run of CpA dinucleotide repeats in the mouse promoter is shown in bold and italic

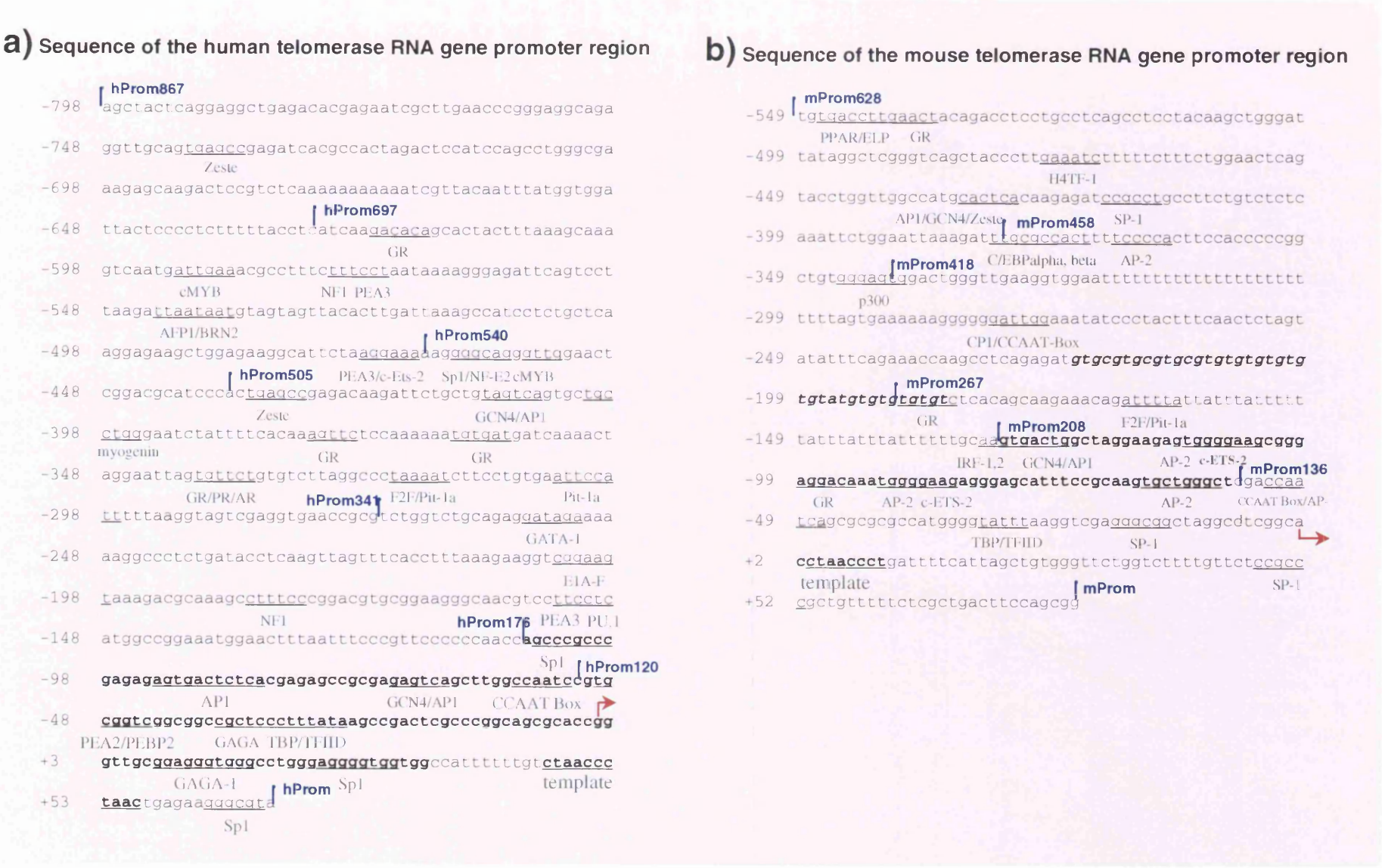


Figure 3.4 Nucleotide Sequence of the h*TERC* and m*Terc* gene 5'-flanking regions

A BLAST search using the 4 kb mouse sequence identified both the published human gene sequences, (HSU85256, HSU86046) and the published sequence for the transcribed region of the mouse gene, MMU33831, (590 bp of sequence) (Blasco et al., 1995; Bryan et al., 1997; Feng et al., 1995). In order to confirm that the genomic sequence obtained from the P1 subclone was genuine, 5'-flanking sequences were cloned using genomic DNA from Balb/c mice in PCR reactions. Sequence analysis of the Balb/c clones was identical to the P1 sequence except for minor polymorphisms. Thus the published sequence for the coding region of the mouse telomerase RNA gene have been extended in both the 3' and 5' flanking regions of m*Terc*. A schematic representation of the 4 kb of sequence information encompassing the mouse telomerase RNA gene is shown in Figure 3.2b.

3.3 Analysis of nucleotide sequence

3.3.1 Genomic cloning sequences of h*TERC* and m*Terc*

The nucleotide sequences of the human and mouse telomerase RNA genes were determined from cloning obtained using PCR products. The human telomerase RNA gene clone is 1765 nucleotides in length and has a base composition of 22.9% A, 26% C, 27.1% G and 24% T. The 450 bp of published h*TERC* RNA sequence (Feng et al., 1995) was found to start at 799 and end at 1248 bp within this 1.7 kb clone. The template sequence of RNA, CTAACCCTAAC, is located between nucleotide 844 and 854 bp. The mouse telomerase RNA gene genomic clone is 4044 nucleotides in length and has a base composition of 25.3% A, 23% C, 24% G and 27.7% T. The 429 base pair of published m*Terc* RNA sequence (Blasco et al., 1995) was found to start at 1524 bp and end at 1952 bp. The template sequence, CTAACCCTG, is located between nucleotide 1560 and 1568 bp. The full sequences have been submitted to GenBank, accession numbers AF047386, AF047387 (See appendix I and II). Thus nucleotide sequences are now available for investigation of the function of the human and mouse telomerase RNA gene promoters to determine the fine mechanisms of transcriptional regulation.

3.3.2 Comparison of h*TERC* and m*Terc* gene sequences

To investigate the similarity of the human and mouse genomic clones, sequence comparisons were carried out. The transcribed regions of the two genes showed 67% identity in keeping with the published estimate (Feng et al., 1995). However, no significant sequence identity could be identified in either the 5'- or 3'-regions flanking the transcribed sequences as shown in Figure 3.3A. The comparisons described here highlight the conserved transcribed regions of two RNA genes as shown in Figure 3.3B

Both the human and mouse sequences were analysed for CpG islands by GRAIL. CpG islands were defined as regions larger than 200 bp, with an average GC content greater than 50% and the ratio of observed versus expected CpGs greater than 0.6 (Gardiner-Garden and Frommer, 1987). Interestingly, both the human and mouse genes lie within CpG islands, (see Figure 3.2). The human gene is covered by a CpG island 733 bp in length, with a GC content of 66% and a ratio of observed versus expected CpGs of 0.89. A CpG island of 659 bp in length, with a GC content of 64%, covers the mouse gene and a ratio of observed versus expected CpGs of 0.81.

3.4 Detection of the h*TERC* and m*Terc* promoter region

3.4.1 Search for the potential transcription factors

The 5'-flanking regions of the human and mouse telomerase RNA genes were analysed for potential transcription factor recognition sites by using computer software described in section 2.6. As shown in Figures 3.4a and b, a number of potential binding sites were identified, including consensus sequences for glucocorticoid/progesterone/androgen receptor binding, AP1 and Ets family members. TATA and CCAAT boxes are found in both genes close to the published transcriptional start sites (Feng et al., 1995; Hinkley et al; 1998).

Analysis of the sequence around the transcriptional start site (TSS) region revealed that the h*TERC* gene promoter consists of a G+C-rich sequence extending from -107 to +69

bp (GC content, 64.7%). This region contains a TATA-box at position -28 to -25 bp, one CCAAT-box at position -58 to -54 bp, three putative Sp1 binding sites and two Ap1 binding sites at position -93 to -83 and -70 to -64 bp (Figure 3.4a). The G+C-rich region of the h*TERC* gene promoter is shorter and its GC content is higher than that of the m*Terc* gene promoter (-129 to +80, 58.2%).

The m*Terc* gene potential promoter also consists of a G+C-rich sequence extending from -129 to +80 bp (GC content, 58.2%) but differs from the h*TERC* promoter in that it lacks a sequence between TSS and template region (Hinkley et al., 1998; Figure 3.4b).A TATA-box is present at -30 to -25 bp, with one inverted Sp1 binding site at position -17 to -12 bp (8 of 10 matching the Sp1 consensus) and one CCAAT-box located at position -53 to -49 bp. Three Ap2 sites are present, two of these overlap with two c-ETS-2 sites. These are clustered between -110 to -79 upstream of the transcription start site. One putative Sp1 binding site is present downstream of TSS. The mouse promoter region also contains a run of CpA dinucleotide repeats that may be of use in developing microsatellite genetic markers for this gene.

3.4.2 Series deletion mutants for the potential promoter

To identify whether the 5'-flanking region of the telomerase RNA genes exhibits promoter activity, sequences were fused to a firefly luciferase reporter gene, (pGL3-Basic). The transcriptional start sites for both the human and mouse telomerase RNA genes have been established (Feng et al., 1995; Hinkley et al., 1998). Various promoter constructs containing the transcriptional start site were therefore generated (Figures 3.2, 3.4 and Table 3.1).

hProm867 contains the h*TERC* promoter from –798 to +69 bp upstream of the luciferase gene of pGL3 basic (Promega). The shortest h*TERC* promoter hProm120 was created by using an oligonucleotide corresponding to the h*TERC* sequence from –51 to +69 bp relative to the transcriptional start site (Feng et al., 1995). Similar luciferase reporter

constructs were created from h*TERC* regions -628/+69, -471/+69, -436/+69, -272/+69 and -107/+69 (Figure 3,2a, Table 3.1). To clone the largest h*TERC* 5'-flanking promoter region (pLhK26, -5.0 Kb/+69), 0.6 Kb Xho I/Not I fragment of hProm(-798/+69) was replaced by about 4.4Kb of Xho I/Not I fragment from Dr Karen Steeghs, (Beatson Institute). pLhE26(-2.5 Kb/+69) was constructed by deletion of EcoR V fragment from pLhK26.

Table 3. 1 The 5' end deletion constructs of h*TERC* and m*Terc*

h*TERC* deletion constructs			m*Terc* deletion constructs		
Name	Other name	Position	Name	Other name	Position
pLhK26	None	-~5.0kb to +69	pL1658	None	-1693 to +80
pLhE26	None	-~2.5kb to +69	pL2730	mProm628	-549 to +80
pLh2023	hProm867	-798 to +69	pL2930	mProm458	-379 to +80
pLh2123*	hProm697	-628 to +69	pL2830	mProm418	-339 to +80
pLh5F23	hProm540	-471 to +69	pL2530*	mProm267	-188 to +80
pLh1417	hProm505	-436 to +69	pL2630	mProm208	-129 to +80
pLh2223*	hProm341	-272 to +69	PL3630	mProm136	-57 to +80
pLh2923	hProm176	-107 to +69	pL2534	mProm196	-188 to -8
pLh26n23	hProm120	-51 to +69	pL2537	mProm154	-188 to -34

* These constructs were used for DNase I footprinting analysis. Position refers to the transcriptional start site.

The mProm628 reporter construct containing -549 to +80 bp of m*Terc* gene promoter was inserted into a pGL3 Basic vector as described above. The shortest m*Terc* promoter mProm136 was created by using an oligonucleotide corresponding to the m*Terc* sequence from –57 to +80 bp relative to the transcriptional start (Hinkley et al., 1998). Similar luciferase reporter constructs were created from m*Terc* regions -379/+80, -339/+80, -188/+80 and -129/-80 (Figure 3.2b, Table 3.1).

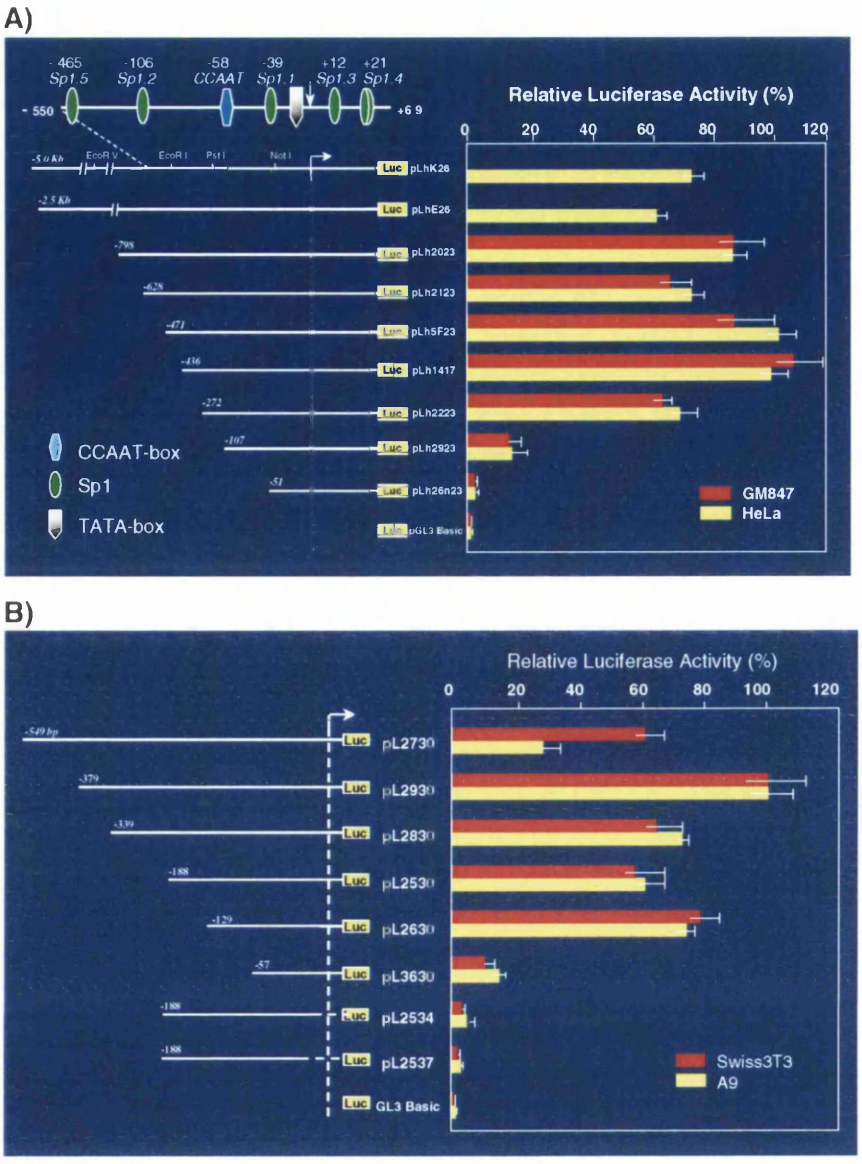


Figure 3.5 Detection of promoter activities in the 5' flanking regions of h*TERC* and m*Terc* genes

Detection of promoter activities in the 5'-flanking regions of human, (A), and mouse, (B), telomerase RNA genes. For each construct having endpoints from -5.0 Kb to -55 bp of h*TERC* gene or having endpoints extending from -549 to -57 bp of m*Terc* gene, the length of sequence upstream from the transcriptional start site is shown to the left and the luciferase activity to the right. pGL3 Basic luciferase vector is shown as negative control.

A) **Diagram comparing luciferase activity from the human promoter constructs in GM847 and HeLa cells.** The predicted promoter structure is shown at the top. The various colours symbols or circles represent the different transcription factor binding sites. The numbers indicate the position of the DNA-binding sites in relation to the TSS. Data for each construct is plotted as a percentage of the hProm505 luciferase activity as this construct consistently gave the highest activity in human cells. Transfection experiment of constructs pLhk26 and pLhE26 in GM847 cell lines did not performed.

B) **Diagram comparing luciferase activity from mouse deletion constructs in Swiss3T3 and A9 cells**. Data for each construct is plotted as a percentage of the mProm458 luciferase activity as this construct consistently gave the highest activity in mouse cells. The name of the deletion constructs is indicated in the centre column and these are also listed in Table 3.1. For each construct the mean and standard deviation for duplicate transfected wells are shown.

3.4.3 Transfection assays detect the promoter activity

3.4.3.1 The h*TERC Luc-reporter construct activity*

The human promoter constructs containing truncated portions of the 5'-flanking region from -5.0 kb to -55 bp were transiently transfected into HeLa and GM847 cells (Figure 3.5A). HeLa is a telomerase positive cervical carcinoma cell line, GM847 is a SV40-immortalized skin fibroblast cell line that expresses the telomerase RNA component but is telomerase-negative (Bryan et al., 1997). As shown in Figure 3.5A, promoter activity was observed in both cell lines with fragments containing 176 bp or more (from position -107/+69, see Figures 3.2a and 3.4a). The highest luciferase activity was observed with construct hProm505(-436/+69) that contains a 505 bp fragment. Construct, hProm120, which contains only 120 bp of 5'-flanking sequence (position -51/+69, see Figures 3.2a and 3.4a), produced a dramatically reduced level of luciferase activity (Figure 3.5A), and is not sufficient on its own for promoter activity. Thus, a minimal promoter sequence can be defined as extending 107 bp upstream of the transcription start site, and that elements responsible for promoter activity may be contained in a 176 bp region.

3.4.3.2 The mTerc Luc-reporter construct activity

Various regions of the mouse promoter (from -549 to +80 bp) were tested for their ability to drive expression of the luciferase reporter gene in transiently transfected Swiss3T3 and A9 cells (Figure 3.5B). Swiss3T3 cells are an embryo derived line and A9 cells are of areolar and adipose origin. Both cell lines are telomerase positive. Results demonstrated that -129, -188 and -379 bp construct showed promoter activity that was significantly higher than the negative control construct (8-fold). As shown in Figure 3.5B, promoter activity was observed in both cell lines with fragments containing from 208 bp or more (from position -129/+80, see Figure 3.2b and 3.4b). Construct mProm136, which contains only 136 bp of 5'-flanking sequence (position -57/+80, see Figure 3.4b), produced low levels of luciferase activity (Figure 3.5B). The promoter activity of mProm196(-188/-8) or mProm154(-188/-34) was completely abolished when sequences between -7 and +80 or -

33 and +80 have been deleted in construct mProm267 (-188/+80, Figure 3.5B), suggesting that this region is necessary for m*Terc* transcription. Thus, a minimal promoter sequence can be defined as extending 57 bp upstream of the transcription start site, and that elements responsible for promoter activity must be contained in a 136 bp region (Figures 3.2b and 3.4b). A 208 bp fragment located at -129 to +80 bp was sufficient for promoter activity, which directs the expression of the reporter gene *in vitro.*

Thus the ability of the upstream DNA to stimulate transcription of a reporter gene in several cell types was demonstrated. Transfection of the human promoter construct hProm867(pLh2023) into mouse cells gave very strong promoter activity, with up to twice that of the strongest mouse construct, and transfection of the mouse promoter construct, mProm628(pL2730) into the human cells also showed luciferase activity at around 25% of the strongest human construct.

3.5 Discussion

The levels of telomerase RNA gene expression vary during normal development and between normal and cancerous cells and tissues (Avilion et al., 1996; Bestilny et al., 1996; Blasco et al., 1995, 1996; Bodnar et al., 1996; Broccoli et al., 1996; Feng et al., 1995; Kuniyasu et al., 1997; Soder et al., 1997a). Knowledge of telomerase RNA gene expression should therefore aid our understanding of the signal transduction pathways linking telomere attrition to proliferation, cellular senescence, differentiation and oncogenesis. As a first step towards this goal, the promoter regions of the human (h*TERC*) and mouse (m*Terc*), telomerase RNA genes have been cloned in order to identify the regulatory elements controlling telomerase RNA gene transcription.

3.5.1 Sequence similarity between the h*TERC* and m*Terc*

In general, there are no significant sequence homologies between the promoter regions of the human and mouse telomerase RNA genes. Indeed, there is considerable debate as to whether telomere length is regulated in a similar fashion in humans and mouse (Blasco et al., 1997; Kipling, 1997a,b; Zakian, 1997). However, mouse models represent a valuable resource with which to study the role of telomerase in cellular senescence and tumour progression and mouse models are likely to be required to investigate new therapies based on telomerase inhibition. In addition, the developmental regulation of telomerase will be more easily approached in mice (Bestilny et al., 1996; Blasco et al., 1995, 1996, 1997; Broccoli et al., 1996; Prowse and Greider, 1995). Thus, knowledge of any differences between the two species may in fact aid our understanding of the function of telomerase in maintaining genome stability and may be important in developing good murine models for human disease or developmental processes. Whether the sequence divergence between the human and mouse gene promoters has any functional consequences can now be studied.

The human and mouse telomerase RNA genes do share an interesting similarity, in that they both lie in CpG islands, and thus their expression may be regulated by methylation. DNA methylation is thought to be important for gene regulation during normal development and cellular senescence, and abnormal methylation patterns may be a fundamental change in tumour progression (Baylin et al., 1991; Bird, 1996; Laird and Jaenisch, 1996; Vertino et al., 1994; Wilson and Jones, 1983). Thus it has been suggested that aberrant CpG island methylation during normal ageing process, could contribute to immortalisation by interfering with expression of 'mortality' genes, of which h*TERC* and m*Terc* can be included (Vertino et al., 1994; Wilson and Jones, 1983).

3.5.2 h*TERC* promoter shares high homology element with b*TERC*

The bovine TERC, (b*TERC*) has recently been cloned, (Tsao et al., 1998). This allows us to carry out a comparative study of the potential promoter functions of these three genes.

The 351 bp of the b*TERC* promoter region has been cloned and sequence in our Lab. But our b*TERC* promoter has additional sequence that was not reported in the previously published sequence (Tsao et al., 1998). There is a sequence GGGTTGCG downstream of TSS (For sequence see appendix VI). Examination of the alignment of h*TERC* and b*TERC* 5' flanking region sequences shows that certain regions are well conserved, particularly between two potential proximal promoter regions. The five potential binding sites were conserved between the h*TERC* and b*TERC* 5'-flanking sequence: a TATA-box element, a CCAAT-box, two GGGTGG motifs (GC-box) and the template region. Despite the lack of sequence similarity between the human and mouse telomerase RNA gene promoter region, h*TERC*, b*TERC* and m*Terc* genes have consensus binding sites such as TATA-box, CCAAT-box and Sp1. Presumably these conserved regions may be of functional significance to the promoter activity. Comparison of proximal promoter from species and the functional divergence will be further discussed in Chapter 5.

3.5.3 h*TERC* minimal promoter is 176 bp in length

Turning to the functional analysis of the cloned sequences, the minimal promoter for h*TERC* resides within a region of 107 bp upstream of the published transcriptional start site (Feng et al., 1995) (Figures 3.2a and 3.4a). There are a number of potential transcription factor binding sites in this region including consensus sequences for AP1, Sp1, PEA2/PEBP2, TATA-box and CCAAT-box. Interestingly, the expression of the fos/jun family of proteins, which determine AP1 activity, are suppressed during the onset of senescence and would be predicted to lead to a reduction in AP1 activity in senescent cells (Campisi, 1997; Irving et al., 1992; Riabowol et al., 1992; Seshadri and Campisi, 1990). AP1 also responds to protein kinase C, and it has recently been demonstrated that h*TERC* expression is induced by protein kinase C during T-cell activation (Bodnar et al., 1996). Extending the promoter region to 436 bp upstream of the transcriptional start site, increases the luciferase activity to its maximum level (Figure 3.5A). This region contains several consensus-binding sites for glucocorticoid/progesterone/androgen receptor binding, which may contribute to the maximal activity demonstrated by hProm505

(pLh1417). Deletion of the sequence from -107 to -51 bp in construct hProm176(-107/69) abolishes the basal promoter activity suggesting requirement of this region for h*TERC* transcription, therefore a minimal functional promoter region is defined in 176 bp fragment at position -107 to +69 bp.

3.5.4 m*Terc* minimal promoter is contained within 136 bp of sequence

The promoter for m*Terc* resides in a 129 bp region upstream of the published transcriptional start site (Hinkley et al., 1998), (Figures 3.2b and 3.4b). A striking feature of this region is the presence of three AP-2 consensus sites, two of which are coupled to c-Ets-2 sites and all these elements are contained in the 73 bp region required for promoter activity (Figure 3.5B). Oncogenic Ras gene signalling has been shown to operate through c-Ets-2 binding sites, thus there is a testable relationship between oncogene activation during tumour progression and telomerase RNA gene transcriptional activity (Galang et al., 1994; Wasylyk et al., 1994). A reduction in promoter activity is observed on extending the promoter fragments to include more 5'-sequence, (Figure 3.5B, mProm628), suggesting that sequences towards the 5'-end of the clone may influence promoter activity in a negative fashion. The shortest construct mProm136(-57/+80) showed a low promoter activity, this minimal promoter region contains a CCAAT-box, TATA-motif and two Sp1 binding sites. Deletion of the sequence from -8 to +80 bp in construct pL2530 (-188/+80) abolishes the m*Terc* promoter activity indicating that this region may be required for m*Terc* gene transcription.

3.5.5 Implications for the study of TERC regulation and gene therapeutics

These studies have a number of implications for the development of new transcription-based therapies for cancer (Cai et al., 1996; Connors, 1995; Miller and Whelan, 1997; Peterson and Baichwal, 1993). Directly down-regulating expression of the telomerase RNA gene through manipulation of transcription factors may be an effective anticancer

therapy and the cloning of the h*TERC* gene promoter will allow the analysis of therapeutic molecules which modulate h*TERC* promoter activity (Cai et al., 1996; Peterson and Baichwal, 1993; Sharma et al., 1997a). Indeed, by using a human cell line which has telomerase activity (HeLa) and one that expresses the hTERC gene but is telomerase negative (GM847) (Bryan et al., 1997), a system has been established in which the specificity of transcriptional manipulation of h*TERC* can be examined. In comparison to HeLa, the growth of GM847 does not appear to be dependant on telomerase expression, thus transcriptional targeting of h*TERC* in GM847 should have no cellular effects, whereas HeLa should be sensitive to the predicted anti-proliferative effects of the transcriptional targeting. In addition, it has recently been shown that there are tumour-specific patterns of h*TERC* gene expression with clear differentials in expression between cancerous and adjacent normal tissue (Soder et al., 1997a,b). Therefore, the h*TERC* gene promoter may be of considerable use in genetic therapies allowing selective expression of therapeutic genes in cancer cells expressing high levels of h*TERC* (Connors, 1995; Miller and Whelan, 1997; Soder et al., 1998).

3.6 Conclusion

These studies have identified potential regions involved in the transcriptional regulation of the human and mouse TERC genes. By defining the h*TERC* and m*Terc* gene promoters it will now be possible to identify the transcription factors which repress or activate these genes transcription. The constructs described in the present study will be ideal for more focused gene therapy studies and the development of transcription based therapeutics. In next chapter, studies that address how the telomerase RNA gene is regulated and which transcriptional regulators are involved in this modulation will be described

Chapter

4

Identification and Mutagenesis of the h*TERC* Transcriptional Regulatory Elements

Key issues

- Identification of protein-DNA interactions at the h*TERC* promoter by DNase I footprinting
- Identification of transcription factor interactions with the h*TERC* promoter by EMSA
- Identification of regulatory sequence elements within the h*TERC* promoter by mutagenesis

Chapter 4 Identification and Mutagenesis of the h*TERC* transcriptional elements

As described in the previous chapter, functional promoter regions have been identified in the 5' flanking sequences of the h*TERC* and m*Terc* genes, and several potential binding sites for transcription factors have been predicted. These studies have provided evidence that both the h*TERC* and m*Terc* gene promoters are regulated at the transcriptional level. The present study focuses on the regulation of the h*TERC* promoter in 5637 and HeLa cell lines and identifies the major sequence elements of the promoter and transcription factors involved in h*TERC* gene expression. The strategy for characterising proteins that bind to a defined control element is summarised in Figure 4.1.

4.1 Introduction

Human telomerase activity can be reconstituted *in vitro* by the essential h*TERC* and hTERT genes (Tesmer et al., 1999; Yi et al., 1999; Autexier et al., 1996), and it is likely that there is a co-ordinated program for the de-repression of telomerase component genes during tumour formation. The complexity of gene regulation in eukaryotes has been well studied in recent years, and it has been shown that transcription, being the first step in the expression of genetic information, is one level at which gene activity is controlled. Transcription is regulated by promoter and enhancer elements recognised by gene-specific DNA-binding proteins, by general transcription factors, and at a higher level, by chromatin structures (Workman & Kingston, 1998). The 5'-flanking regulatory regions of the h*TERC*, hTERT (Zhao et al., 1998; Cong et al., 1999; Horikawa et al., 1999; Oh et al., 1999; Wick et al., 1999; Takakura et al., 1999) and the mouse (Zhao et al., 1998, 1999; Hinkley et al., 1998) telomerase RNA genes have been partially characterised. Studies of the 5' flanking region of h*TERC* and m*Terc* have identified the minimal promoter regions for these genes (Zhao et al., 1998; Chapter 3), however the specific transcriptional elements and molecular basis for this regulation has not yet been defined.

Transient expression of 5'-end truncated h*TERC* promoter-reporter constructs identified the elements responsible for proximal promoter activity are contained in -107 to 69 bp region relative to the transcriptional start site (TSS). In this study, I showed that mutation or deletion of the h*TERC* CCAAT-box abolishes the proximal promoter activity completely, whereas mutation of the h9 region of sequence from GCTC to ATAG (-36/-33 bp) results in an increased Luc-reporter activity.

4.2 Results

4.2.1 DNase I Footprinting to detect DNA regions bound by nuclear proteins

To investigate trans-acting factors that bind to the h*TERC* promoter in the -628 to +69 bp region, HeLa nuclear extracts were allowed to bind to 312 bp (-628/-306) and 375 bp (-306/+69) DNA fragments which were then used for DNase I footprinting analysis (see Figure 2.2 for strategy). DNase I footprinting of this region revealed 7 protected regions designated H2 (-545 GATTAATAA -537 bp), H3 (-155 CTTCCTC -149 bp), H4 (-110 ACCAGCCCGCCCGAGAGAGT -95 bp), H5 (-467 AAGGGGCAGGGTTGGAAC -450 bp), H9 (-40 GGCCGCTCCCTTTATAA -24 bp), H10 (-60 GGCCAATCCG -50 bp) and H11 (+6 CGGAGGGTGGGCCTGGGAGGGGTGGTGG +34 bp). Only the H2, h5 and h11 protection regions were shown in Figure 4.2. The 3' end of the promoter was not well defined because of its proximity to the labelled end of the probe, and the presence of proteins that bind non-specifically to the ends of DNA. Although several DNA elements were protected, the three regions shown in Figure 4.2 (H2, H5 and H11) were the most strongly protected sequences. In following section, regions H4, H9, H10 and H11 were investigated further using the electrophoretic mobility shift assay (EMSA) as they lie in the minimum h*TERC* promoter region as identified in chapter 3.

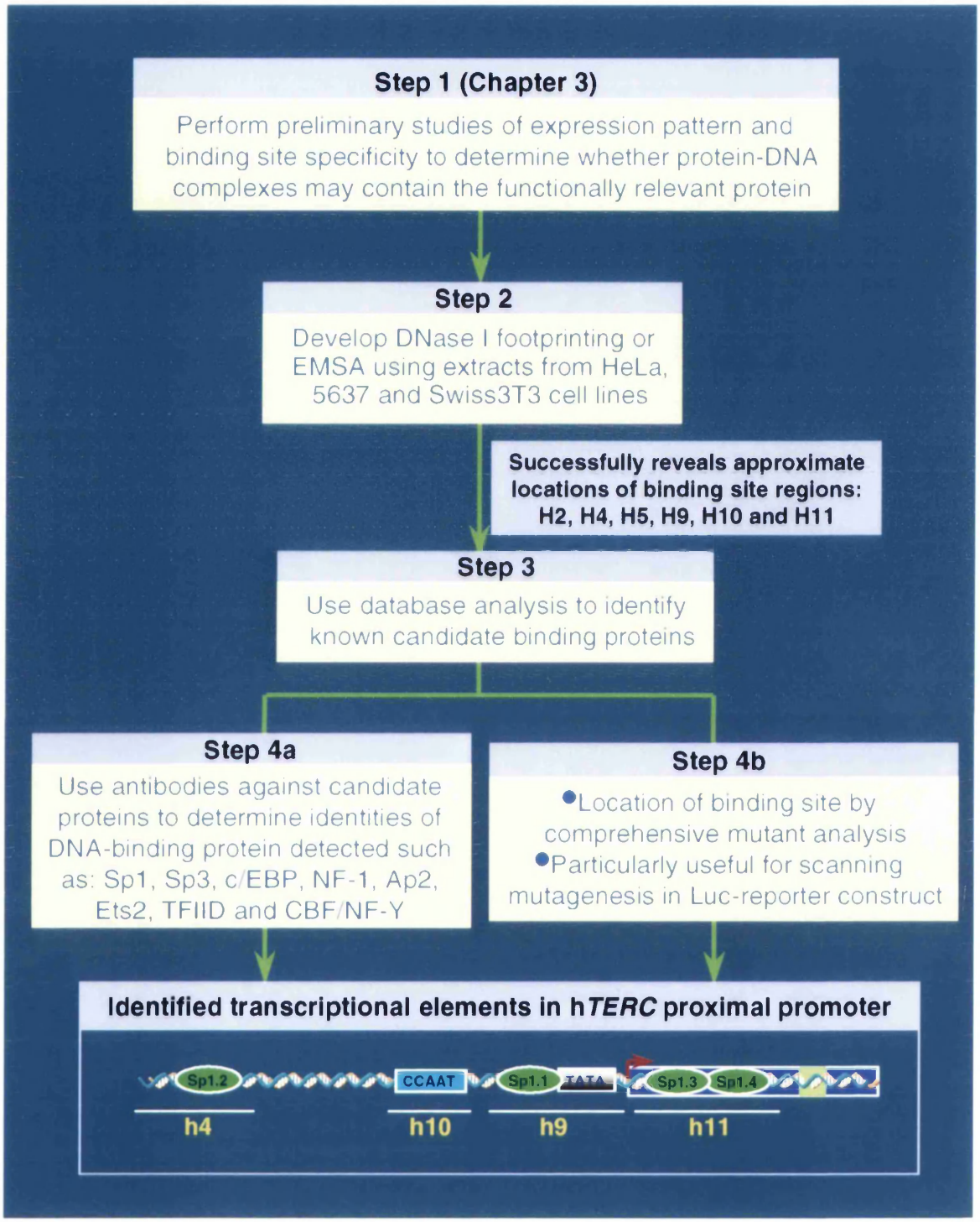


Figure 4.1 Strategy for characterising proteins that bind to a defined control element

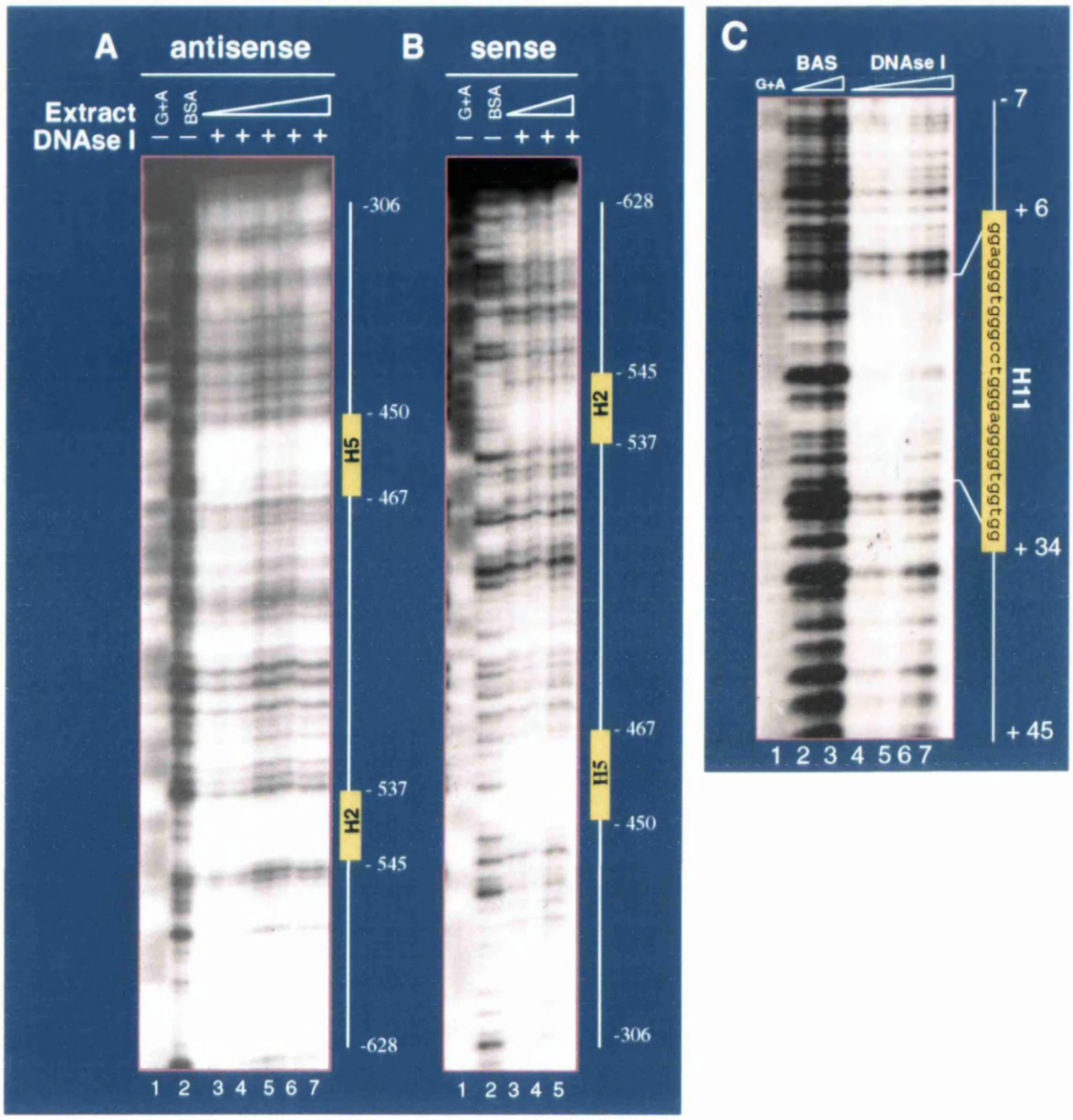


Figure 4.2 DNase I footprinting analysis of h*TERC* promoter region

The strategy for DNase I footprinting analysis is shown in Figure 2.2. The antisense (**A**) and the sense (**B**, **C**) DNA fragments were labelled by ^{32}P-ATP at either end and incubated with 50 ug of HeLa nuclear extract in a footprinting reaction. DNAse I footprint analysis reveals protein binding regions in the h*TERC* promoter (from -628 to -306 bp, or -7 to +45 bp) which are indicated by yellow rectangles. Maxam-Gilbert G+A sequence results from the probe are shown in lane 1. BSA control is shown in lane 2. Footprints were produced with increasing quantities of DNase I (A; lanes 3 to 7, B; lanes 3 to 5, C; lanes 4 to 7). DNA protection region indicated as H2, H5 and H11 correspond to the promoter sequence.

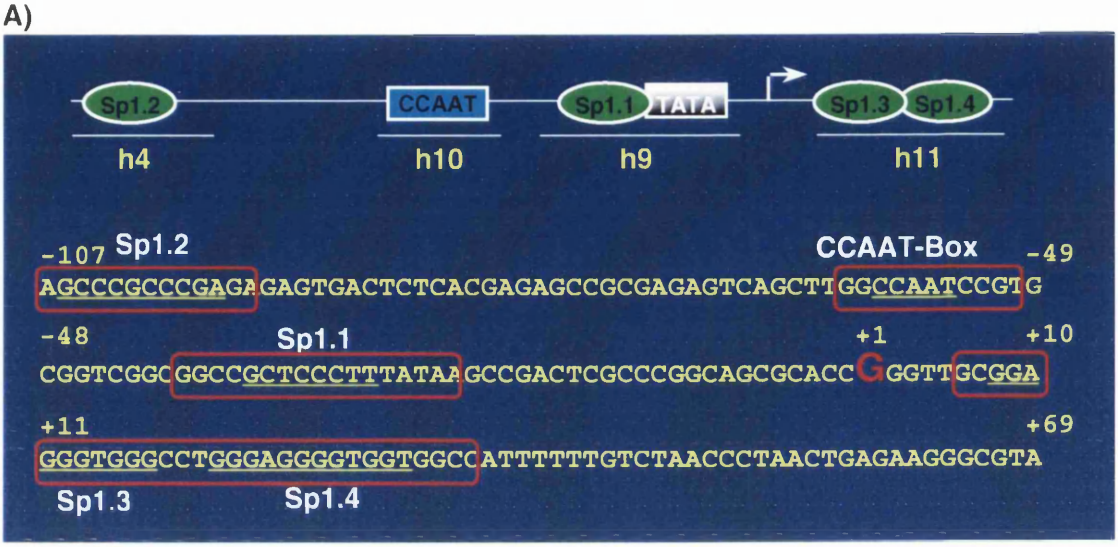
Figure 4.3A h*TERC* proximal promoter sequence and identified transcription factor binding sites

176 bp of h*TERC* proximal promoter sequence (-107/+69) is shown. The transcriptional start site (TSS) is marked as "+1" . The numbers at either end of the sequence relate to the TSS. The four red boxes indicated are protein protected regions detected by DNase I footprinting analysis. Four identified Sp1 binding sites are underlined and termed Sp1.1 to Sp1.4. Sp1.4 contains two overlapping Sp1 binding sites. The promoter structure is also depicted at the top of diagram. Lines marked h4, h9, h10 and h11 indicate oligonucleotides used for EMSA.

Figure 4.3B Oligonucleotides used in EMSA and mutagenesis studies of the h*TERC* promoter.

The sequence of the wild type oligonucleotides covering potential transcription factor recognition sites are shown. Oligonucleotide h9 covers Sp1.1 and the adjacent TATA-box, h10 covers the CCAAT-box, h4 covers Sp1.2 and h11 covers the overlapping Sp1.3 and Sp1.4 sites. Mutations, h10m1 and h10m2, were introduced into the wild type oligonucleotides for use in EMSA and the construction of mutant reporter constructs. Oligonucleotide mutations are shown below wild type sequences. Regulatory motifs underlined on the wild type sequence are determined by mutation analysis or previously reported (Kim et al., 1992; Thiesen et al., 1990). These oligonucleotides are also listed in Table 2.4

A)



B)

Sp1.1	h9(wt:-44/-17)	CGGCGGCCGCTCCCTTTATAAGCCGACT
	h9m1 (-43/-40)	:TTAC::::::::::::::::::::::::
	h9m11(-39/-36)	:::::TAAA::::::::::::::::::::
	h9m2 (-36/-33)	::::::::ATAG::::::::::::::::
	h9m21(-32/-29)	::::::::::::ATGC::::::::::::
	h9m3 (-28/-25)	::::::::::::::::CGAC::::::::
Sp1.2	h4(wt:-110/-91)	ACCAGCCCGCCCGAGAGAGT
	h41m(-101/-100)	:::::::::AA:::::::::
Sp1.3/4	h11(wt:-2/+36)	CCGGGTTGCGGAGGGTGGGCCTGGGAGGGGTGGTGGCC
	h111 (+11/+13)	::::::::::::AAA:::::::::::::::::::::::
	h112 (+24/+26)	:::::::::::::::::::::::::::TAA::::::::::
	h113	::::::::::::AAA:::::::::::TAA::::::::::
	h112c	:::::::::::TAA::TAA:::::
CCAAT Box	h10(wt:-63/-42)	CTTGGCCAATCCGTGCGGTCGG
	h10m1 (-58/-55)	:::::AGTC::::::::::::::
	h10m2 (-51/-48)	::::::::::::TGAT::::::

Figure 4.3A

Figure 4.3B

4.2.2 Potential binding sites in the proximal promoter

Eukaryotic gene expression is controlled by a combination of effects from various *cis*-acting elements present in the promoter and enhancer regions. In general basal transcription is regulated by binding sites located in the proximal region of promoters close to the transcriptional start site.

As described in section 3.4.1 and 3.5.3, minimum h*TERC* promoter has been narrowed down to 176 bp in length. Using the Transcription Element Search Software (TESS) (web address see appendix V) to examine this region (+107/+69 bp, Figure 3.3a) several potential transcription factor binding sites were identified. These include one CCAAT-box and TATA-box element, three Sp1 sites, two GAGA and Ap1 sites, one PEA2/PEBP2 binding motif and 90.5% identity for a YY1 site. Four regions, H4, H9, H10 and H11 indicated by the red box in Figure 4.3A, detected by DNase I footprinting match the Sp1, GAGA/TATA-box, CCAAT-box, and GAGA-1/Sp1 potential site, respectively.

4.2.3 Sp factors bind to the h*TERC* promoter at multiple sites

4.2.3.1 Sp1 binds to the h9 region

To identify the factors binding to the H9 region, oligonucleotides containing binding sites for Sp1, TFIID, Ap2 and Ets2 were tested as competitors for h9 (-44/-17, for sequence see Table 2.2 and Figure 4.3B) binding in an electrophoresis mobility shift assay (EMSA). The results of the EMSA are shown in Figure 4.4A. In all but two instances the complexes remained unchanged. The exceptions were competition with oligonucleotides corresponding to Sp1 and h9 itself (Figure 4.4A, lanes 2 and 4). The Sp1 consensus oligonucleotide competed with the three slowest migrating complexes (Sp1 and Sp3) suggesting that Sp1 or related factors together with one or more unknown proteins are responsible for the h9 binding characteristics (Figure 4.4A, lane 2). In contrast, a consensus TFIID oligonucleotide did not compete for h9 binding activity (Lane 3). Ap2, a transcription factor that binds to the consensus GCGGGGGCG, may compete with some

of the slowest migrating complexes suggesting an association with Ap2. Indeed the core h9 sequence shows a 6/10 match with the Sp1 consensus and 7/9 matches with Ap2 consensus (Figure 4.4B).

As shown in Figure 4.5A, an antibody against the Sp1 protein causes a super-shift of the upper band, indicating that Sp1 is part of this protein complex (Figure 4.5A, lane 9). The second and third lower bands were super-shifted by antibodies against Sp3 (lane 10). Antibodies against Ap2 or Ets2 failed to interact with any of the protein complexes binding to h9 (lanes 11 and 12). Depending on antibodies being effective in this assay, these studies demonstrate that transcription factors Sp1 and Sp3 specifically bind to the h*TERC* h9 region (termed Sp1.1)

4.2.3.2 Mutation of the h9 sequence prevents Sp1 binding

Since the h9 region (-44/-17 bp) containing the Sp1-binding site was present in the critical promoter region, additional studies were carried out using site-directed mutagenesis to clarify the specific nucleotides responsible for Sp1 binding activity. Mutations were created that spanned the h9 region with 4 sequential nucleotide substitutions (mutants from h9m1 to h9m3) as shown in Figure 4.3A. EMSA was performed and results are shown in Figure 4.5A, lanes 3-7. The three major DNA-protein binding complexes were disrupted by a 100-fold molar excess of unlabelled h9 oligo itself (lane 2). Mutants h9m1, h9m11 and h9m3 each retained the ability to displace nuclear protein binding (lanes 3, 4 and 7), suggesting that regions -43 to -36 bp and -28 to -25 bp were not critical for the DNA-protein interaction. Mutants' h9m2 and h9m21 did not compete for Sp1 binding activity suggesting (lanes 5 and 6) that the region -36 to -29 bp is important for h9 binding to protein. To demonstrate that this sequence change did not introduce a new protein binding site, EMSA was performed using labelled h9m2^{a} oligonucleotide as a probe, as shown in Figure 4.4A. The results show that no specific complexes can be formed with h9m2 oligonucleotide and HeLa nuclear extract protein (Figure 4.4A, lanes 7 to 12).

These data clearly indicate that the specific binding of Sp1 and Sp3 nuclear proteins to the h*TERC* promoter primarily involves nucleotides -39 to -29 bp and that each of the residues -36 to -29 bp (5'-GCTCCCTT-3') are essential. The TATAA sequence (-28/-24 bp) does not appear to be responsible for protein binding under these experimental conditions.

4.2.3.3 Two Sp1 binding sites in h11 region

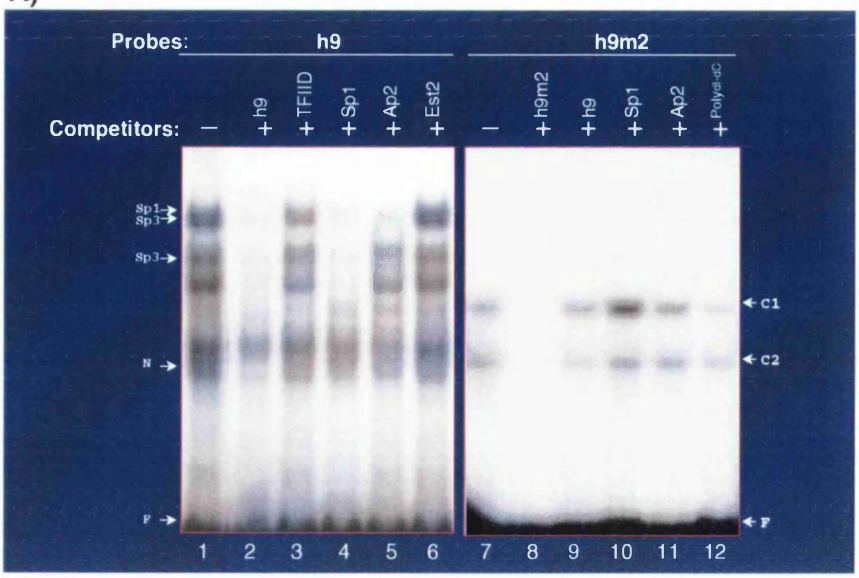
The two putative binding sites for GAGA-1 and Sp1, at positions +9 to +18 bp and +23 to +32 bp within the H11 region, have 96% and 100% identity with the Sp1 binding sites described by Kim (1992) and Thiesen (1990). Interestingly, this sequence region, +1 5'-GGGTTGCGGAGGGTGGGCCTGGGAGGGGTGGTGGCC-3' +36, also contains two copies of GGGTGG motif (also called retinoblastoma control element, termed RCE) identified in the promoter of the *c-fos* proto-oncogene (5'-CCCGCGCGCCACCCC-3', core element underlined)(Pai & Bird, 1997; Felzien et al., 1997; Udvadia et al., 1992 and 1993). This motif in the *c-fos* gene has recently been shown to bind both Sp1 and Sp3 (Adnane et al., 1999; Udvadia et al., 1993 and 1995; Kennett et al., 1997). It would therefore be interesting to test whether the Sp-family of factors bind to these elements and whether pRb is involved in the regulation of h*TERC* transcription. These issues will be discussed further in section 5.2.3.

To clarify which specific transcription factors target this region, an EMSA was performed with a ^{32}P-labelled h11 oligonucleotide corresponding to the -2/+36 bp region (see sequence in Figure 4.3B). As shown in Figure 4.5C, EMSA revealed the formation of several specific DNA-protein complexes (lanes 1 and 12). The combination of Sp1 and Sp3 antibodies caused a super-shift of almost all of the DNA-protein complexes (lane 15), whereas antibodies against Ets2 did not cause any super-shifts (lane 16). These experiments identify Sp1 and Sp3 transcription factors as binding to the h11 region.

To further map the exact position of these Sp1 binding sites in h11 region several oligonucleotides containing different site-directed mutations spanning the h11 region (termed h111, h112 and h113, Figure 4.3B) were tested for their ability to compete with ^{32}P-labeled h11 for protein binding. As shown in Figure 4.5C, the oligonucleotides h111 and h112 (lanes 3-8) competed as effectively as the unlabeled h11 oligonucleotide itself (lane 2), whereas oligonucleotide h113 (lanes 9-11) was a less effective competitor than unlabeled h11. These results indicate that both the GGG(+11/+13) and the AGG(+24/+26) within the Sp1 elements are necessary for the formation of DNA-protein complexes. The oligonucleotide h113 shows weak competition that may be due to the remaining GGTGG motif (+27/+31 bp), and may therefore bind Sp1 protein (Sabath et al., 1998). This was proved by mutation of the GG to AA at position +30/+31 in oligonucleotide h112c (for sequences see Table 2.3 and Figure 4.3B) that totally abolishes the ability to bind Sp1.

To summarise, these results show that at least two distinct Sp1 elements termed Sp1.3 and Sp1.4 are present between the TSS and the RNA template region. Mutations in either one of these sites (oligo's h111 and h112) reduce the affinity of the binding to Sp1 proteins; and mutations in both sites (mutant h113) causes a decrease in binding ability, however it is still not totally abolished, suggesting the presence of an other potential binding sequence in this region, possibly the GGTGG motif at +27 to +31.

A)



B)

```
Ap2cons          5'-GCCCGCGGC-3'
                 5'-GCCNNNGGC-3'
                    ||||||  |      (Match 7/9)
h9 (-44/-17) 5'-CGGCGGCCGCTCCCTTTATAAGCCGACT-3'
             3'-GCCGCCGGCGAGGGAAATATTCGGCTGA-5'
                      ||  |  |||
Sp1cons            3'-GGGGCGGGGC-5'(Match 6/10)
```

Figure 4.4 Sp1 Protein binding to h*TERC* h9 region (-44/-17)

A) Protein binding to h*TERC* h9 region (-44/-17) EMSA was performed using HeLa nuclear extract and the radiolabelled double-stranded oligonucleotides h9 or h9m2. Competition was performed in the presence of 100-fold molar excess of h9 (lane 2), or consensus DNA-binding oligonucleotides (lanes 3-6). Specific and unspecific DNA-protein complexes are indicated with arrows corresponding to Sp1/Sp3, Sp3 and N, respectively. F is free probe. Double-stranded mutant oligonucleotide h9m2 probe was used in EMSA analysis as shown on the right hand side. Competition was performed in the presence of 100-fold molar excess of the non-radioactive h9m2 oligonucleotide (lane 8), h9 oligonucleotide (lane 9), consensus Sp1 binding-site sequence (lane 10), Ap2 (lane 11), or 5 ug poly(dI-dC) (lane 12). C1 and C2 are indicated by arrows as unspecific DNA-binding complexes.

B) h9 region sequence An alignment of the h9 sequence with Sp1 and Ap2 consensus sequence. The h9 sequence shows a 6 to 10 bp match with the Sp1 consensus and 7 to 9 bp match with Ap2 consensus sequence. The core sequence responsible for h9 DNA binding activity is underlined.

Figure 4.5 Identification of Sp-family factors binding to the h*TERC* promoter

HeLa nuclear extract was mixed with radiolabelled oligonucleotide probes and analysed by EMSA. Specific DNA/protein complexes are indicated by arrows on the left. Competition was performed in the presence of 100-fold molar excess of the cold-probe alone (lane 2), or site-replaced mutation oligonucleotides (Figure A: lanes 3-7; Figure B: lane 4), or increasing concentrations of mutant oligonucleotides (25, 50 and 100-fold molar excess, Figure C: lanes 3-11). In Figure 4.5C, complexes supershifted by preincubation with antibodies are shown on the right by arrows marked 'ss'. The oligouncleotides used as probes are indicated at the top of each figure.

A) Protein complex formation at the Sp1.1 site in h9 region.

B) Protein complex formation at the Sp1.2 site in h4 region.

C) Protein complex formation at the Sp1.3/Sp1.4 sites in h11 region. Sequences of all oligonucleotides are shown in Figure 4.2B and Table 2.4.

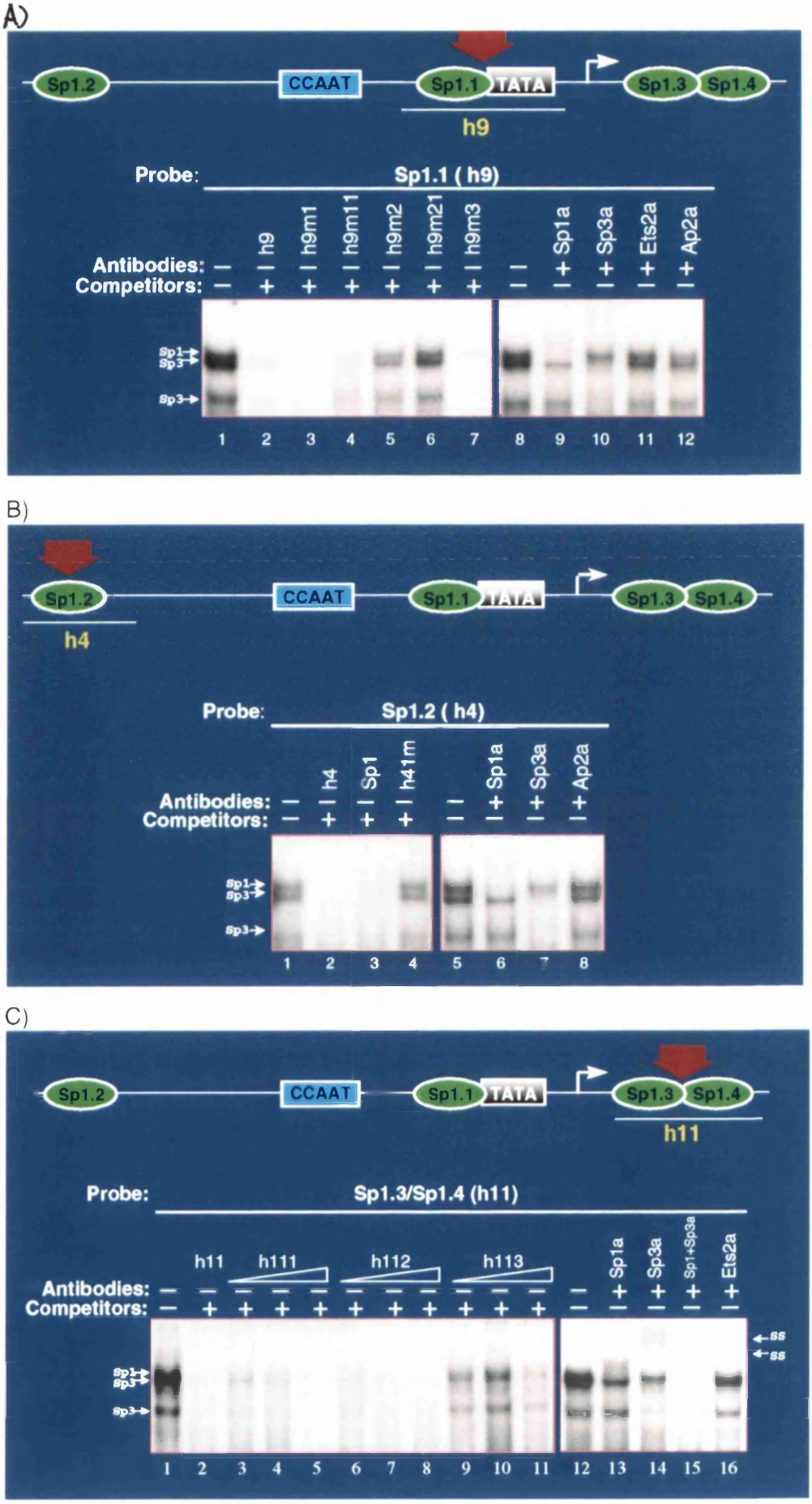
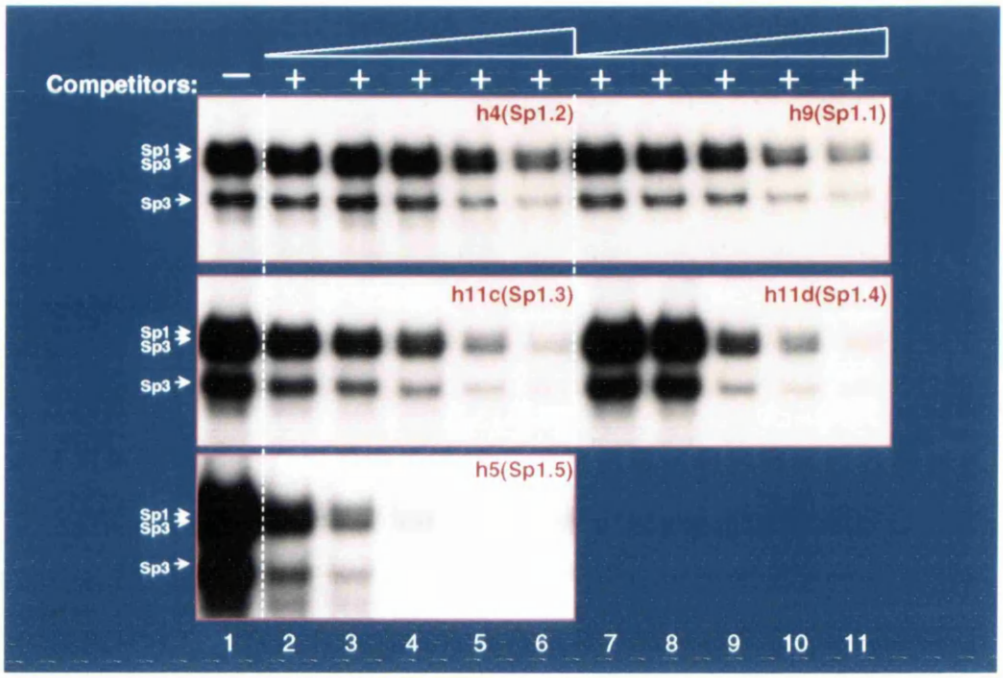

A)
Sp1.2
CCAAT
Sp1.1
TATA
Sp1.3
Sp1.4
h9
Probe:
Sp1.1 (h9)
Antibodies:
Competitors:
h9
h9m1
h9m11
h9m2
h9m21
h9m3
Sp1a
Sp3a
Ets2a
Ap2a
Sp1
Sp3
1 2 3 4 5 6 7 8 9 10 11 12
B)
Sp1.2
CCAAT
Sp1.1
TATA
Sp1.3
Sp1.4
h4
Probe:
Sp1.2 (h4)
Antibodies:
Competitors:
h4
Sp1
h41m
Sp1a
Sp3a
Ap2a
Sp1
Sp3
1 2 3 4 5 6 7 8
C)
Sp1.2
CCAAT
Sp1.1
TATA
Sp1.3
Sp1.4
h11
Probe:
Sp1.3/Sp1.4 (h11)
Antibodies:
Competitors:
h11
h111
h112
h113
Sp1a
Sp3a
Sp1+Sp3a
Ets2a
ss
ss
Sp1
Sp3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 4.6 Differential Sp1 binding affinity

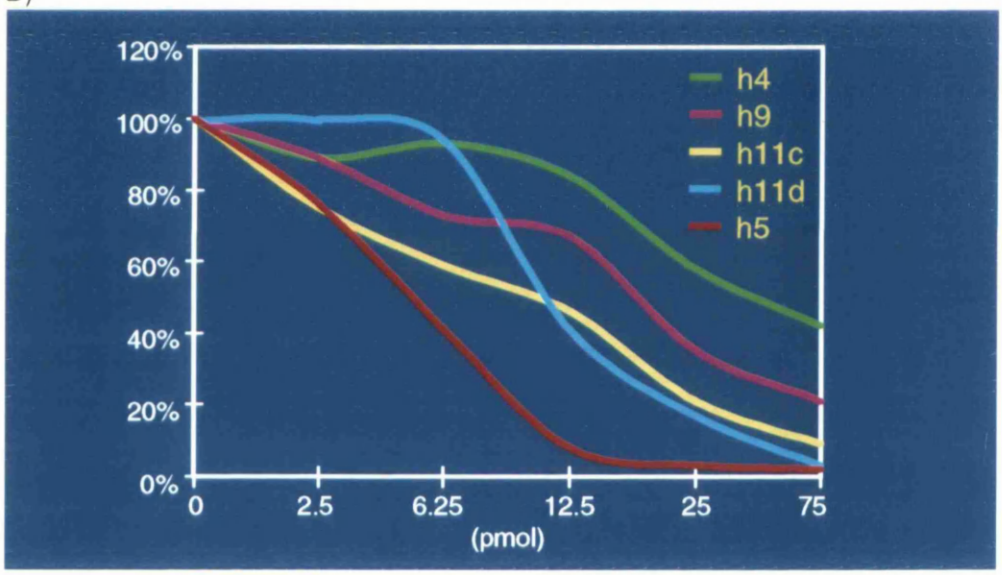
A) Consensus Sp1 oligonucleotide (Promega) was radiolabelled by kinase treatment using [γ-^{32}P] dATP. 5.5 ug of HeLa nuclear extract was incubated with increasing concentration of each unlabelled oligonucleotide for 10 minutes followed by incubation with the Sp1 probe and electrophoresed on a 5% polyacrylamide gel. Molar excess (increasing from 0, 2.5, 6.25, 12.5, 25 to 75 pMol present in lanes 2-6 or lanes 7-11) of unlabelled h4 (Sp1.2), h9(Sp1.1), h11c(Sp1.3), h11d(Sp1.4) and h5(Sp1.5) oligonucleotides are indicated. Lane 1 without competitors was used as a control. Three major DNA-protein complexes are indicated with arrows.

B) Diagram shows the affinity of each Sp1 element to the Sp1 protein. Quantitative analysis of the dried gel was performed using both a computing PhosphorImager with ImageQuant software (Molecular Dynamics) and autoradiography on Kodak XAR-5 films.

C) Alignment of identified GC box in h*TERC.* The numbers indicate the sequence position in the promoter region. The core Sp1 binding sequences are underlined in bold and differential Sp1 affinity is shown at the bottom.



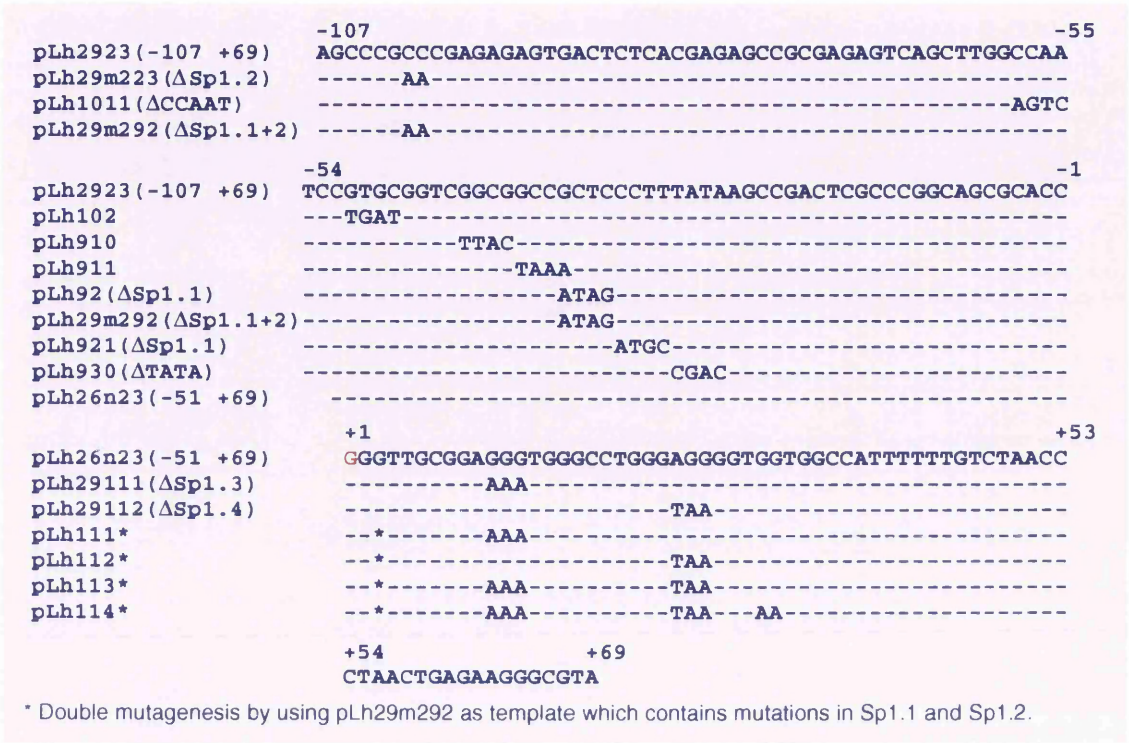
B)



C)

Sp1	ATTCGAT**CGGGGCGGGG**CGAGC	Consensus Sp1 binding sequence
h5	-471 GAAAA**AGGGGCAGGG**TTGGA -452	Sp1.5 in this study
h4	-91 ACTCTC**TCGGGCGGGC**TGGT -110	Sp1.2 in this study
h9	-17 AGTCGGCTTATAA**AGGGAGCGGC**CGCCG -45	Sp1.1 in this study
h11c	-2 CCGGGTTGCG**GAGGGTGGGC**CTGGG +23	Sp1.3 containing RCE (Kim, 1992)
h11d	+14 GCCTGG**GAGGGGTGGT**GGCC +36	Sp1.4 containing 12t (Thiesen, 1990)

Affinity of Sp1: h5 > h11d > h11c > h4 > h9



* Double mutagenesis by using pLh29m292 as template which contains mutations in Sp1.1 and Sp1.2.

Figure 4.7 Scanning mutation analysis of h*TERC* promoter (-107/+69).

The sequence of the wild-type h*TERC* proximal promoter is shown at the top, and identified regulatory motifs are underlined. The name of each mutant construct with mutation site is indicated on the left hand side. The number on either side of the sequence is related to the transcriptional start site. Dashes indicate an identical sequence to the wild type, mutated nucleotides are shown below the wild type sequence. The multiple site-replaced mutations were created by using construct pLh29m292 (ΔSp1.1/Δ Sp1.2) as template and indicated as '*'. Each mutant was confirmed by sequencing.

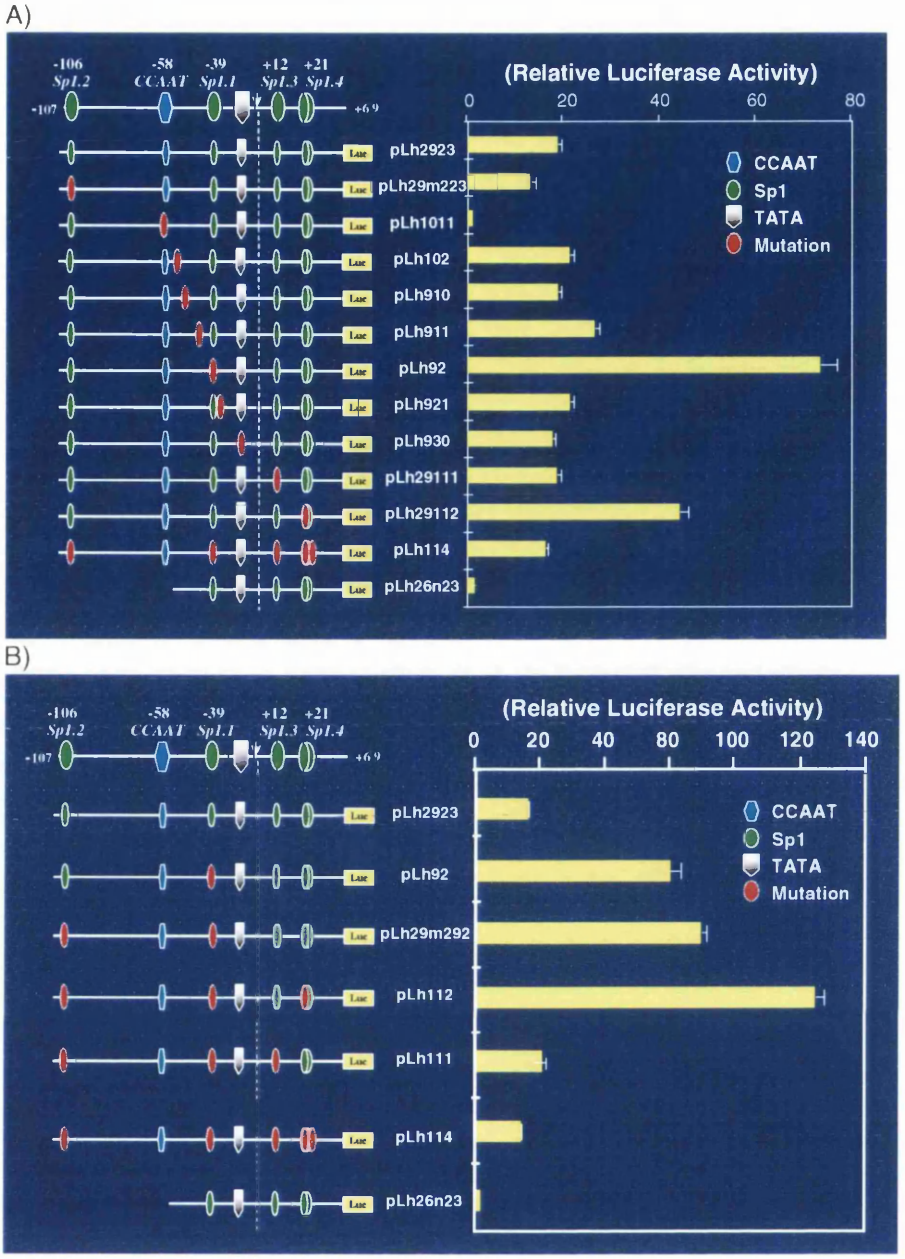


Figure 4.8 Promoter activities of the site-replaced mutant constructs

The predicted promoter structure is shown at the top. The various colours symbols or circles represent the different transcription factor binding sites. The numbers indicate the position of DNA-binding sites related to the transcriptional start site. The constructs are shown with red circles indicating a site-replaced mutation in one or more positions. The names of the Luc-reporter constructs are shown in the middle column. The result of the promoter activity is shown on the right hand side. Three micrograms of each plasmid were used for transfection analysis in 5637 cells. (For DNA quantitation, transfection and luciferase assays see section 2.3.4 or 2.4.4). Promoter activities of the mutant constructs were assayed by transfection and compared to the wild-type promoter (pLh2923). For each transfection the mean and standard error for duplicate samples are shown. Hatched bars show relative luciferase activities, error bars show standard deviations. The pSEAP2 Control plasmid was used as an internal control to normalise the transfection efficiency.

A) Promoter activities of single site-replaced mutant constructs

B) Promoter activities of double or multiple site-replaced mutant constructs

4.2.3.4 h4 region is recognised by Sp1

EMSAs were also performed with DNA probe spanning footprint H4 region, containing potential Sp1 site termed Sp1.2. HeLa nuclear extract formed three major complexes on the h*TERC* DNA probe h4 (Figure 4.5B, lane 1). These three complexes were competed by the Sp1 consensus oligonucleotide (lane 3) and cold h4 oligonucleotide itself (h4, lane 2). These complexes were not competed when the oligo's contained the double-point mutation in the potential Sp1 site (h41m, lane 4, for sequence see Figure 4.3B). After incubation with antibodies to Sp1 or Sp3, the number of complexes formed with the nuclear extract was reduced (Figure 4.5B, lanes 6 and 7) respectively. Antibodies against Ap2 failed to interact with any of the protein complexes binding h4. The faster migrating minor complex formed by nuclear proteins with h4 oligo's appears to be non-specific. These experiments therefore demonstrate that Sp1 and Sp3 are two of the proteins that bind to motifs within the h4 regions in the h*TERC* promoter. An Sp1 site was also predicted in H5 footprint region; this region was also investigated by EMSA even though it lies upstream of the proximal promoter region. The result was identical to those for h4.

4.2.3.5 Sp1 sites show different affinities to Sp1 protein

As shown in the preceding section, five different Sp1 binding sites were identified each with different sequences, it seemed interesting to determine if there were any differences in their binding affinities for Sp1. To approach this question, an end-labelled Sp1 consensus sequence oligonucleotide probe was utilised in competition EMSAs with excess unlabelled h*TERC* Sp1 element oligonucleotides. As shown in Figure 4.6A and B, the Sp1.5 (h5) oligonucleotide was able to efficiently compete the labelled Sp1 probe at a low level molar excess concentration, whereas the Sp1.1(h9) and Sp1.2(h4) oligonucleotides required at least 100- to 1000- fold excess concentrations, respectively, for efficient competition (Figure 4.6B). The two cold oligonucleotides, h11c and h11d, designed separately from h11 sequence, competed well with a radio-labelled commercial Sp1 consensus oligonucleotide binding activity as shown in Figure 4.6A. Since h11 contains the elements that show similarity to Sp1 sites, the ability of a consensus Sp1

(CGGGGCGGGG) oligonucleotide to compete with h11 oligonucleotide for binding to nuclear proteins was assessed. These data indicate that the relative affinities of the h*TERC* GC-box elements as determined by EMSA are Sp1.5(h5) > Sp1.3(h11c) > Sp1.4(h11d) > Sp1.2(h41) > Sp1.1(h9) (Figure 4.6C). In conclusion, at least five Sp1 binding sites have been identified in the h*TERC* promoter region and those sites show a differential binding affinity to Sp1 family of transcription factors.

4.2.4 Refining the functional regions in the h*TERC* promoter

A number of putative sequence elements were identified in the h*TERC* proximal promoter (Figure 4.3A, -107/+69 bp) described above. These motifs were subsequently focused on as the promoter elements most likely to be supporting h*TERC* transcription and for further analysis by site-directed mutation. To examine the involvement of each of these elements in the h*TERC* gene transcription, a series of single site-directed mutant Luc-reporter constructs were created and their corresponding luciferase activities were monitored in 5637 cells. The mutant sequences of each construct were shown in Figure 4.7.

A comparison of promoter activities between this series of mutant constructs is shown in Figure 4.8A. The wild-type (pLh2923, -107/+69) and neutral mutant constructs (pLh102, pLh910, pLh911, pLh921 and pLh29111) show similar promoter activities. Mutation or deletion of the CCAAT-box sequence element [constructs pLh1011 (ΔCCAAT-box), and pLh26n23(del-CCAAT-box, -51/+69) respectively] completely abolishes the h*TERC* promoter activity suggesting that this element is the site of action for an activator for the h*TERC* promoter and central to h*TERC* transcriptional efficiency. By comparison, mutation of the TATA-box had no effect on reporter gene activity (construct pLh930, Δ TATA).

Turning to the Sp1 sites, mutation of the Sp1.1 site (pLh92 ΔSp1.1) significantly increases basal promoter activity by 3-4 fold, suggesting that this element is the site of action for a repressor of h*TERC* promoter activity. A similar up-regulation is shown by the single-site

mutant construct, pLh29112(ΔSp1.4), which showed about a 2-fold increase of basal promoter activity. Mutation of Sp1.2 (construct pL29m223, ΔSp1.2) did not significantly alter promoter activity.

To further determine how these Sp1 elements interact or relate to each other, double and multiple site-directed mutant constructs were created. As shown in Figure 4.8B, the stimulation of promoter activity by mutation of site Sp1.1 was still observed upon mutation other Sp1 sites within the promoter constructs pLh29m292 (ΔSp1.1+ΔSp1.2) and pLh112 (ΔSp1.1+ΔSp1.2+ΔSp1.4). However, combination of mutation of Sp1.1 with mutation of Sp1.3 reduces promoter activity back to control levels in construct pLh111 (ΔSp1.1+Δ Sp1.2+ΔSp1.3). Thus it is possible that site Sp1.3 is a positive regulatory element of h*TERC* promoter where activation is revealed by mutation of Sp1.1 site. However mutation of all four Sp1 sties in construct pLh114 (ΔSp1.1+ΔSp1.2+ΔSp1.3+ΔSp1.4) did not abolish promoter activity completely suggesting another unknown transcriptional mechanisms. The same results of constructs pLh92, pLh1011, pLh26n23 and pLh114 were obtained by transient transfection analysis in HeLa cells. These results demonstrate that the Sp1 family of transcription factors, through its differential interactions with different regions of the h*TERC* promoter, play an important part in the h*TERC* gene regulation. Thus several regulatory elements involved in either the activation or repression of the h*TERC* promoter have been identified. The major conclusions of this part of the study are the CCAAT-box is essential for h*TERC* promoter activity; Sp1.1 acts as negative regulatory element and Sp1.3 as positive regulatory element.

4.3 Discussion

In the present study, the h*TERC* promoter was investigated by DNase I footprinting, EMSA, site-directed mutagenesis and transient transfection experiments to identify regulatory elements. The major findings are as follows: (i) at least five discrete sequence elements were identified, namely one CCAAT-box and four Sp1 elements. These elements constitute the 176 bp proximal promoter of the h*TERC* gene; (ii) the CCAAT-box

element was found to be essential for h*TERC* gene transcription; (iii) an unusual Sp1 site was found overlapping with the TATA-box which appears to inhibit h*TERC* promoter activity.

4.3.1 Regulation of h*TERC* through Sp1 sites

The 176 bp of the h*TERC* proximal promoter was able to drive promoter activity in 5637 and HeLa cells. Transient transfections with a series of progressive site-directed mutants of the h*TERC* proximal promoter fused to the Luc-reporter gene implicated a sequence element between -40 to -17 bp region, which harbours a TATAA motif closely linked to an Sp1.1 binding site. Interestingly, Sp1.1 may be a negative regulatory element of h*TERC* activity. Evidence to support this conclusion involves mutation of the Sp1.1 sequence from GCTC to ATAG, (-36/-33 bp), in different constructs, pLh92, pLh112 and pLh29m292 (Figure 4.8B). Whereas, mutation of the nearby TATA sequence to CGAT, (-28/-25 bp), did not affect the DNA-binding complex or Luc-reporter activity. Therefore Sp1.1 motif is perhaps the target for the transcriptional repressor Sp3.

It is noteworthy that Sp1.1 shows low-affinity for Sp1 binding and this may be due to differences in the h*TERC* Sp1.1 sequence to the consensus Sp1 sequence. In addition the spatial alignment of the multiple Sp1 sites along the h*TERC* promoter may be important. Sp1.1 is only 49 bp 5' to Sp1.3 and 67 bp 3' to Sp1.2 . Sp1.1 also overlaps with a TATA-box consensus sequence and only 19 bp 3' to CCAAT-box. Several studies have shown that small changes in the separation of Sp1 binding sites can have significant effects. For example, movement of an Sp1 binding site from 8 bp downstream of a TATA box in the adenovirus E1B early region to 30 bp upstream of the box was sufficient to abolish its transcriptional function (Wu & Berk, 1988). Other studies with the E1B early region showed that separation of two Sp1 binding sites by <30 bp did not alter expression levels, but these levels dropped when the separation was = 40 bp (Segal & Berk, 1991). It has also been shown that bending of DNA induced by Sp1 binding relative to a TATA box can be influenced by an insertion as small as 5 bp (Sjøttem et al., 1997).

Multiple site-directed mutation analysis releases a positive regulatory element Sp1.3 although reduction is not observed in single mutation of Sp1.3. Evidence to support this conclusion involves different constructs pLh92, pLh112 and pLh29m292 (Figure 4.8B). This is probably due to the removal of the nearby negative regulatory Sp1.1 sites which may renders up Sp1.3 for Sp1 activation. It was found in this study that the affinity of Sp1 protein binding to Sp1.3 site is higher than Sp1.1. Presumably protein binding to Sp1.1 will be more active to repress Sp1.3 activation. However it is unclear how the h*TERC* promoter is organised and selectively recognised by Sp3 (causing repression) and Sp1 (which might cause activation) and whether the negative/positive regulation is dependent on either the precise sequence element or the critical position of the Sp1 site.

While the presence of Sp1 binding site in a promoter region is a good indication that they play a role in regulating gene transcription, mutation of the Sp1 sites can up- or down-regulate gene promoter activity (Conn et al., 1996; Peters et al., 1997; She and Taylor, 1995). It was reported that mutation of RCE site (A typical Sp1 site) results in an up-regulation of elastin promoter activity of about 2-3 fold (Conn et al., 1996). Perhaps more relevant to the current study is work by She and Taylor (1995) which showed that the three Sp1 binding sites found in the hamster aprt promoter region are not required for expression, though all three sites can bind Sp1 protein *in vitro*. These studies demonstrated that mutation of Sp1 sites in a promoter sometimes do not correlate with transfection analysis *in vitro* and it is unclear how those genes are regulated when Sp1 sites are destroyed. It may suggest the presence of other unknown transcriptional mechanisms. However, it seems that multiple transcriptional elements form the minimal h*TERC* promoter by directing Sp-family factors to up- or down-regulate h*TERC* gene. Thus h*TERC* gene expression may be due to a combination of Sp-family and CCAAT-binding factors.

4.3.2 h*TERC* transcription requires the CCAAT-box

The regulation of the h*TERC* gene through the CCAAT-box is discussed in full in the next chapter. Deletion of the sequence from -107 to -51 bp in construct pLh2923(-107/69) abolishes the basal promoter activity suggesting requirement of this region for h*TERC* transcription. The mutation sequence from CCAA to AGTC at position -58 to -55 bp of CCAAT-box disrupts the h*TERC* transcription therefore demonstrating that CCAAT-box is a key controlling element. The data discussed above suggests that the h*TERC* gene contains multiple transcriptional elements and the CCAAT-box may be essential for promoter activity. The CCAAT-box motif is recognised by a variety of CCAAT-binding transcription factors (for example; NF-Y/CBP, c/EBP, CTF, NF-1). The selective binding mechanism by which multiple factors bind to the same target sequence in different genes is not clear. It may involve competition among the factors, influenced by sequences flanking the consensus motif, or interactions with other proteins binding to adjacent sites. Identification of the specific CCAAT-binding factors will be an important issue for understanding regulatory mechanism of the h*TERC* gene transcription.

4.4 Conclusions and Implications

In conclusion, the h*TERC* promoter posesses sequence elements that allow interactions with several different transcription factors. The interplay between CCAAT-binding factor, Sp1 and Sp3 within the architecture of the h*TERC* promoter may combine to enable a wide variety of cell types from mortal to immortal to regulate h*TERC* expression through transcriptional control, and the protein binding to CCAAT-box element is essential. Use of different types of promoters to drive synthesis of telomerase RNA genes between species could reflect distinct pathways of telomerase RNA gene regulation. It is interesting to know which protein binds to the CCAAT-box in h*TERC* and how those regulators act on h*TERC* promoter, and that will be discussed in chapter 5.

Chapter

5

Identification of Transcriptional Complexes Regulating the h*TERC* Gene Promoter

Key issues

- Characterisation of transcriptional regulators of the h*TERC* gene
  - NF-Y is a key activator of h*TERC* transcription
  - Sp1, pRb and E1A are positive regulators of h*TERC* promoter activity
  - Sp3 is a repressor of h*TERC* transcription
- Sequence comparison and alignment of mammalian TERC gene promoters
- Functional comparison of the transcriptional regulation of the human, mouse and bovine *TERC* genes

Chapter 5 Identification of Transcriptional Complexes Regulating the h*TERC* Gene Promoter

The previous chapter identified several transcription factor binding sites in the h*TERC* proximal promoter region. This chapter will discuss the identification of NF-Y as the h*TERC* CCAAT-box binding complex. In addition, the role of NF-Y, Sp1, Sp3, pRb and E1A proteins in h*TERC* promoter regulation will be demonstrated (see Figure 5.1 for overview). Functional divergence of TERC gene regulation between species will also be discussed.

5.1 Introduction

Reconstitution of telomerase activity *in vitro* by the essential h*TERC* and h*TERT* gene has been reported, although it is likely these core subunits are augmented by additional factors to function optimally (Holt & Shay 1999; O'Reilly et al., 1999). h*TERC* gene expression is regulated during normal human development, with the high levels of expression found during embryogenesis decreasing as tissue differentiation occurs. By the 10^{th} postnatal week the adult pattern of h*TERC* expression is evident with only primary spermatocytes retaining high levels of expression (Yashima et al., 1998). Although h*TERC* expression is down-regulated from most adult somatic cells, low h*TERC* expression can be detected in some cells such as the regenerative regions of epithelium and activated lymphocytes (Weng et al., 1997; Ogoshi et al., 1998; Yashima et al., 1998). However, during carcinogenesis the repression of h*TERC* in the cancer cell is lost or deregulated, thus contributing to the almost universal reactivation of telomerase in cancer cells (Holt and Shay 1999; Sarvesvaran et al., 1999). Recent studies have indicated that transcriptional regulation may have a role to play in the control of h*TERC* gene expression (Zhao et al., 1998; Yi et al., 1999). It is important to identify the molecular basis for the transcriptional regulation.

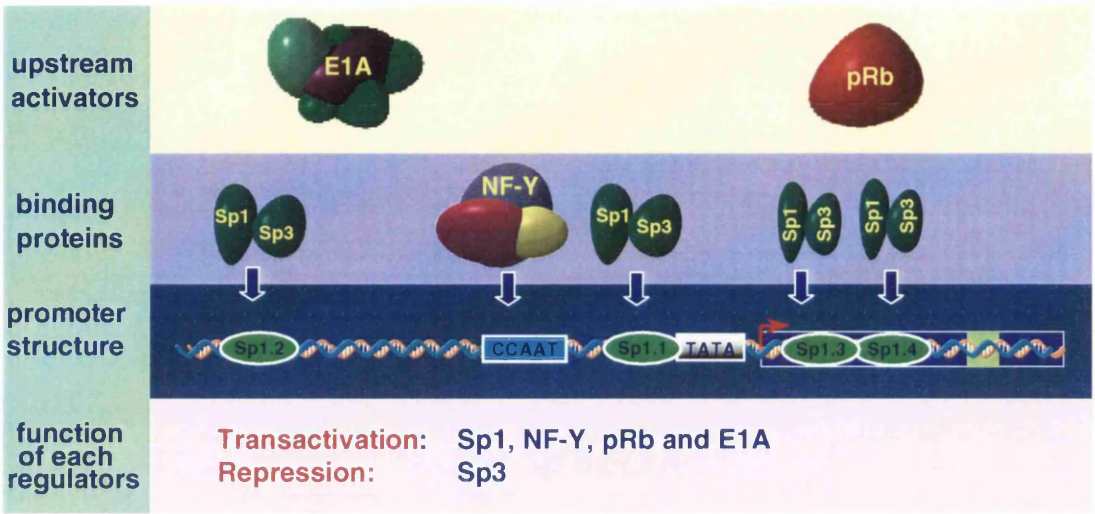


Figure 5.1 Model illustrating the h*TERC* proximal promoter control elements and the functionally relevant transcriptional regulators.

h*TERC* proximal promoter structure with identified transcription factor binding site is shown. NF-Y complexes are indicated as grey, yellow and red ellipsoid shapes joined together, and Sp1/Sp3 is illustrated as green ellipsoid shapes. E1A and pRb as activators are shown at the top of diagram.

Studies on h*TERC* regulation suggest that two mechanisms determine the steady-state level of h*TERC* in cells; the rate of synthesis (the transcription rate) and the rate of degradation (the half-life). Elevated h*TERC* levels in tumour cells might be due to an increased transcription rate, an increased half-life as a result of the association of h*TERC* with the hTERT or other regulatory modifications, or a combination of these factors (Yi et al., 1999). The promoter of the h*TERC* has been cloned and partially characterised (Zhao et al., 1998; Chapter 3 and 4) and several transcriptional elements have been identified. These studies provide some evidence to suggest that the 5'-flanking sequence region plays a role in the h*TERC* transcriptional control.

The RNA component of telomerase has been characterized in a variety of species, including ciliates, yeast, and mammals (Greider, 1995). Ciliate telomerase RNAs are RNA polymerase III (Pol III) transcripts of ~160 to ~190 nucleotides. The studies of these genes demonstrate a well-conserved secondary RNA structure although the primary sequence appears to be poorly conserved (Lingner et al., 1994; Romero & Blackburn, 1991). A conserved proximal sequence element (PSE) located at the proximal promoter region of ciliates telomerase RNA has also been identified suggesting transcriptional regulation (Hargrove et al., 1999). Telomerase RNAs from the budding yeasts are much larger than their ciliate counterparts, they are transcribed by Pol II and processed at their 3' ends from polyadenylated precursor forms (Chapon et al., 1997). To the date, 35 telomerase RNA genes have been cloned and sequenced from a variety of vertebrate species including 21 mammals, 2 birds, 1 reptile, 7 amphibians, and 4 fishes. Biochemical evidence suggests that the vertebrate telomerase RNA gene is transcribed by RNA polymerase II (Avilion, 1995 ; Feng et al., 1995; Chen et al., 2000; Zhao et al. 1998; Hinkley et al., 1998) and contains potential promoter regions at the 5' end. However, transcriptional regulation has yet to be fully investigated.

Telomerase function is conserved across species and is involved in telomere maintenance. Interestingly, the regulation of telomerase expression may differ between species. Telomerase activity is low or negative expression in the normal human somatic tissue but is readily detectable in the mouse. This suggests that telomerase is regulated in different ways in human and mouse (Broccoli et al., 1995, 1996; Zhao et al., 1998). Knowledge of the genomic organisation of the telomerase RNA gene and isolation of upstream regulatory sequence would be valuable in understanding species differences in expression of *TERC*. Differential expression between different species could reflect distinct pathways of telomerase gene regulation. It is important to identify whether different pathways of telomerase gene regulation exist because this has significant implication for generation of animal models for human disease.

In this chapter, I describe the identification and partial characterisation of the promoter-regulatory region of the human, bovine and mouse TERC gene and compare the sequence of 18 other mammalian TERC genes. Functional comparison of the human, bovine and mouse TERC promoters demonstrate that TERC genes are indeed differentially regulated in different species.

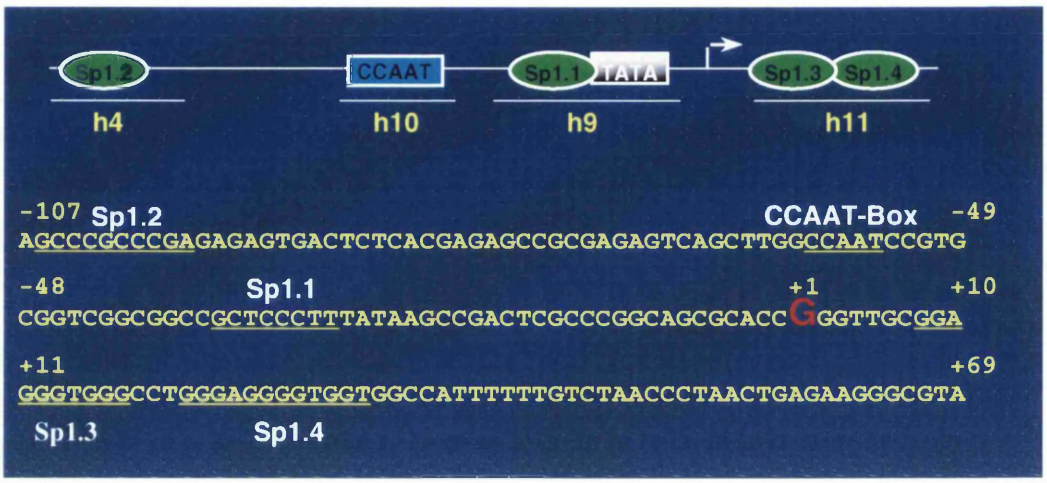
5.2 Results

5.2.1 NF-Y activation of the h*TERC* gene transcription

5.2.1.1 NF-Y factor binds to the h*TERC CCAAT-box*

As described previously (section 4.2.2), a CCAAT-box is found upstream of the transcriptional start site for h*TERC* (Figure 4.3A), and corresponds to the protected H10 region identified by DNase I footprinting (see section 4.2.1). In order to identify which cellular proteins interact with this motif, protein extracts from the HeLa cell line were used in an electrophoretic mobility shift assay (EMSA) with an oligonucleotide corresponding to the wt h*TERC* sequence (termed h10, Figure 5.2B). Figure 5.3A shows that the h10 oligonucleotide binds proteins in a specific fashion as competition for binding with the h10 oligonucleotide itself abolishes the complex, as does an oligonucleotide with mutations outside the CCAAT-box region (sequence h10m2 see Figure 5.2B). Addition of oligonucleotide with mutation introduced into the CCAAT site does not compete for protein binding (Figure 5.3A, lane 3; see h10m1 sequence in Figure 5.2B). This suggests that the CCAAT-box is the functional region for DNA-binding proteins that might regulate the h*TERC* promoter.

A)



B)

```
h10(wt:-63/-42) CTTGGCCAATCCGTGCGGTCGG
h10m1 (-58/-55) :::::AGTC:::::::::::::
h10m2 (-51/-48) ::::::::::::TGAT::::::
CBFcons    AGACCGTAGGTGATTGGTTAATCTCTT
NF-Ycons      ATTTTTCTGATTGGTTAAAAGT
c/EBPcons   TGCAGATTGCGCAATCTGCA
NF-1cons TTTTGGATTGAAGCCAATATGATAA
```

Figure 5.2 h*TERC* proximal promoter sequence and identified transcription factor binding sites

176 bp of h*TERC* proximal promoter sequence (-107/+69) is shown in (**A**), and identified regulatory motifs are underlined. The transcriptional start site is marked as "+1". Four Sp1 sites are shown and termed Sp1.1 to Sp1.4. Sp1.4 contains two overlapping Sp1 binding sites. The promoter structure is also depicted at the top of diagram. Lines with h4, h9, h10 and h11 indicate oligonucleotides used for EMSA.

Consensus oligonucleotides for definition of CCAAT binding protein (**B**). Oligonucleotide sequences used in EMSA competition experiments to define h*TERC* CCAAT-binding complexes. The c/EBP, CBF and NF-1 oligonucleotides were obtained from Santa Cruz, and the NF-Y oligonucleotide was from Geneka Biotechnology Inc. These sequences are also listed in Table 2.2. Forward or inverted CCAAT-boxes are shaded in light purple.

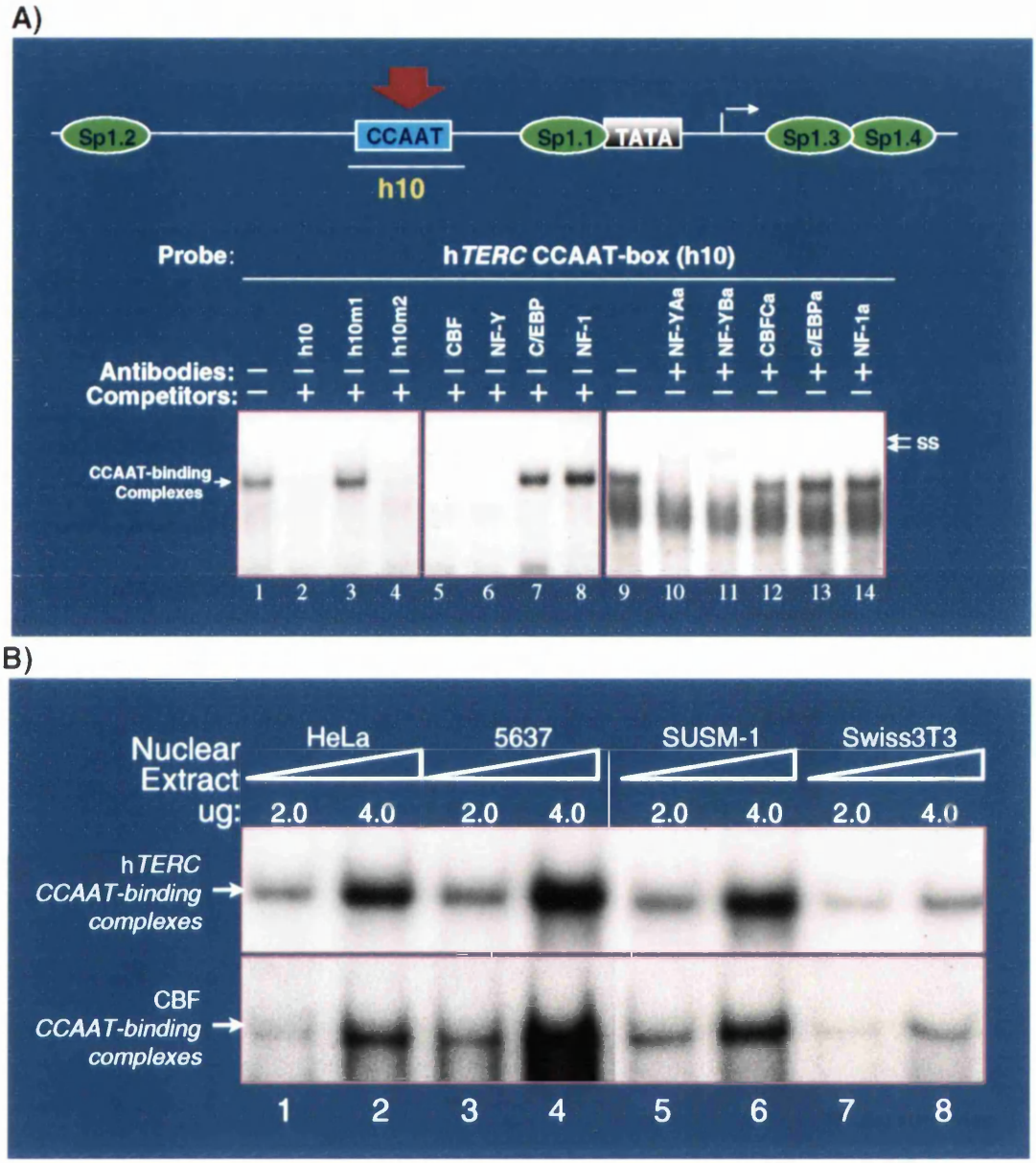
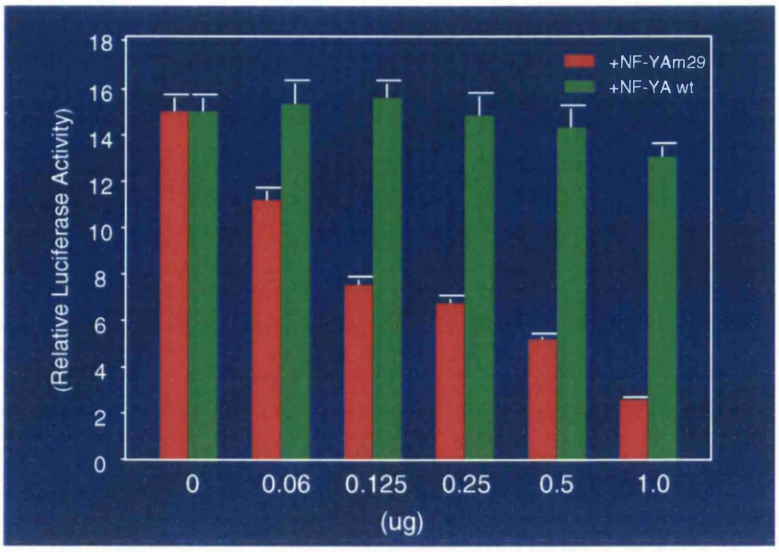


Figure 5.3 h*TERC* CCAAT-box binding to NF-Y factor

A) DNA-binding complexes in HeLa cells 5.5 µg of HeLa cells nuclear extracts were mixed with radiolabelled h10 oligonucleotide probe and analysed by EMSA. Specific DNA-protein complexes are indicated by arrows on the left. Competition was performed in the presence of 100-fold molar excess of the cold h10 oligonucleotide alone (lane 2), or site-replaced mutation oligonucleotides (lanes 3 and 4), or consensus CCAAT-binding sequence (lanes 5 to 8) as indicated. Complexes supershifted by preincubation with antibodies are shown on the right by arrows marked 'ss' (lanes 10-14). Sequences of all oligonucleotides are shown in Figure 5.2B and Table 2.4.

B) DNA-binding complexes in 5637, SUSM-1 and Swiss3T3 cell lines Nuclear extracts from 5637, SUSM-1 and Swiss 3T3 cell lines were mixed with radiolabelled h10 or CBF consensus oligonucleotide probe and analysed by EMSA as described above.

A)



B)

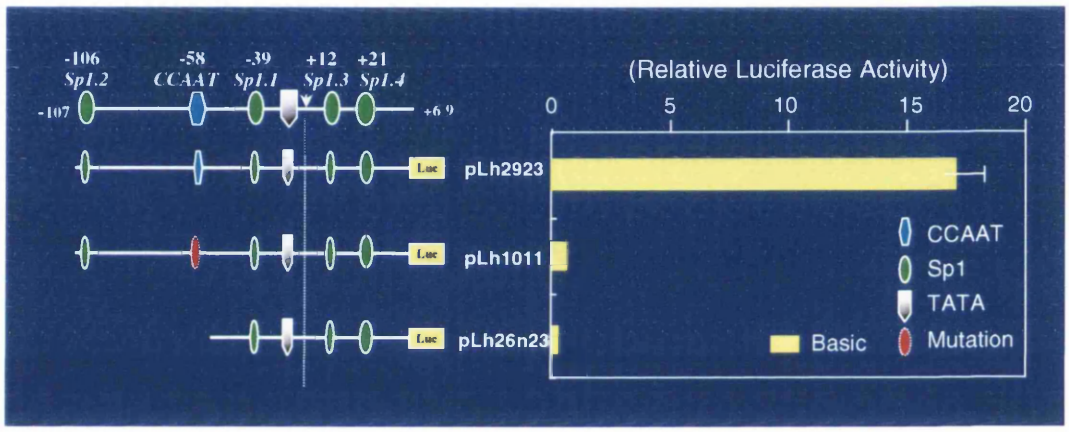


Figure 5.4A Inhibition of h*TERC* promoter activity by expression of the dominant-negative-mutant protein NF-YA

5637 cells were co-transfected with 3.0 µg of the pLh2923(-107/+69) construct and increasing concentrations of the NF-YAm29 dominant-negative expression vector (red bars) or wt NF-YA expression vector (green bars). For each transfection the mean and standard deviation for duplicate samples is shown. Hatched bars show relative luciferase activities, error bars show standard deviations. The pSEAP2 Control plasmid was used as an internal control to normalise the transfection efficiency.

Figure 5.4B Mutation or deletion of CCAAT-box inhibits h*TERC* promoter activity

The predicted promoter structure is shown at the top. The various colours symbols or circles represent the different transcription factor binding sites. The numbers indicate the DNA-binding sites related to the transcriptional start site. The constructs are shown with red circles indicating a site-replaced mutation in one or more positions. The names of the Luc-reporter constructs are shown in the middle column. Three micrograms of each plasmid were used for transfection analysis in 5637 cells. The result of the promoter activity is shown on the right hand side. Promoter activities of the mutant constructs were assayed by transfection and compared to the wild-type promoter (pLh2923). For each transfection the mean and standard deviation for duplicate samples are shown. Hatched bars show relative luciferase activities, error bars show standard deviations. The pSEAP2-Control expression system and PCR semi-quantitation were used to normalise transfection efficiency.

CCAAT-binding proteins encompass a heterogeneous group of transcriptional regulators that differ in their sequence requirements and fulfil distinct biological roles (Maity & de Crombrugghe, 1998). It was therefore important to ascertain the likely components of the h*TERC* CCAAT-box binding complex. Three candidate CCAAT-binding complexes were investigated: NF-Y, (also called CBF or CP1), c/EBP, and NF-1 (consensus binding sequences are shown in Figure 5.2B and Table 2.2). As shown in Figure 5.3A, two consensus oligonucleotides for NF-Y (termed NF-Y and CBF), compete for h*TERC* CCAAT-binding activity whereas consensus oligonucleotides for c/EBP and NF-1 do not (lanes 5-8). Also, antibody supershift analysis revealed that the h*TERC* CCAAT-binding complexes contain two subunits of the NF-Y protein complex, NF-YA and NF-YB (lanes 10 and 11), but not the distinct CCAAT-binding factors NF-1 or c/EBP (lanes 13-14). As shown by others, antibodies to the NF-YC subunit of NF-Y did not disrupt the complex, possibly due to inaccessibility on complex formation (Faniello et al., 1999).

To clarify whether h*TERC* CCAAT-box binds to NF-Y protein from other cell lines, EMSAs were also performed using 5637, Swiss 3T3, and Susm-1 cell nuclear extracts as shown in Figure 5.3B. The radiolabelled consensus CBF probe was used as a control oligonucleotide and the CCAAT-box/NF-Y binding complexes detected in these cell lines. The antibody supershift also detected NF-YA and B complexes in 5637 cells as described above in HeLa cells. Taken together, these data indicate that the h*TERC* CCAAT-box is recognised by the NF-Y complex.

5.2.1.2 Inhibition of NF-Y abrogates h*TERC promoter activity*

As previously described in section 4.3.2, mutation or deletion of the CCAAT-box element abolishes the h*TERC* promoter activity completely, suggesting that this element is the site of action for an activator of the h*TERC* promoter. Furthermore, a mutation of CCAAT-box in the large version of hTERC construct, pLh471(ΔCCAAT, -272/+69, Table 2.5.), lead to dicrease wt promoter activity about 88%. In order to evaluate the functional significance of NF-Y-CCAAT complex formation on the h*TERC* promoter, the dominant-negative NF-Y

vector (NF-YA13m29) developed by Mantovani (1994) was used. Upon transfection into mammalian cells, it expresses a mutant protein containing a triple amino acid substitution in the NF-YA DNA-binding subdomain that still enables the subunit to interact with the NF-YB-NF-YC dimer, but renders the resulting trimer inactive in terms of CCAAT recognition. This vector is highly diagnostic, as it has been tested in many different systems and has been shown to be specific for NF-Y-dependent promoters (Mantovani, 1994). 5637 cells were co-transfected with the h*TERC* Luc-reporter constructs (pLh2923, -107/+69) and increasing amounts of the dominant-negative NF-YA construct or wild-type NF-YA. Figure 5.4A shows that the dominant-negative vector is indeed inhibitory to h*TERC* promoter activity starting at 60 ng . At higher concentration, h*TERC*-Luc transcription is decreased by 5-7-fold, a level that corresponds to the relative contribution of NF-Y binding to the CCAAT-box, as tested in transfections with CCAAT element mutants (Figure 5.4B, constructs pLh1011 and pLh26n23). Co-transfection of the wt h*TERC* Luc-reporter construct with wt NF-YA expression vector had no effect on promoter activity. Similar experiments were also performed to verify the specificity of the dominant-negative mutant by using a larger h*TERC* promoter construct, pLh2023 (-798/+69)(Figure 5.8). These results confirm that NF-Y is the h*TERC*-dependent factor that transactivates the h*TERC* promoter via the CCAAT-box.

5.2.2 h*TERC* promoter is activated by Sp1 and repressed by Sp3

The identification of Sp1 sites in the h*TERC* promoter (Chapter 4) suggests the involvement of the Sp1 family of transcription factors in the regulation of the h*TERC.* In order to investigate this further expression vectors for candidate regulators were co-transfected into the bladder carcinoma cell line 5637 (which is functionally negative for pRb) along with the h*TERC* Luc-reporter constructs. Fluctuations in reporter activity due to expression of the candidate proteins were then monitored.

As shown in Figure 5.5A, Sp1 is an activator of the h*TERC* promoter. Interestingly, Sp1-mediated induction of h*TERC* transcription was abrogated by combined mutation of all

identified Sp1 sites in construct pLh114 (Figure 5.5A). In contrast, Sp3 is an efficient repressor of h*TERC* promoter activity, even having small effect on construct pLh114 (Figure 5.5B). As shown in Figure 5.5A and B, constructs pLh1011 (ΔCCAAT-box) and pLh26n23(-51/+69) were not stimulated by expression of the Sp1 protein, the interaction of these constructs with Sp3 could not be tested due to the very low basal activity. These studies suggest that Sp3 is repressor and Sp1 is activator of the h*TERC* promoter, and Sp1 activation requires functional Sp1 binding sites and a functional CCAAT-box for their effect.

To further analyse the functional combination of Sp-family transcription factors in h*TERC* promoter regulation, constant amount of the pLh2023, pLh29m292 or pLh2923 Luc-reporter constructs, containing the different versions of the h*TERC* promoter context (see Table 3.1 and Figure 4.7), was co-transfected with 3.0 μg of expression construct for Sp1 and varying amounts of the Sp3 expression construct in 5637 cells. As shown in Figure 5.5C and consistent with the above findings, expression of Sp1 alone increased the activity of constructs pLh2023 and pLh2923 about 2-fold, but construct pLh29m292 with two mutated Sp1 sites (ΔSp1.1 and ΔSp1.2) did not show clear stimulation. Co-expression of Sp3 caused a dose-dependent decrease in luciferase activity. Furthermore, repression of the promoter activity was seen in all three constructs when the Sp3 expression vector was transfected at quantities as low as 0.25 μg, with a plateau of repression (-10 fold) being reached at 2.0 μg. These results demonstrate that Sp3 repression appears to override Sp1 activation of the h*TERC* promoter.

A combination of the Sp-family of factors and NF-Y would appear to play an important role in the control of h*TERC* gene transcription. Co-transfection experiments were also performed with several different h*TERC* Luc-reporter constructs and the dominant-negative NF-YA expression vector and with Sp1/Sp3 expression vectors. The results indicate that h*TERC* transcription was almost totally repressed by expression of dominant-negative NF-YA, the resulting level of transcription being approximately the same as the shortest deletion construct, pLh26n23 (-55/+69). Taken together, these studies suggest that Sp-family of transcription factors activate or repress the h*TERC* gene

transcription, and this interaction depends on NF-Y binding to CCAAT-box as no Sp1 activation was seen when the CCAAT-box was mutated.

5.2.3 pRb regulates the h*TERC* transcription

The retinoblastoma protein (pRb) is considered to have important roles in normal transcriptional regulation and in the progression of a normal mortal cell to an immortal cancer cell (Seller and Kaelin 1996; Holt and Shay 1999). It has also been suggested that Sp1-mediated transcription can be stimulated by pRb (Kim et al., 1992b; Udvadia et al., 1993; Chen et al., 1994a). The h*TERC* gene promoter contains *cis*-acting elements for the transcription factor Sp1, therefore, the ability of pRb to modulate the h*TERC* promoter was assessed in the pRb/p53 null bladder cell line, 5637.

To test whether the h*TERC* promoter is regulated by the tumour suppresser protein pRb, an 867 bp fragment of the h*TERC* promoter construct pLh2023 (-798/+69) was used. Transient expression of pRb from a CMV-pRb expression vector induced h*TERC* promoter activity as shown in Figure 5.6A. This activation was dose dependent ranging from 1.5 fold induction when 62.5 ng CMV-pRb vector was introduced up to 4-7 fold induction with 250 ng. As CMV is a strong promoter and h*TERC* activation is very sensitive to the dose of CMV-pRb vector used, a SV40 promoter-driven pRb expression vector was also tested. When 0.5 to 1.0 μg of the SV40-Rb expression vector were co-transfected with the h*TERC* Luc-reporter, very similar results were obtained.

To determine whether pRb activation is specific to the h*TERC* promoter, a m*Terc* Luc-reporter construct, pL2730 (-549/+80), was also used. The m*Terc* promoter shows no significant sequence homology to the h*TERC* promoter (Zhao et al., 1998; section 3.3.2) although 3 potential Sp1 sites are detected in the m*Terc* promoter region. As shown in Figure 5.6A, the m*Terc* promoter was not activated by pRb under the same experimental conditions as the human promoter, thus demonstrating a functional difference between the human and mouse telomerase RNA gene promoters in terms of their response to pRb. This study demonstrates for the first time that pRb specifically stimulates h*TERC* promoter activity.

It has been reported that pRb can transactivate a number of gene promoters by interaction with a variety of transcriptional complexes including MyoD, c/EBP, NF-IL6, YY1 and Sp1 (Sellers et al., 1998; Chen et al, 1996a,b; Kim et al., 1992b; Udvadia et al., 1993; Petkova et al., 2000). There are two Sp1 sites (Sp1.3 and Sp1.4) which contain GGGTGG elements, which has be reported as an indicator of a pRb responsive gene (Kim et al., 1992b; Udvadia et al., 1993), located at position +11 to +16 and +26 to +31 bp within the h*TERC* proximal promoter. To determine whether these or other transcriptional elements are involved in pRb-mediated induction of hTERC, co-transfection experiments were performed using 0.5 μg of SV40-Rb expression vector with 3.0 μg of the h*TERC* proximal promoter (pLh2923 -107/+69) or the shortest deletion promoter (pLh26n23 -51/+69) which still retains Sp1.3 and Sp1.4 sites but lacks the CCAAT-box. As shown in Figure 5.6B, pRb activates transcription of 176 bp of the h*TERC* proximal promoter by 4-fold but is unable to activate the 120 bp minimal promoter.

Next h*TERC* Luc-reporter constructs with mutations of the four Sp1 sites (construct pLh114; ΔSp1.1, ΔSp1.2, ΔSp1.3 and ΔSp1.4) or with mutation of the CCAAT-box (construct pLh1101 ΔCCAAT) were used. In co-transfection experiments, pRb is able to increase pLh114 promoter activity by 2-fold, but it is unable to activate pL1101 promoter activity. As described previously (section 4.2.4), the promoter activity was not fully abolished in construct pLh114 despite multiple mutations. To further confirm that pRb was still able to activate this construct, the sequence of this individual plasmid preparation was verified by restriction enzyme mapping and DNA sequencing, and the experiments were repeated with separately prepared preparations of plasmid DNA. The same result was obtained in each case. Thus, mutation of all four Sp1 sites including GGGTGG motifs in the h*TERC* proximal promoter region did not abolish pRb transactivation.

Figure 5.5 Sp1/Sp3 factors regulate h*TERC* gene transcription

The predicted promoter structure is shown at the top. The various colours symbols or circles represent the different transcription factor binding sites. The numbers indicate the DNA-binding sites related to the transcriptional start site. The constructs are shown with red circles indicating a site-replaced mutation in one or more positions. The names of the Luc-reporter constructs are shown in the middle column. The result of the promoter activity is shown on the right hand side. Three micrograms of each plasmid were used for transfection or co-transfection with the Sp1/Sp3 expression vector in 5637 cells. Promoter activities of the mutant constructs were assayed by transfection and compared to the wild-type promoter (pLh2923). For each transfection the mean and standard deviation for duplicate samples are shown. Hatched bars show relative luciferase activities, error bars show standard deviations. The pSEAP2 Control plasmid was used as an internal control to normalise the transfection efficiency.

A) The Sp1 transcription factor activates the h*TERC* promoter.

B) The Sp3 transcription factor represses the h*TERC* promoter.

C) Suppression of Sp1-induced h*TERC* transcription by Sp3 Indicated amounts of pCMVSp3 were transfected into 5637 cells along with a constant amount (3.0 μg) of pCMVSp1, in addition to either pLh2023 (-798/+69, 1.0 μg), pLh29m292 (ΔSp1.1/Δ Sp1.2, 3.0 μg) or pLh2923 (-107/+69, 3.0 μg) luciferase reporter constructs. The results are expressed as relative luciferase activity from one of three independent experiments.

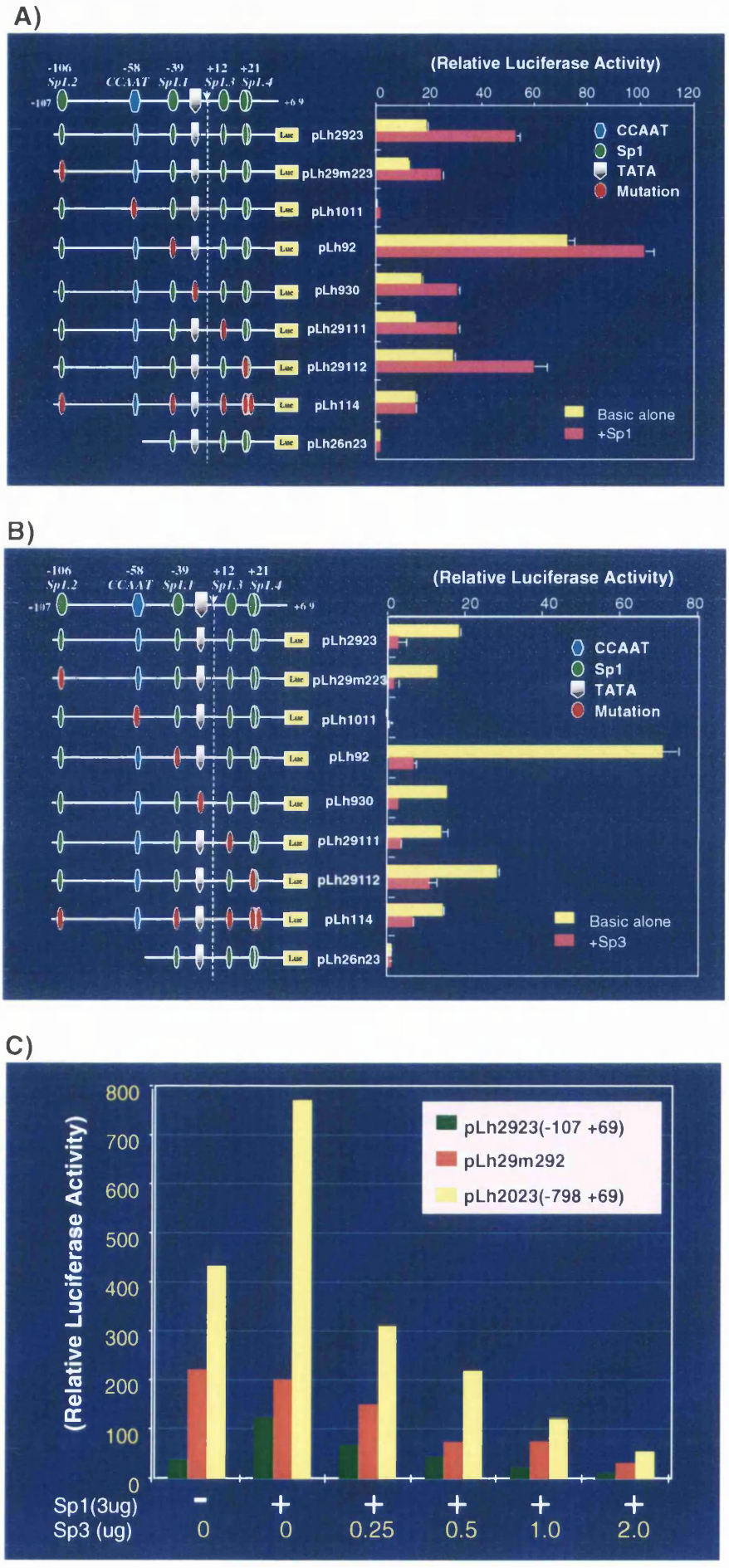

A)
-106 Sp1.2
-58 CCAAT
-39 Sp1.1
+12 Sp1.3
+21 Sp1.4
-107
+69
pLh2923
pLh29m223
pLh1011
pLh92
pLh930
pLh29111
pLh29112
pLh114
pLh26n23
(Relative Luciferase Activity)
0 20 40 60 80 100 120
CCAAT
Sp1
TATA
Mutation
Basic alone
+Sp1
B)
-106 Sp1.2
-58 CCAAT
-39 Sp1.1
+12 Sp1.3
+21 Sp1.4
-107
+69
pLh2923
pLh29m223
pLh1011
pLh92
pLh930
pLh29111
pLh29112
pLh114
pLh26n23
(Relative Luciferase Activity)
0 20 40 60 80
CCAAT
Sp1
TATA
Mutation
Basic alone
+Sp3
C)
(Relative Luciferase Activity)
800
700
600
500
400
300
200
100
0
pLh2923(-107 +69)
pLh29m292
pLh2023(-798 +69)
Sp1(3ug) - + + + + +
Sp3 (ug) 0 0 0.25 0.5 1.0 2.0

Figure 5.5

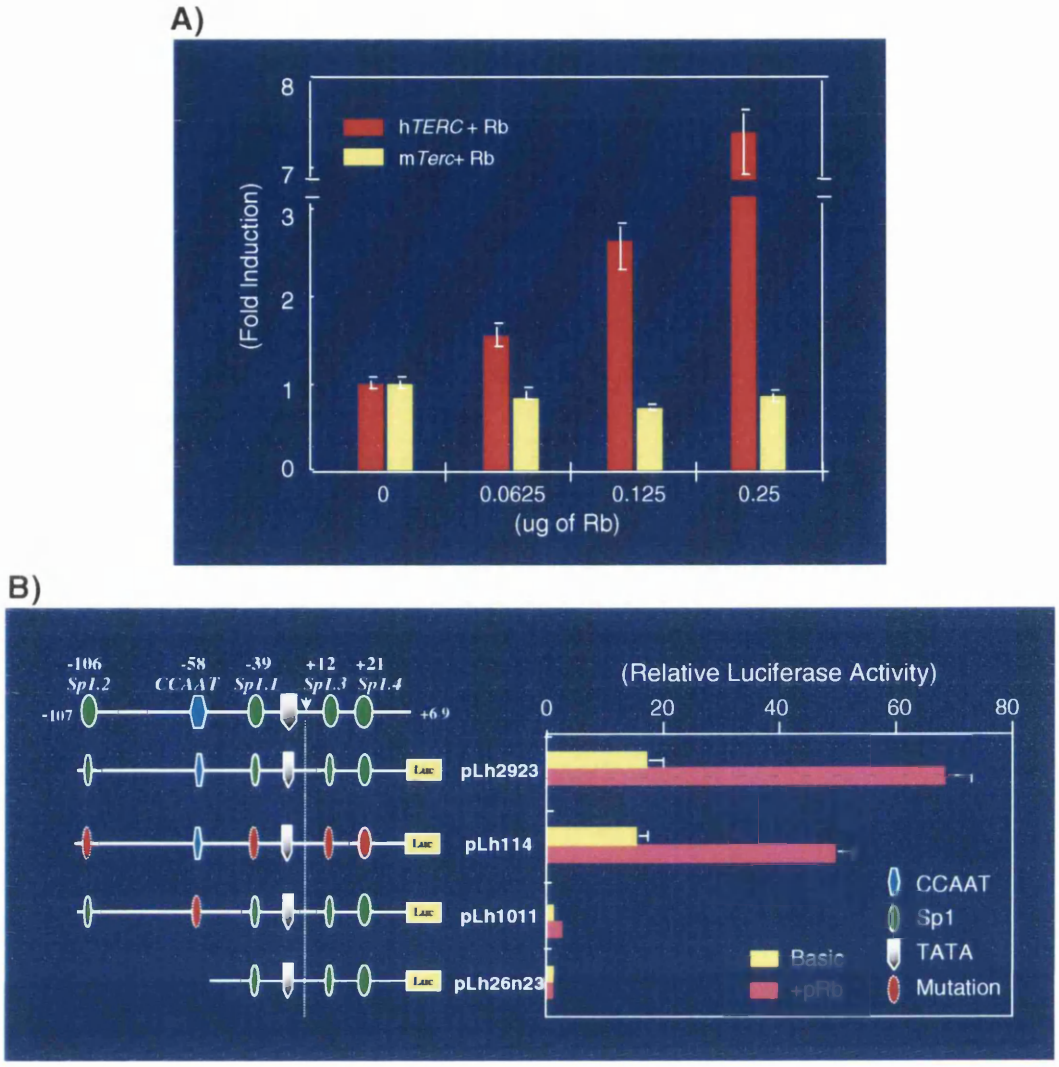


Figure 5.6 pRb transactivates h*TERC* gene transcription

A) pRb activates h*TERC* promoter activities The pRb/p53 negative cell line 5637 was transfected with a fixed amount of the pLh2023 (-798/+69, 1.5 µg) construct and increasing concentrations of CMV-Rb expression vector. In the control experiment, the mouse telomerase (m*Terc*) promoter-luciferase construct pL2730 (-549/+80, 1.5 µg) was analysed for its response to pRb. This experiment has been performed at least three times. For each transfection the mean and standard deviation for duplicate samples is shown.

B) Mutation or deletion of the CCAAT-box blocks the pRb activation of h*TERC* gene transcription The predicted promoter structure is shown at the top. The various colours symbols or circles represent the different transcription factor binding sites. The numbers indicate the DNA-binding sites related to the transcriptional start site. The constructs are shown with red circles indicating site-replaced mutations in one or more positions. The names of the Luc-reporter constructs are shown in the middle column. The result of the promoter activity is shown on the right hand side. Three micrograms of each plasmid were used for transfection or co-transfection with 0.5 µg pSV40-Rb vector in 5637 cells. The result of the promoter activity is shown on the right hand side. Promoter activities of the mutant constructs were assayed by transfection and compared to the wild-type promoter (pLh2923). For each transfection the mean and standard deviation for duplicate samples are shown. The Luciferase activity was normalised by co-transfection with the pSEAP control vector. Hatched bars show relative luciferase activities, error bars show standard deviations.

As described above, pRb activation could not be observed in mutation construct pLh1101 (ΔCCAAT), or deletion construct, pLh26n23 (-51/+69). Removal of the h*TERC* CCAAT-box from the proximal promoter region abolishes the promoter activity totally and this can not be rescued by pRb. These studies suggest that the h*TERC* promoter can be positively regulated by pRb, a functional CCAAT-box is necessary for this activation, but Sp1 sites including the GGGTGG motif are not essential, at least in 5637 cells.

5.2.4 The h*TERC* and hTERT promoter activity in mortal and immortal cells

To investigate the regulation of the human telomerase genes (h*TERC* and hTERT) transcriptional regulation in mortal and immortal cells, 3.0 μg of Luc-reporter constructs, h*TERC* and hTERT, were transfected into the mortal or immortal cells and the promoter activities were monitored. 5637 cells are a pRb/p53 null immortal cell line that is telomerase-positive, whereas WI38 and IMR90 are telomerase-negative mortal cell lines (Meyerson et al., 1997). As shown in Figure 5.7A, WI38 and IMR90 cells have very low h*TERC* and hTERT promoter activity. In contrast, high levels of reporter gene expression were detected in 5637 cells with the h*TERC* exhibiting a 3-fold higher activity than the hTERT promoter. In this experiment, pSEAP2-Control vector (Clontech) was used as internal control for transfection efficiency within cell line. But in across cell lines, the transfected plasmid DNAs were measured by PCR semi-quantitation, identical amount of luciferase gene PCR products were detected in all transfected cells after luciferase assay (for methods described in section 2.4.5). These results show that low telomerase gene promoter activity in two mortal cells is not due to low transfection efficiency.

5.2.5 E1A induces the h*TERC* and hTERT promoter activity

The Adenovirus 5 early region 1A (E1A) oncogene induces progression through the cell cycle by binding to the products of the p300/CBP and retinoblastoma gene families.

To test whether the oncoprotein E1A affects the transcriptional regulation of the human telomerase genes, the two constructs, h*TERC* (pLh2923, -107/+69) and hTERT (phTERT19, -526/+9), were used in co-transfection experiments with wild-type E1A. As shown in the Figure 5.7B, transient expression of wild-type E1A could efficiently increase both h*TERC* and h*TERT* promoter activity. The activities of the hTERT and h*TERC* promoters were increased about 5- and 2.5-fold respectively by E1A. These preliminary results suggest for the first time that the adenovirus protein E1A can induce both the RNA and protein components of telomerase transcription. To summarise, human telomerase genes show high promoter activity in immortal cells but not in mortal cells, and the hTERT and h*TERC* promoters are capable of up-regulation by E1A in 5637 cells.

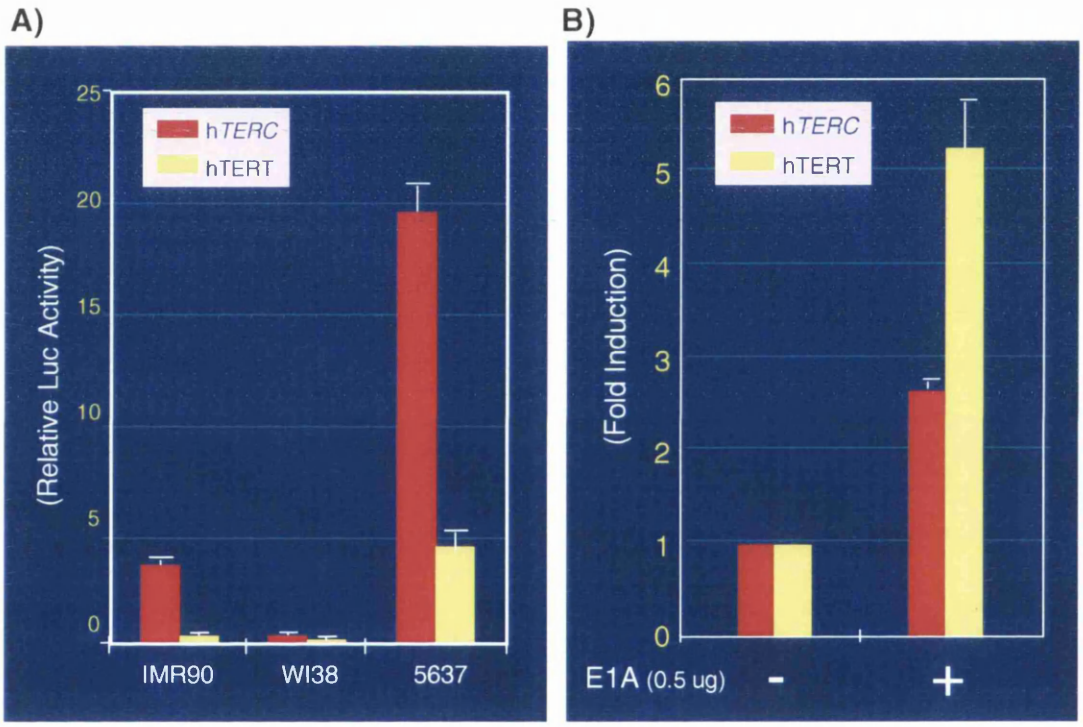


Figure 5.7 Comparing regulation of hTERC and hTERT promoter transcription

Three micrograms of the luciferase reporter constructs; h*TERC*, (pLh2923, -107/+69, red bars), and hTERT, (phTERT19, -526/+9, yellow bars) were transfected by using SuperFect reagents with or without the E1A expression vector (for hTERT promoter sequence see Appendix III). Related luciferase activity was detected 48 hours post-transfection. For each transfection the mean and standard deviation for duplicate samples are shown.

A) h*TERC* and hTERT promoter activity in mortal and immortal cells. Two mortal cell lines (IMR90 and WI38) and one immortal cell line (5637) were used for the transfection assay. Luciferase activity was normalised to the SEAP activity of a co-transfected internal control plasmid, pSEAP-control(1.5 µg) (Clonetech). In this study, the semi-quantitation PCR system (see section 2.4.4) was used to detect the transfected plasmid DNA in tested cells to normalise the transfection efficiency.

B) E1A induction of hTERC/hTERT gene transcription in 5637 cells

5.2.6 Functional divergence in the transcriptional regulation of the TERC gene between species

Despite conservation in telomerase enzyme function across species, there may be fundamental differences in telomerase gene regulation between mouse and human (Zhao et al., 1998; Blasco et al., 1997). In order to gain a clearer insight into the regulation of telomerase RNA genes, and to understand TERC transcriptional mechanisms whether different exist in differences species, the human, mouse and bovine TERC gene promoters cloned in our Lab were analysed for comparative transcription. The response of these three TERC gene promoters to the transcriptional regulators pRb, NF-Y, Sp1 and Sp3 were compared in transient co-transfection experiments in 5637 cells. The bovine telomerase RNA gene promoter was included in this study as it is more closely related to the human gene in terms of sequence and may be of value in dissecting promoter function (Tsao et al., 1998). As shown in Figure 5.8, the human and bovine promoters were activated by pRb whereas the mouse promoter was not; the result from m*Terc* is consistent with an observation described in section 5.2.3. Transcription factor Sp1 activates and Sp3 represses all three promoters. The dominant negative NF-YA mutant, NF-YAm29, is diagnostic for NF-Y-dependent gene transcription. Transient expression of NF-YAm29 repressed promoter activities of the h*TERC* but not b*TERC* and m*Terc* suggesting NF-Y regulation appears to be essential to human gene promoter. Titration experiments have been performed in each individual promoter construct to further prove this point. These studies demonstrate that the TERC genes are differentially regulated at the transcription level in different species.

5.2.7 Comparison of different mammalian TERC promoter region

A recent study of the vertebrate TERC genes (Chen et al., 2000) allows comparison of the proximal promoter regions. This may provide insight into the transcription regulation of different species. As shown in Figure 5.9, alignment analysis of the mammalian TERC gene proximal promoter sequence realised a phylogenic tree indicating the bovine and pig sequences are closely related to the human. Since the h*TERC* proximal promoter has

been detected within 107 bp upstream of the TSS, a sequence comparison of 176 bp of the h*TERC* and b*TERC* promoter was performed. As shown in Figure 5.10, an alignment of the h*TERC* proximal promoter region sequence shares 70.5% identity with the b*TERC*. The sequence element between TSS and the template region of h*TERC* and b*TERC* is not found in the m*Terc* proximal promoter. Among promoter elements thought to be important for the regulation and expression of h*TERC*, the CCAAT-box and Sp1.3 binding sites, were also found in the b*TERC* potential promoter region. A YY1 site, and the TATA-box are also conserved between the h*TERC* and b*TERC* proximal promoter region.

The sequence analysis of the three TERC promoters revealed an interesting divergence between the human and mouse. It was therefore of interest to analyse the promoter sequence relationship of a larger group of mammalian TERC genes. In order to identify potential clusters of related sequences, by using the human TERC proximal promoter as the control sequence, the 5' flanking region sequences of 21 mammalian TERC genes spanning -106 to +63 bp region were aligned by using the Vector NTI Suite program (InforMax) to detect conserved promoter elements. As shown in Figure 5.11, from this study, the mammalian TERC proximal promoter sequence was predicted. The following major points were found; the mammalian TERC promoters contain (i) a single forward CCAAT-box element which is located between -40 bp and -66 bp relative to the TSS; (ii) a TATA-box is located about 25 bp up-stream of the TSS; (iii) a conserved template sequence downstream of the TSS from 2 to 51 bp away. The mouse, hamster, rat and vole TERC genes appear to lack a typical sequence region between TSS and template region (termed 5'-FTR). The results of sequencing studies demonstrate that the TATA box, an upstream forward CCAAT-box and template region are conserved in all species, and a conserved promoter structure for rodent TERC genes can be predicted as shown in Figure 5.12.

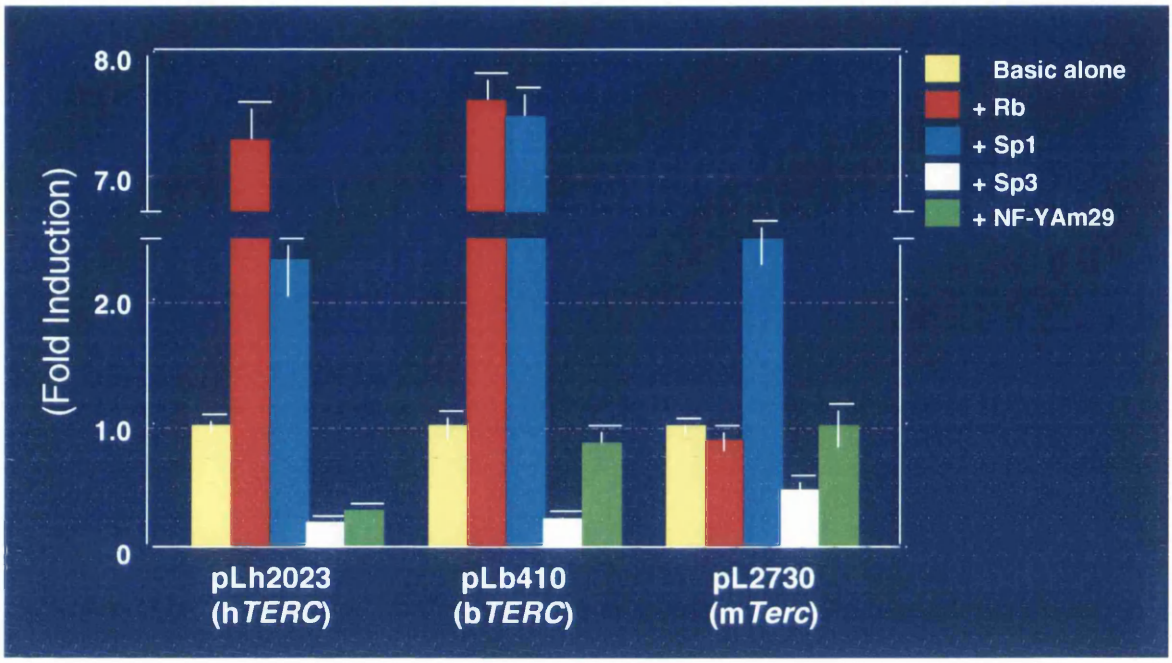
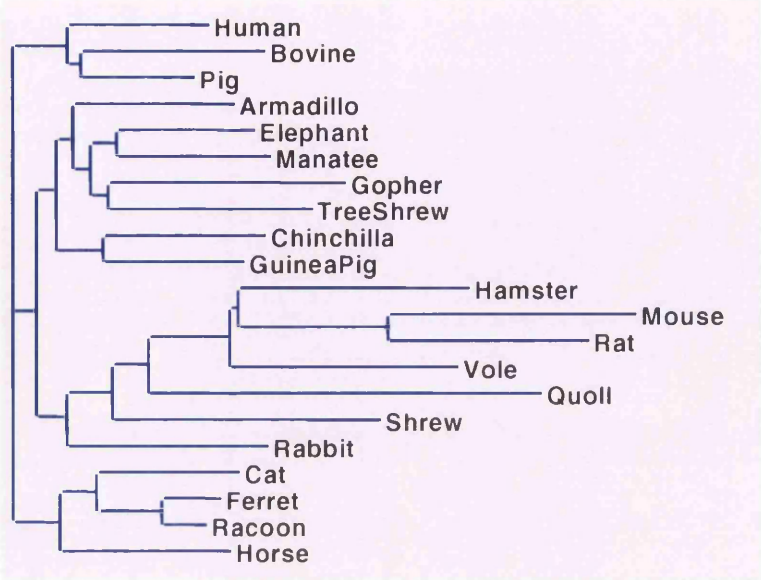


Figure 5.8 Functional divergence in the transcriptional regulation of *TERC* genes between species

Promoter-luciferase constructs for the human, mouse and bovine *TERC* genes were compared to assess their regulation by NF-YAm29, Sp1, Sp3 and pRb. The human promoter (h*TERC*) construct shown is the full-length 867 bp plasmid (pLh2023, -798/+69 bp). The mouse telomerase (mTerc) promoter-luciferase construct used was pL2730 (-514/+114 bp), and the luciferase-reporter construct containing the bovine telomerase RNA gene promoter (b*TERC*) was pLb410 (-274/+69 bp; for sequence see Appendix VI). The activity of the promoters in response to co-transfection with the transcriptional regulation is shown as fold inductions relative to the promoters alone. For each transfection the mean and standard deviation for duplicate samples are shown.

Figure 5.9 *TERC* promoter phylogentic tree

Phylogenetic analysis using proximal promoter sequence in the 5'-flanking transcriptional regulatory region from 21 mammalian TERC genes. The phylogenetic tree is derived from minimum-evolution using the vector NTI Suite program (InforMax).



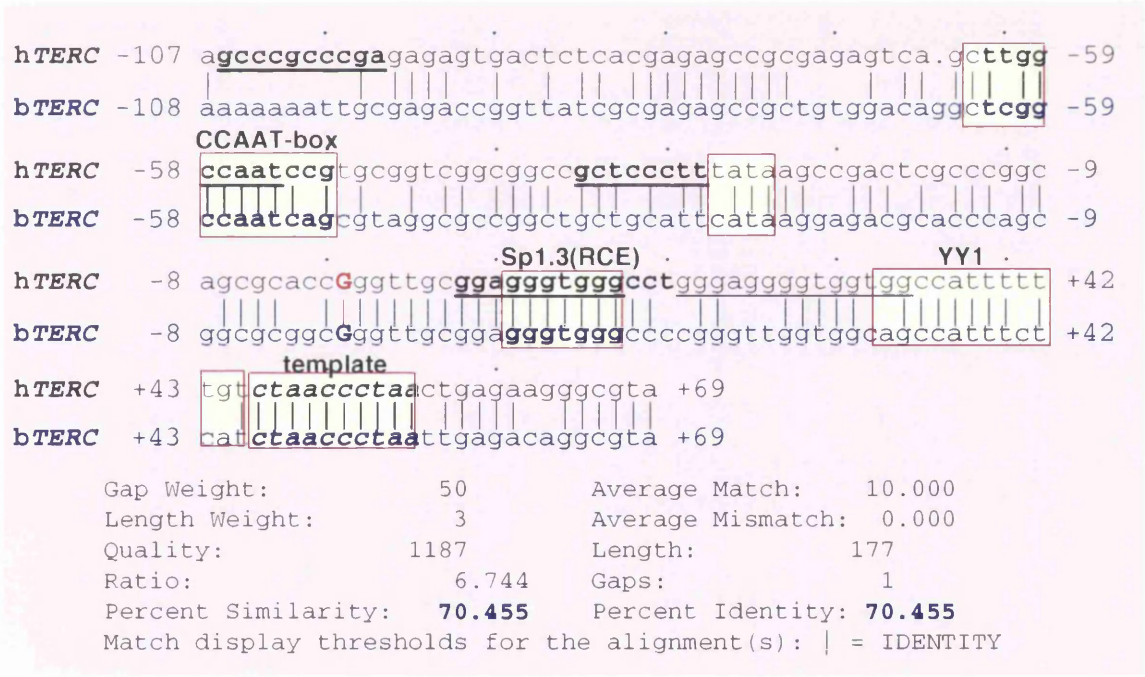


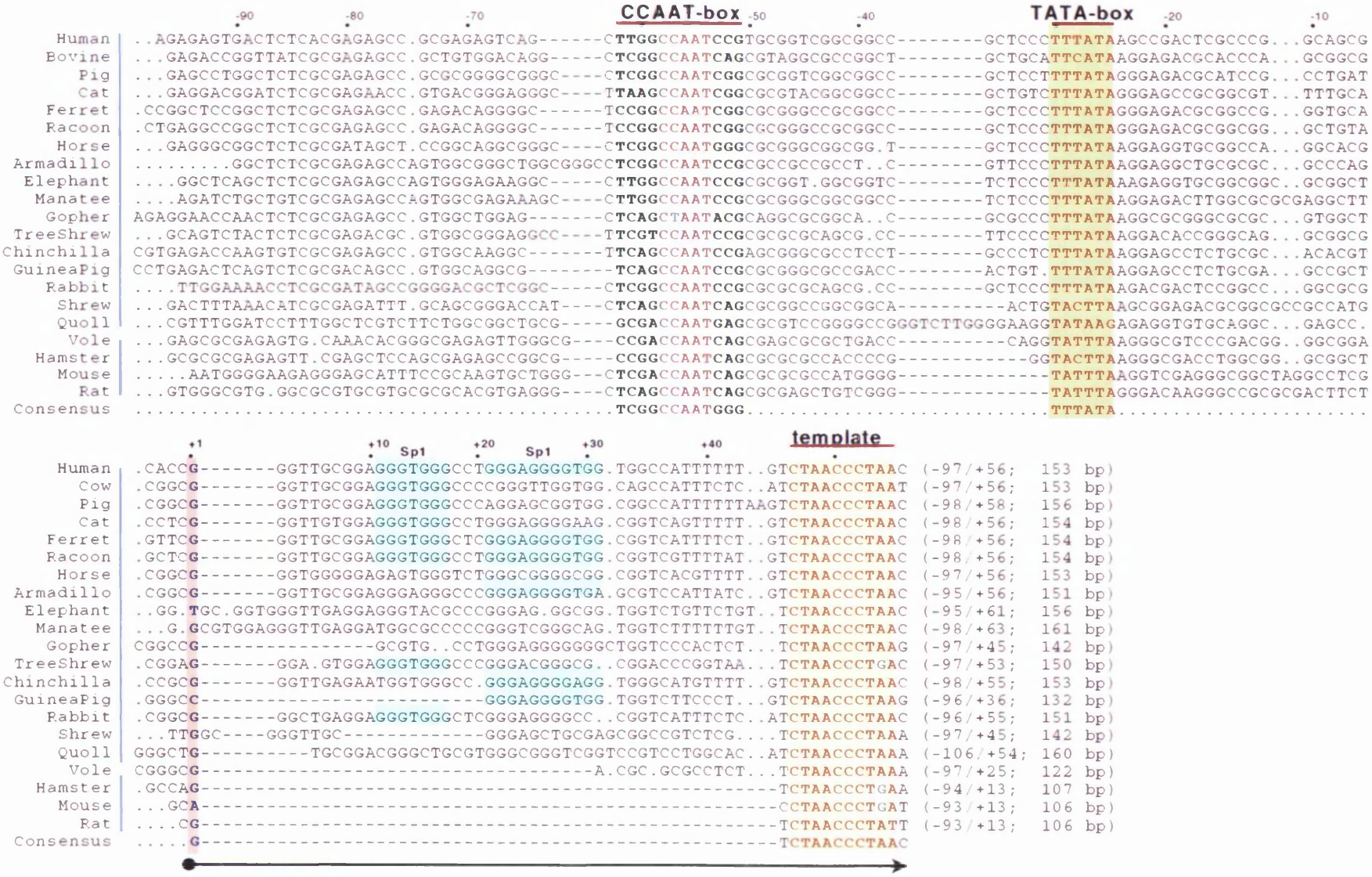
Figure 5.10 Sequence alignment of the h*TERC* and b*TERC* proximal promoters

Conserved nucleotides are shown as 'I'. There exists 70.5% sequence identity between h*TERC* and b*TERC* promoters, the five conserved elements are highlighted with red boxes. The identified binding sites for transcription factors are underlined in the h*TERC* sequence. The transcriptional start site is shown as red capital "G", numbers on either side are in relation to the TSS. The template regions are in bold Italic.

Figure 5.11 Alignment of mammalian telomerase RNA gene proximal promoter sequences

Three conserved sequence elements (CCAAT-box, TATA-box and template) are indicated with red lines above the aligned sequences; identical nucleotides are indicated as red bold and shaded in white (CCAAT-box), green (TATA-box), or yellow (template). Transcription start site (TSS) described by Chen (2000) is shaded in red and indicated as "+1". Two identified Sp1 elements located between TSS and template regions of the human promoter are shaded in light blue. Gaps between the promoter region and potential TSS are filled with dashes, while gaps in the promoter and RNA gene sequence alignment are represented with dots. The coding region of the telomerase RNA genes is indicated with an arrow under the sequence alignment. Every tenth nucleotide of the human sequence with respect to the TSS is marked with a dot above the alignment. The range of each proximal TERC promoter and its size is indicated at the end of the respective sequence. Two blue vertical lines at the left of the sequences indicate two subgroups of mammalian TERC promoter. These are determined by presence or absence of sequence between TSS and template region.

Class Mammalia includes *Homo sapiens* (human), *Trichechus manatus* (manatee), *Elephas maximus* (elephant), *Dasypus novemcinctus* (armadillo), *Oryctolagus cuniculus* (rabbit), *Tupaia glis belangeri* (tree shrew), *Chinchilla brevicaudata* (chinchilla), *Cavia porcellus* (guinea pig), *Equus caballus* (horse), *Bos taurus* (bovine), *Sus scrofa* (pig), *Felis catus* (cat), *Procyon lotor* (raccoon), *Mustela putorius furo* (ferret), *Suncus murinus* (shrew), *Geomys breviceps* (gopher), *Microtus ochrogaster* (vole), *Cricetulus griseus* (hamster), *Mus musculus* (Mouse), *Rattus norvegicus* (rat), and *Dasyurus hallucatus* (quoll). (For reference see Chen et al., 2000).


CCAAT-box
TATA-box
-90
-80
-70
-50
-40
-20
-10
Human ..AGAGAGTGACTCTCACGAGAGCC.GCGAGAGTCAG------CTTGGCCAATCCGTGCGGTCGGCGGCC--------GCTCCCTTTATAAGCCGACTCGCCCG...GCAGCG
Bovine ...GAGACCGGTTATCGCGAGAGCC.GCTGTGGACAGG-----CTCGGCCAATCAGCGTAGGCGCCGGCT--------GCTGCATTCATAAGGAGACGCACCCA...GCGGCG
Pig ...GAGCCTGGCTCTCGCGAGAGCC.GCGCGGGGCGGGC----CTCGGCCAATCGGCGCGGTCGGCGGCC--------GCTCCTTTTATAGGGAGACGCATCCG...CCTGAT
Cat ...GAGGACGGATCTCGCGAGAACC.GTGACGGGAGGGC----TTAAGCCAATCGGCGCGTACGGCGGCC--------GCTGTCTTTATAGGGAGCCGCGGCGT...TTTGCA
Ferret .CCGGCTCCGGCTCTCGCGAGAGCC.GAGACAGGGGC------TCCGGCCAATCGGCGCGGGCCGCGGCC--------GCTCCCTTTATAGGGAGACGCGGCCG...GGTGCA
Racoon .CTGAGGCCGGCTCTCGCGAGAGCC.GAGACAGGGGC------TCCGGCCAATCGGCGCGGGCCGCGGCC--------GCTCCCTTTATAGGGAGACGCGGCGG...GCTGTA
Horse ...GAGGGCGGCTCTCGCGATAGCT.CCGGCAGGCGGGC----CTCGGCCAATGGGCGCGGGCGGCGG.T--------GCTCCCTTTATAAGGAGGTGCGGCCA...GGCACG
Armadillo .........GGCTCTCGCGAGAGCCAGTGGCGGGCTGGCGGGCCTCGGCCAATCCGCGCCGCCGCCT..C--------GTTCCCTTTATAAGGAGGCTGCGCGC...GCCCAG
Elephant ....GGCTCAGCTCTCGCGAGAGCCAGTGGGAGAAGGC-----CTTGGCCAATCCGCGCGGT.GGCGGTC--------TCTCCCTTTATAAAGAGGTGCGGCGGC..GCGGCT
Manatee ....AGATCTGCTGTCGCGAGAGCCAGTGGCGAGAAAGC----CTTGGCCAATCCGCGCGGGCGGCGGCC--------TCTCCCTTTATAAGGAGACTTGGCGCGCGAGGCTT
Gopher AGAGGAACCAACTCTCGCGAGAGCC.GTGGCTGGAG-------CTCAGCTAATACGCAGGCGCGGCA..C--------GCGCCCTTTATAAGGCGCGGGCGCGC...GTGGCT
TreeShrew ...GCAGTCTACTCTCGCGAGACGC.GTGGCGGGAGGCC----TTCGTCCAATCCGCGCGCGCAGCG.CC--------TTCCCCTTTATAAGGACACCGGGCAG...GCGGCG
Chinchilla CGTGAGACCAAGTGTCGCGAGAGCC.GTGGCAAGGC-------TTCAGCCAATCCGAGCGGGCGCCTCCT--------GCCCTCTTTATAAGGAGCCTCTGCGC...ACACGT
GuineaPig CCTGAGACTCAGTCTCGCGACAGCC.GTGGCAGGCG--------TCAGCCAATCCGCGCGGGCGCCGACC--------ACTGT.TTTATAAGGAGCCTCTGCGA...GCCGCT
Rabbit ....TTGGAAAACCTCGCGATAGCCGGGGACGCTCGGC-----CTCGGCCAATCCGCGCGCGCAGCG.CC--------GCTCCCTTTATAAGACGACTCCGGCC...GGCGCG
Shrew ...GACTTTAAACATCGCGAGATTT.GCAGCGGGACCAT----CTCAGCCAATCAGCGCGGCCGGCGGCA----------ACTGTACTTAAGCGGAGACGCGGCGCCGCCATG
Quoll ...CGTTTGGATCCTTTGGCTCGTCTTCTGGCGGCTGCG-----GCGACCAATGAGCGCGTCCGGGGCCGGGTCTTGGGGAAGGTATAAGAGAGGTGTGCAGGC...GAGCC.
Vole ...GAGCGCGAGAGTG.CAAACACGGGCGAGAGTTGGGCG----CCGACCAATCAGCGAGCGCGCTGACC----------CAGGTATTTAAGGGCGTCCCGACGG..GGCGGA
Hamster ...GCGCGCGAGAGTT.CGAGCTCCAGCGAGAGCCGGCG-----CCGGCCAATCAGCGCGCGCCACCCCG------------GGTACTTAAGGGCGACCTGGCGG..GCGGCT
Mouse .....AATGGGGAAGAGGGAGCATTTCCGCAAGTGCTGGG---CTCGACCAATCAGCGCGCGCCATGGGG--------------TATTTAAGGTCGAGGGCGGCTAGGCCTCG
Rat ...GTGGGCGTG.GGCGCGTGCGTGCGCGCACGTGAGGG----CTCAGCCAATCAGCGCGAGCTGTCGGG--------------TATTTAGGGACAAGGGCCGCGCGACTTCT
Consensus .....TCGGCCAATGGG.....TTTATA.....
+1
+10
Sp1
+20
Sp1
+30
+40
template
Human .CACCG-------GGTTGCGGAGGGTGGGCCTGGGAGGGGTGG.TGGCCATTTTTT..GTCTAACCCTAAC (-97/+56; 153 bp)
Cow .CGGCG-------GGTTGCGGAGGGTGGGCCCCGGGTTGGTGG.CAGCCATTTCTC..ATCTAACCCTAAT (-97/+56; 153 bp)
Pig .CGGCG-------GGTTGCGGAGGGTGGGCCCAGGAGCGGTGG.CGGCCATTTTTTAAGTCTAACCCTAAC (-98/+58; 156 bp)
Cat .CCTCG-------GGTTGTGGAGGGTGGGCCTGGGAGGGGAAG.CGGTCAGTTTTT..GTCTAACCCTAAC (-98/+56; 154 bp)
Ferret .GTTCG-------GGTTGCGGAGGGTGGGCTCGGGAGGGGTGG.CGGTCATTTTCT..GTCTAACCCTAAC (-98/+56; 154 bp)
Racoon .GCTCG-------GGTTGCGGAGGGTGGGCCTGGGAGGGGTGG.CGGTCGTTTTAT..GTCTAACCCTAAC (-98/+56; 154 bp)
Horse .CGGCG-------GGTGGGGGAGAGTGGGTCTGGGCGGGGCGG.CGGTCACGTTTT..GTCTAACCCTAAC (-97/+56; 153 bp)
Armadillo .CGGCG-------GGTTGCGGAGGGAGGGCCCCGGGAGGGGTGA.GCGTCCATTATC..GTCTAACCCTAAC (-95/+56; 151 bp)
Elephant ..GG.TGC.GGTGGGTTGAGGAGGGTACGCCCGGGAG.GGCGG.TGGTCTGTTCTGT..TCTAACCCTAAC (-95/+61; 156 bp)
Manatee ...G.GCGTGGAGGGTTGAGGATGGCGCCCCCGGGTCGGGCAG.TGGTCTTTTTTGT..TCTAACCCTAAC (-98/+63; 161 bp)
Gopher CGGCCG----------------GCGTG..CCTGGGAGGGGGGGCTGGTCCCACTCT...TCTAACCCTAAG (-97/+45; 142 bp)
TreeShrew .CGGAG-------GGA.GTGGAGGGTGGGCCCGGGACGGGCG..CGGACCCGGTAA...TCTAACCCTGAC (-97/+53; 150 bp)
Chinchilla .CCGCG-------GGTTGAGAATGGTGGGCC.GGGAGGGGAGG.TGGGCATGTTTT..GTCTAACCCTAAC (-98/+55; 153 bp)
GuineaPig .GGGCC-------------------------GGGAGGGGTGG.TGGTCTTCCCT...GTCTAACCCTAAG (-96/+36; 132 bp)
Rabbit .CGGCG-------GGCTGAGGAGGGTGGGCTCGGGAGGGGCC..CGGTCATTTCTC..ATCTAACCCTAAC (-96/+55; 151 bp)
Shrew ...TTGGC----GGGTTGC-------------GGGAGCTGCGAGCGGCCGTCTCG....TCTAACCCTAAA (-97/+45; 142 bp)
Quoll GGGCTG----------TGCGGACGGGCTGCGTGGGCGGGTCGGTCCGTCCTGGCAC..ATCTAACCCTAAA (-106/+54; 160 bp)
Vole CGGGCG-----------------------------------A.CGC.GCGCCTCT...TCTAACCCTAAA (-97/+25; 122 bp)
Hamster .GCCAG---TCTAACCCTGAA (-94/+13; 107 bp)
Mouse ...GCA---CCTAACCCTGAT (-93/+13; 106 bp)
Rat ....CG---TCTAACCCTATT (-93/+13; 106 bp)
Consensus .....G---TCTAACCCTAAC

5.3 Discussion

Reactivation of telomerase is an almost universal event in carcinogenesis. Though other proteins are involved in optimal function of the telomerase enzyme, the RNA subunit (TERC) and the catalytic protein components (TERT) are sufficient to reconstitute this enzyme activity. Consistent with telomerase activity, both components are expressed at high levels in the majority of cancers. The general goal of this study is to define candidate molecules that regulate the h*TERC* gene expression and to further understand mechanisms of transcriptional regulation. Several factors capable of modulating h*TERC* promoter activity in cell culture have been identified (overview in Figure 5.1). (i) h*TERC* transcription requires binding of nuclear factor NF-Y to the CCAAT-box. (ii) The promoter is activated by Sp1, and repressed by Sp3. (iii) The tumour suppressor protein pRb stimulates h*TERC* transcription. (iv) The transcriptional regulation of telomerase RNA gene appears to exhibit divergence between species.

5.3.1 NF-Y is essential for the h*TERC* expression

The sequences that control basal transcription are common to many genes and include the TATA-box, the CCAAT-box and the initiator sequence. Mutation of TATA-box did not affect promoter activity, however mutation of the CCAAT-box abolished h*TERC* transcription suggesting that this CCAAT motif is an essential element. Several pieces of evidence provided in this study indicate that the CCAAT-box bound by NF-Y plays a significant role in the transcriptional regulation of the h*TERC* gene.

In higher eukaryotic promoters the CCAAT-box is most usually found between 60 and 100 bp upstream of the transcriptional start site (TSS) of a gene (Maity & Crombrugghe, 1998; Mantovani, 1998). The h*TERC* CCAAT-box is located -58 to -54 bp upstream of the TSS, within the minimal promoter region that was narrowed down to 176 bp (ranging from position -107 to +69 bp) with the aid of 5´-deletion constructs (section 3.5.3). Secondly, *in vitro* DNase I footprinting analysis revealed that there were proteins binding to the region centred around the CCAAT-box from -60 to -50 bp (designated the H10 region). Thirdly,

in EMSAs, similar CCAAT-binding complexes were detected from four different immortal cell lines (HeLa, 5637, Swiss 3T3 and Susm-1; Figure 5.3B). These complexes could be supershifted by NF-Y antibodies or disrupted by mutation the CCAAT-box. These results indicate that the major retarded band was due to the binding of NF-Y to the CCAAT element in the promoter. Fourthly, deletion and site-directed mutagenesis of the CCAAT-box drastically reduced the Luc-reporter activity. Furthermore, inhibition of NF-Y by transient expression of a dominant-negative mutant NF-YA protein also specifically reduced Luc-reporter activity. In summary, NF-Y appears to be the major transcription factor that binds to h*TERC* CCAAT-box, and regulates the h*TERC* transcription.

NF-Y is a heterotrimeric transcription factor composed of three subunits (NF-YA, B, and C), which complexes with DNA (Maity & Crombrugghe, 1998; Mantovani, 1998). NF-Y subunit sequences are highly conserved among eukaryotes, and the yeast homologue heme-activated proteins can form heterotrimeric complexes with the corresponding mammalian subunits (Chodosh et al., 1988). Both NF-YB and NF-YC contain conserved putative histone fold motifs (HFM)(Baxevanis et al.,1995). This motif shows most similarity to histones H2B and H2A (Motta et al., 1999), and is responsible for the formation of the histone octamer. NF-YB and NF-YC form a tight association that is a prerequisite for NF-YA binding and sequence-specific DNA interactions (Sinha et al., 1995). Therefore, NF-Y may be important for organising promoter architecture leading to gene expression.

Recent studies from several laboratories have suggested that NF-Y interacts, either functionally or physically, with other transcription factors or regulatory proteins both *in vitro* and *in vivo* (Framson & Bornstein, 1993; Ericsson et al., 1996; van Ginkel et al., 1997; Wright et al., 1995a; Zwicker et al., 1995). Mutation of the CCAAT motifs of promoters, including that of the mouse 1 collagen gene, the mouse albumin gene, the human multidrug resistance gene, and the human blood coagulation factor X gene, decreases the basal promoter activity (Maity & de Crombrugghe, 1998) suggesting important role of NF-Y regulation in those genes. As discussed by Fucharoen studies (1990), removal of Y-box from the MHC class II Ea promoter dramatically decreases

transcription, altering start site selection (Fucharoen et al., 1990). NF-Y might have distinct roles depending on the promoter context; in Ea it could directly participate in the formation of a pre-initiation complex and ultimately help in selection of the TSS, in conjunction with an Initiator element (Mantovani et al., 1992; Bellorini et al., 1996,1997a; Caretti et al., 2000). In the globin promoter it might help build the overall architecture and allow the proper interactions of additional DNA binding activators (Liberati et al., 1998). Mutation in the NF-Y-binding site of the mouse α2(1) collagen promoter abolished the NF-Y -dependent transcriptional activation (Coustry et al., 1996). Studies by Hu et al (2000) reported that stable expression of a dominant negative NF-Y mutant protein in the mouse fibroblast cells inhibit expression of the E2F1, topo IIα, and α2(1) collagen genes, thus indicating that NF-Y directly in the regulation of these genes *in vivo* (Hu & Maity, 2000).

NF-Y specifically bound to the h*TERC* CCAAT-box, whereas other proteins which have been reported to bind CCAAT-boxes such as c/EBP, CTF and NF-1 did not bind to the h*TERC* CCAAT-box, suggesting that in addition to the CCAAT sequence other specific nucleotides are also required for NF-Y binding. Inhibition of NF-Y binding to CCAAT-box abrogates the h*TERC* transcription. Thus, the results presented so far strongly suggest that NF-Y is the h*TERC*-dependent CCAAT-box activator.

In general the vast majority of the CCAAT boxes have been shown to significantly contribute to overall promoter strength and, indeed, sometimes to be strictly required for activity (Mantovani, 1998). The h*TERC* gene promoter contains a TATA sequence which is not required for h*TERC* gene expression in the tested cells, others have also demonstrated that other promoters containing a TATA-like sequence might actually work without it (for review see Mantovani, 1998). In the case of the Ea promoter, for example, a TATA-like sequence binding TBP and TFIID is present at -25 bp (same as h*TERC*), but is functionally irrelevant (Viville et al., 1991; Bellorini et al., 1996). A direct interaction of NF-YB and NF-YC with TBP has recently been demonstrated *in vitro* (Bellorini et al., 1997a). NF-Y can serve a structural role by recruiting TBP and/or TAFIIs to connect upstream activators with the general polymerase II transcription machinery (Coustry et al., 1998; Matuoka & Chen, 1999). It has also been reported that NF-Y can interact with the

nucleosome. NF-Y, itself not posessing very strong transcriptional activation domains, play a dominant role in determining gene promoter architecture (Motta et al., 1999). Therefore, NF-Y might play an important role in h*TERC* gene transcription by organising promoter architecture. The significance of the architectural factor, NF-Y, in the differential expression of mortal and immortal cells should be considered and further elucidated.

Primary human cells have a limited life span in culture and undergo replicative senescence after a certain number of divisions. CBP/tk (equivalent to NF-Y) protein binding to the inverted CCAAT-box in the thymidine kinase gene is detected in serum-stimulated young (low PDL) IMR-90 cells, but not in senescent cells (Good & Chen, 1996), this indicates that the expression of CBP/tk protein is cell cycle- and age-dependent. Both NF-YA and NF-YB are constitutively expressed at the mRNA level in IMR-90 cells, however, expression of NF-YA and NF-YB at the protein level are clearly age-dependent (Good & Chen, 1996). NF-Y may also have a rule in cell senescence (Matuoka & Chen, 2000).

NF-Y also binds to and controls a number of cell cycle regulated promoters and may be directly involved in oncogenesis (Pang et al., 1996; Maity & Crombrugghe, 1998; Gu et al., 1999). NF-Y activity is found to change during senescence and in response to inducing agents (Good & Chen 1996; Farina et al., 1999; Marziali et al., 1999; Maity & Crombrugghe, 1998). It has been discussed that the reactivation or expression of telomerase may be a major mechanism by which cancer cells overcome normal cellular senescence (Bodnar et al., 1998; Holt & Shay, 1999; Parkinson et al., 1997). Therefore, NF-Y may be part of the molecular mechanism linkage cell senescence and telomerase expression.

5.3.2 Sp-family factors regulate telomerase gene transcription

The Sp1 binding sites within the h*TERC* promoter appears to be two distinct functions, one to weakly promote transcription and other to strongly repress telomerase RNA gene

activation. Competitive binding between different trans-factors at the same or nearby promoter elements, often having opposing effects, is a mechanism of transcriptional regulation seen in many promoters. This type of regulation may be a particularly important mode for the h*TERC* promoter. Sp1 and Sp3 represent just two members of a growing family of mammalian Kruppel-like transcription factors which bind GC-rich sequence elements, and are involved in the regulation of tissue specific and housekeeping genes. These factors have been shown to compete with similar affinities for binding at the same GC-box elements (Kingsley & Winoto, 1992; Hagen et al., 1992, 1994). Although Sp1 has long been known to function as an activating transcription factor, in many instances over-expression of Sp3 has indicated that Sp3 may serve as an inhibitory member of the Sp family, causing repression of Sp1-mediated promoter activation (Hagen et al., 1994; Majello et al., 1994, 1995; Kumar & Butler, 1997). Therefore, the relative levels of Sp1 and Sp3 proteins in a given cell are likely to be important determinants of the transcriptional activity of a target promoter (Kubo et al., 1995; Apt et al., 1996).

The multiple Sp1 elements found within the h*TERC* promoter suggest a complex interactions between Sp1 and Sp3. It has been demonstrated that the repression of promoter activity by Sp3 is strictly dependent on the promoter context of the DNA-binding sites and the number of Sp1 sites. It was discussed that Sp3 is functional when targeted to a promoter-proximal-RNA sequence and Sp3 may serve as important regulatory targets in setting the mRNA expression levels in various cell types (De Luca et al., 1996; Majello et al., 1997). Sp3-mediated repression may act directly on the general transcription machinery. Removal of the all identified Sp1 sites in the h*TERC* promoter abolished the Sp1 activation but did not abolish Sp3 repression (Figure 5.5B) suggesting other unknown mechanism. That may act through another as yet unidentified mechanisms (for example; a potential Sp1 site close to the 3'-end of proximal promoter region, Figure 3.4a).

Recent studies identified a 181 bp proximal promoter region of h*TERT* around the transcription start site which contains five Sp1 sites. Each of the five Sp1 sites co-operatively functioned as a *cis*-acting element and abrogation of all Sp1 sites resulted in

almost complete loss of transcriptional activity. In addition, Sp1 function was required for Myc-mediated transactivation of h*TERT* (Kyo et al., 2000). As described in section 5.2.2, removal of all four Sp1 sites in the h*TERC* proximal promoter region abolishes the Sp1 transactivation. These findings suggest a central role for Sp1 in activation of h*TERC* and h*TERT* transcription in an overlapping, though not necessarily co-ordinated control mechanism. Sp1 knockouts show that Sp1 is required for early mouse development (Lania et al., 1997). Sp1 is considered to be a ubiquitous factor, however greater than 100-fold differences in its expression level have been found between different cell types (Saffer et al., 1991), It has been demonstrated that levels of Sp1 expression were much higher in cancer than normal cells, correlating with telomerase activity (Kyo et al., 2000). Thus, despite its widespread expression, the level of Sp1 expression may be a critical determinant of telomerase activity not only in cancer but also in normal cells.

Co-operation between NF-Y and Sp1 has been demonstrated in the regulation of several genes including the MHC class II-associated invariant chain (Wright et al., 1995a), the $p27^{KIP1}$ gene (Inoue et al., 1999), the hamster thymidine kinase gene (Sorensen & Wintersberger, 1999), and the rat fatty acid synthase gene (Roder et al., 1997). The promoters of these genes all have in common one or several Sp1 binding sites located in close proximity (20 to 30 nucleotides) with an inverted CCAAT motif. Cooperation between these two transcription factors is involved in the insulin response of the fatty acid synthase gene, the vitamin D3 response of the $p27^{KIP1}$ promoter, and the serum response of the thymidine kinase promoter. The molecular mechanism responsible for this cooperative activity has been recently partially elucidated by the demonstration of the cooperative DNA binding of NF-YA and Sp1 and the presence of specific protein-protein interaction domains in NF-YA and Sp1 (Roder et al, 1997, 1999). A physical interaction between NF-YA and Sp1/Sp3 has been demonstrated (Yamada et al., 2000; Roder et al., 1999). In the h*TERC* promoter, there are multiple Sp1 binding sites, one Sp1 site located 38 nucleotides upstream, three Sp1 sites located 17, 35 and 48 nucleotides downstream of the NF-Y binding site, respectively. These Sp1 sites alone did not mediate h*TERC* transcription with a mutated CCAAT motif suggesting that Sp1 family factors modulation

of h*TERC* transcription is subject to a functional NF-Y binding CCAAT-box. Thus the functional co-operation between NF-Y and Sp1 remains to be tested. A proximal promoter structure is now emerging composed of a functional CCAAT-box, four Sp1 sites and template region (Figure 5.1).

5.3.3 pRb activates the h*TERC* transcription

Our finding that pRb is an activator of the h*TERC* promoter was somewhat surprising given its well-known function as a tumour suppresser. pRb negatively regulates transcription of a number of genes that play a role in cell cycle progression by forming complexes with members of the E2F transcription family. However, pRb also has an ability to co-activate a number of genes including TGF-β2, the retinoblastoma gene itself, cyclin D1, YY1 and the Werner helicase gene is well described (Kim et al., 1992a; Muller et al., 1994; Yamabe et al., 1998; Chen et al., 1994a: Petkova et al., 2000). Rb can also stimulate the c-fos, c-Myc, TGF-β1 (Kim et al., 1991; Udvadia et al., 1993) promoters through stimulation of Sp1-mediated transcription.

As described in previous sections, h*TERC* regulation involves Sp1-mediated transcription. Promoters with Sp1 sites containing GGGTGG sequence have been shown to be positively regulated in a cell type-specific manner by the retinoblastoma gene product (Kim et al., 1992b; Udvadia et al., 1992; Yamabe et al., 1998; Seller et al., 1996). The GGGTGG motif may therefore be an indicator for gene transactivation by pRb. In agreement with this, both the h*TERC* and b*TERC* promoters which contain these motifs (Figure 5.10) are stimulated by pRb, whilst m*Terc* dose not contain this motif and fails to respond to pRb activation. A study by Noe et al. provided evidence that Sp1 can be physically associated with pRb (Noe et al., 1998). Recombinant pRb protein enhances the DNA-binding activity of Sp1 (Kim et al., 1992b; Udvadia et al., 1993; Chen et al., 1994a) thus stimulating Sp1-mediated transcription. pRb might also act by liberating Sp1 from a negative regulator (Chen et al., 1994a). However, these mechanisms may not be acting in the case of h*TERC* regulation since pRb still activates promoter activity when all four Sp1

sites have been mutated. This is consistent with the fundings of others (Udvadia et al., 1992, 1995) and suggests that pRb regulation in the context may not involve DNA binding.

pRb has been shown to enhance the binding activity of both c/EBP (Chen et al, 1996a) and NF-IL6 (Chen et al, 1996b), members of the c/EBP family of transcription factors, to their cognate DNA sequences *in vitro*, and to transactivate c/EBPβ- and NF-IL6-responsive promoters in cells. It has been reported that Rb inhibits YY1 binding to DNA and blocks YY1-dependent transcription in vitro. YY1-Rb interaction causes the activation of genes that free YY1 repress and repression where free YY1 functions as an activator (Petkova et al., 2000).The present study suggests that pRb transactivation of the h*TERC* promoter requires a functional CCAAT-box. However, since the h*TERC* CCAAT-box has been shown to be recognised by NF-Y rather than c/EBP, it is unlikely that pRb activation of the h*TERC* transcription is through interaction with the CCAAT-binding factor c/EBP. It has been reported that over-expression of pRB alone in RB/p53-defective tumor cells causes cell senescence decrease in telomerase activity (Xu et al., 1997), and Nguyen et al., also demonstrated that functionally intact Rb was required for cell cycle-dependent downregulation of telomerase activity in SCC lines (Nguyen et al, 1999). Here transient expression pRb has been shown to transactivate h*TERC* promoter but to have no effect on the hTERT promoter (data not shown) suggesting differential regulatory mechanisms may be involved in transcriptional control between h*TERC* and hTERT gene expression. In recent years growing evidence has been accumulating for a more general function of pRb at both the transcriptional level and the cellular level. pRb not only regulates the activity of certain protein-encoding genes but also the activity of RNA polymerase pol II and pol III transcription (Herwig & Strauss, 1997). How pRb activates transcription is not fully understood but in keeping with the results of Sellers (1998) the ability of pRb to activate the h*TERC* promoter is independent of its ability to bind E2F (unpublished data generated in our Lab). It is unknown whether pRb can activate endogenous h*TERC* gene expression in cells, or whether pRb acts through an interaction with NF-Y and further research is clearly required to answer this question.

To summarise, the h*TERC* promoter is responsive to transactivation by pRb, which is not mediated through identified Sp1 sites. By analogy with other studies pRb possibly activates h*TERC* transcription by interaction with other nuclear proteins rather than directly binding to the DNA sequence. The present findings provide evidence for a functional link between pRb and the h*TERC* gene, and the identification of h*TERC* as another gene that is activated by pRb may contribute to the investigation of this aspect of pRb function.

5.3.4 E1A activates telomerase genes transcription

Since active telomerase requires the presence of both the protein and RNA components, it is intuitive to expect that the synthesis of the components may be co-ordinated. Transcriptional up-regulation of h*TERC* is observed in immortalised cell types that express endogenous hTERT (Yi et al., 1999). In addition, I have shown that activity of both the h*TERC* and hTERT promoters is higher in immortal cells than in two mortal cell strains. This observation is consistent with other studies (Cong et al., 1999) showing that transcription-rate of the telomerase genes is different between normal and tumour cells and that these parallel telomerase enzyme activity (Yi *et al.*, 1999).

Expression of telomerase has been reported to be regulated at the transcription level (Zhao et al., 1998; Hinkley et al., 1998; Fujimoto et al., 2000; Kyo et al., 1999, 2000; Wick et al., 1999; Cong et al., 1999; Horikawa et al., 1999; Hargrove et al., 1999). A variety of molecules may potentially be involved in telomerase regulation (section 1.5.2, and 1.5.4). In this study, transcriptional regulation of the h*TERC* promoter was further investigated by introducing a viral transforming protein E1A. The E1A proteins of small DNA tumour viruses are able to disrupt normal cell growth control leading to uncontrolled cell proliferation by their abilities to associate with a number of cellular growth regulatory proteins through multiple domains. The E1A proteins alter the cell growth regulation through at least two independent protein binding domains; one domain binds members of the pocket-containing protein family, the most characterised of which is the Rb tumour

suppresser protein. The second domain binds members of the CBP/p300 family of co-activators (Jones, 1995; Moran, 1993). It has also been reported that E1A protein induces the expression and assembly of a heteromeric complex consisting of the 110-kDa protein CBF/NF-Y, and mediates optimal transactivation of the human cdc2 promoter by promoting interaction of NF-Y with the two CCAAT motifs of the cdc2 promoter (Kao et al., 1999; Tanimoto et al., 1998). The adenovirus E1A protein is also be able to alter YY1 function from a repressor to a trans-activator (Shi et al., 1991) and trans-activates gene promoters through induction of c-MYC (Jayachandra et al., 1999). Since the oncoprotein c-Myc up-regulates telomerase activity by targeting h*TERT* promoter E-box and NF-Y is a major activator of h*TERC* transcription by binding h*TERC* promoter Y-box, these could provide the mechanisms of activation of both telomerase components by E1A. Further investigation using E1A mutants will be useful in dissecting the mechanism of E1A activation of telomerase.

Taken all together, transcriptional regulation of the h*TERC* promoter involves NF-Y and Sp1 family factors. This promoter architecture also allows other onco- or cellular-proteins (such as E1A and pRb) to transactivate the h*TERC* promoter activity in certain circumstances. The data for NF-Y, Sp1 factors, pRb and E1A suggest a possible role of chromatin in the h*TERC* regulation.

5.3.5 Chromatin modification and the h*TERC* gene regulation

Transcription factors not only bind to the cognate DNA sequences, but also interact with one another to regulate gene transcription. In addition, these factors interact with non DNA-binding proteins, such as co-activators and co-repressors. They, in turn, form a ternary complex, interact with basic transcription machinery, and confer 3-dimensional structure which results in stimulation or repression of the gene promoter. Information accumulated over the past several years has demonstrated that transcription is the first step in eukaryotic gene expression and that chromatin structure plays a vital role in the regulation of transcription. Chromatin structure can restrict the access of transcription-

associated proteins to promoters and histone acetylation is a major mechanism for antagonism of chromatin-mediated repression.

A number of transcription factors can associate with histone acetyltrasferases (HAT) which stimulate transcription by acetylating histones and disrupting nucleosome structure. Conversely, several other factors have been shown to interact with histone deacetylases (HDAC) whose activity leads to nucleosome formation and promoter repression (Owen et al., 1998; Motta et al., 1999; Faniello et al., 1999; Jin & Scotto, 1998; Currie, 1998). Sp1 interacts with p300/ CBP which has HAT activity (Owen et al., 1998). The activities of Sp1 and NF-Y are altered by the treatment of cells with an inhibitor of HDAC, trichostatin A (Jin & Scotto, 1998; Sowa et al., 1997). NF-Y has been demonstrated to associate with nucleosomes (Motta et al., 1999) and to exert its effect through alterations in promoter architecture. It can also interact with HAT, p300, P/CAF and GCN5 (Faniello et al., 1999; Jin & Scotto, 1998; Currie, 1998). These studies demonstrate that Sp1 and NF-Y may regulate gene transcription by chromatin modification. It would be of interest to examine whether the regulation of telomerase RNA gene in development and in mortal and immortal cells is related to chromatin structure and if so, whether this is mediated by NF-Y.

5.3.6 Regulation of mammalian TERC promoter

The major transcriptional regulatory elements are usually present in the proximal promoter region. Comparative studies have demonstrated that the conserved 5'-flanking sequences often constitute *cis*-acting elements that are involved in the regulation of gene expression (Thacker et al; 1999), and that important regulatory regions can be discovered by comparison of the human and rodent sequences (Fickett & Wasserman, 2000). As more data accumulates, it will be valuable to examine the extent of the conservation within evolutionary tree of mammalian telomerase RNA gene proximal promoter sequences looking, firstly, at a particular sequence element, secondly, the proteins binding that element, and finally, a possibly correlated phenotype. I have identified several elements conserved across mammals in the h*TERC* promoter (Figure 5.11).

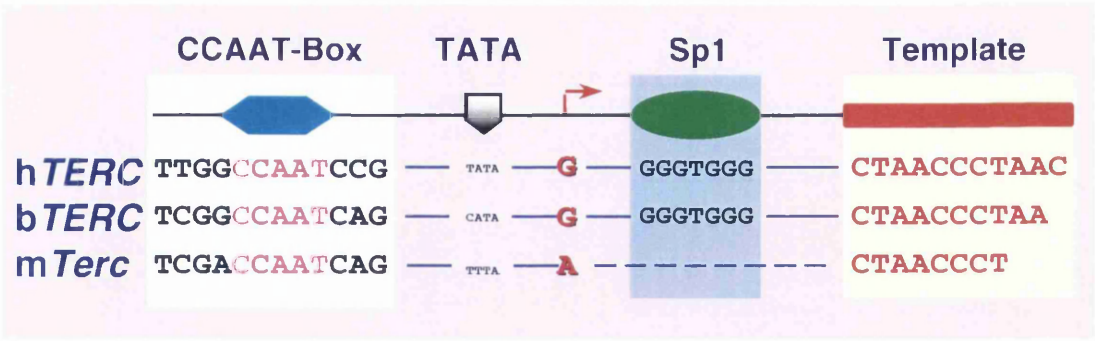


Figure 5.12 Speculative model of mammalian *TERC* proximal promoter structure

The highly conserved CCAAT-box, TATA-box and TERC template regions are indicated. Sp1 motifs are indicated in the 5'-flanking transcriptional region (5'-FTR) between the transcriptional start site and template regions of the human and bovine TERC gene promoters. The 5'-FTR absent in the m*Terc* gene promoter is indicated as a broken line.

Table 5.1 The list of CCAAT-box elements in mammalian TERC

Class	Gene	CCAAT-box	MAT	POS	TATA	DIS	REF
	Consensus	YYRRCCAAT(C/G)(A/G)G	-------	-----	-----	-----	Chen,1997; Matuoka,1999
	NF-Y	TTAACCAATCAG	12/12	-----	-----	-----	Vourio et al., 1990
	CBF	TTAACCAATCAC	11/12	-----	-----	-----	Lum et al., 1990
Mammalian (21)	Bovine	TCGGCCAATCAG	12/12	-58/-54	a	24	AF221936
	Pig	TCGGCCAATCGG	12/12	-58/-54	a	24	AF221920
	Horse	TCGGCCAATGGG	12/12	-57/-53	a	23	AF221925
	Ferret	CCGGCCAATCGG	12/12	-58/-54	a	24	AF221931
	Racoon	CCGGCCAATCGG	12/12	-58/-54	a	24	AF221917
	Mouse	TCGACCAATCAG	12/12	-53/-49	b	18	AF221922
	Rat	TCAGCCAATCAG	12/12	-53/-49	b	18	AF221916
	Hamster	CCGGCCAATCAG	12/12	-55/-51	b	20	AF221928
	Vole	CCGACCAATCAG	12/12	-57/-53	b	22	AF221909
	Human	TTGGCCAATCCG	11/12	-58/-54	a	24	AF221907
	Rabbit	TCGGCCAATCCG	11/12	-57/-53	a	23	AF221918
	Elephant	TTGGCCAATCCG	11/12	-56/-52	a	23	AF221932
	Armadillo	TCGGCCAATCCG	11/12	-56/-52	a	22	AF221906
	Chinchilla	TCAGCCAATCCG	11/12	-58/-54	a	24	AF221937
	GuineaPig	TCAGCCAATCCG	11/12	-57/-53	a	23	AF221929
	Manatee	TTGGCCAATCCG	11/12	-44/-40	-16/-11	24	AF221923
	Cat	TAAGCCAATCGG	11/12	-58/-54	a	24	AF221939
	Shrew	TCAGCCAATCAG	12/12	-57/-53	b	22	AF221921
	Gopher	TCAGCTAATACG	9/12	-57/-53	b	22	AF221930
	TreeShrew	TCGTCCAATCCG	10/12	-57/-53	a	23	AF221912
	Quoll	GCGACCAATGAG	11/12	-66/-62	a	32	AF221919

The name of the gene is indicated as is the CCAAT sequence in the promoter. The CCAAT-box consensus sequence (Matuoka & Chen, 1999; Chen et al., 1997; Pang et al., 1993) is shown (Y=pyrimidines and R=purines). Bold sequence indicates a proven binding site for transcription factor NF-Y. MAT is the number of base pairs matching the consensus CCAAT-box sequence and the pink colour shows a mismatch. POS is the position of the CCAAT motif with respect to the +1 transcriptional start site (TSS). TATA indicates whether the promoter has a consensus TATA signal in the -30/-25 bp region shown as "a" or in the -31/-26 bp region shown as "b". DIS indicates the distance between the CCAAT and the TATA signals. REF is the reference or GeneBank accession number.

The distances between TSS and template region vary from 2 to 51 bp in mammalian TERC genes. The h*TERC* TSS lies 45 bp upstream of the template, whereas the mouse appears only 2 bp upstream of the template (Feng et al., 1995; Hindely et al., 1998; Chen et al., 2000). The mouse, rat, hamster and vole TERC genes lack this sequence region suggesting that it is not essential for telomerase function. This region is part of TERC gene promoter defined as the 5' flanking transcriptional regulatory region (5'-FTR). Comparison 5'-FTR sequence (+1 to +45 bp) of the human with other species shows that, the ferret has the highest identity of 93%, the racoon, pig and bovine have 91%, 87% and 76% respectively. Phylogenetic analyses of proximal promoter (Figure 5.9) demonstrate that the bovine and pig TERC gene are more similar to the human. Depending on whether they contain the 5'-FTR, mammalian TERC gene promoters can be divided into two groups, the m*Terc* promoter is amongst those which lack this feature. Presence of the Sp1 site with GGGTGG sequence element in the TERC 5'-FTR region indicates pRb activates gene transcription. The GGGTGG motif in h*TERC* 5'-FTR region is also present at the same position in 11 of 21 mammalian genes including the bovine, pig, cat, ferret, racoon, treeshrew, rabbit and possible horse (see Figure 5.11). We hypothesise that the 5'-FTR regions of TERC gene may be responsible for differential transcriptional mechanisms which may alternatively explain the different phenotype. Therefore, this is important as the divergence in the regulation of telomerase RNA genes between different species needs to be taken into account when studying telomerase in animal models of development and disease states (Blasco et al., 1997).

TATA-box and CCAAT-box binding site and the template sequence in the proximal region are highly conserved across mammalian species even though considerable variation is tolerated in other potential transcription factor binding sites. A typical TERC promoter structure is predicted (Figure 5.12). The alignments demonstrate constraints on sequence that are not explained by simple binding of the known factors. The TATA box, although it was shown to be non-functional in h*TERC*, is fixed at position -30 to -25 bp, but shows a varying distance to the template. The CCAAT-box is located position at -58 to -49 bp in 19 of 21 genes, with the exception of two genes (manatee is in -44/-40 and quoll is in -66/-

62, Table 5.1). Interestingly, h*TERC* NF-Y binding sequence, 5'-TTGGCCAATCCG-3', is also found in elephant and manatee genes. It has been reported that NF-Y binding motif in a promoter of the same gene from different species is strictly conserved. In all cases so far reported [MHC class II, γ-globin, α1 (I) collagen, albumin, MDR1, topoisomerase II α] this has been formally proven (Matovani, 1998). Statistical analysis of the binding sequence around NF-Y revealed a consensus YYRRCCAAT(C/G)(A/G)G (Matuoka & Chen, 1999; Pang et al., 1993; Bucher et al., 1990), which it seems to fit well with the mammalian TERC gene CCAAT-boxes. Thus it would be of interest to examine the interaction of NF-Y with TERC CCAAT-box in more detail.

5.4 Summary

Taking these findings collectively, a model for h*TERC* gene regulation can be developed (Figure 5.1). In this model, direct DNA-protein interaction is shown for NF-Y, Sp1 and Sp3. These and other factors may then be modulated by transcriptional regulators to interfere directly or indirectly with transactivation. Thus, permutations in signals including NF-Y, Sp1, Sp3, pRb and E1A are able to contribute to the regulation of h*TERC* gene expression. Alterations in the relative contributions of those factors in a cell may be relevant to h*TERC* gene expression. These data raise the possibility of cross-talk between the h*TERC* and hTERT gene expression at the transcriptional level as Sp1 is known to regulate hTERT. This is an important issue and mechanism of co-regulation should be a focus of future work. In particular, the study of NF-Y expression in mortal and immortal cells will be key issue for understanding h*TERC* regulation. This may perform an important biological function *in vivo*, which may link the global way of chromatin modification and telomerase regulation.

Chapter

6

Summary

- Summary of results
- Discussion of results
- Concluding remarks
- Future study

Chapter 6 Summary and Future Study

Over the past few years, the human telomerase RNA gene (h*TERC*) has been cloned and sequenced and the functional region for reconstitution of telomerase activity has been identified. In this study, the h*TERC* and m*Terc* gene promoters have been cloned and the transcriptional mechanism regulating h*TERC* gene expression has been investigated. Several regulators have been identified as being involved in this regulation (Figure 6.1). Analysis of promoter-reporter constructs has provided information concerning individual transcription factors, leading to the conclusion that Sp1, NF-Y and pRb activate, Sp3 represses the h*TERC* promoter.

6.1 Summary of results

6.1.1 h*TERC* promoter analysis

About 5.9 kb of h*TERC* genomic sequence has been cloned and 1.76 kb encompassing the h*TERC* gene promoter region was sequenced. No significant sequence similarity could be detected in comparisons between human and mouse 5' and 3' -flanking regions. Range between -5.0 kb and -51 bp in the h*TERC* gene is responsible for high promoter activity, the minimal promoter region has been defined as 176 bp of h*TERC* (-107 to +69 bp). Four Sp1 binding sites termed Sp1.1(-29/-36), Sp1.2(-106/-97), Sp1.3(+8/+17) and Sp1.4(+21/+32) have been identified in the h*TERC* proximal promoter region and were found to have different affinities for the Sp1 protein. A TATA-box closely overlapping the Sp1.1 site was not shown to bind any protein. Mutation of the Sp1.1 site resulted in a 3-4-fold induction of promoter activity suggesting it to bind to a negative regulator. Single or multiple site-directed mutation analysis demonstrated that the Sp1.3 might interact with positive regulators of h*TERC*. Mutation of the TATA-box alone had no significant effect on reporter gene activity. Transient co-transfection analyses demonstrated that Sp1 stimulates and Sp3 represses h*TERC* gene transcription. These data illustrate that Sp-family of factors involve in the regulation of h*TERC* transcription.

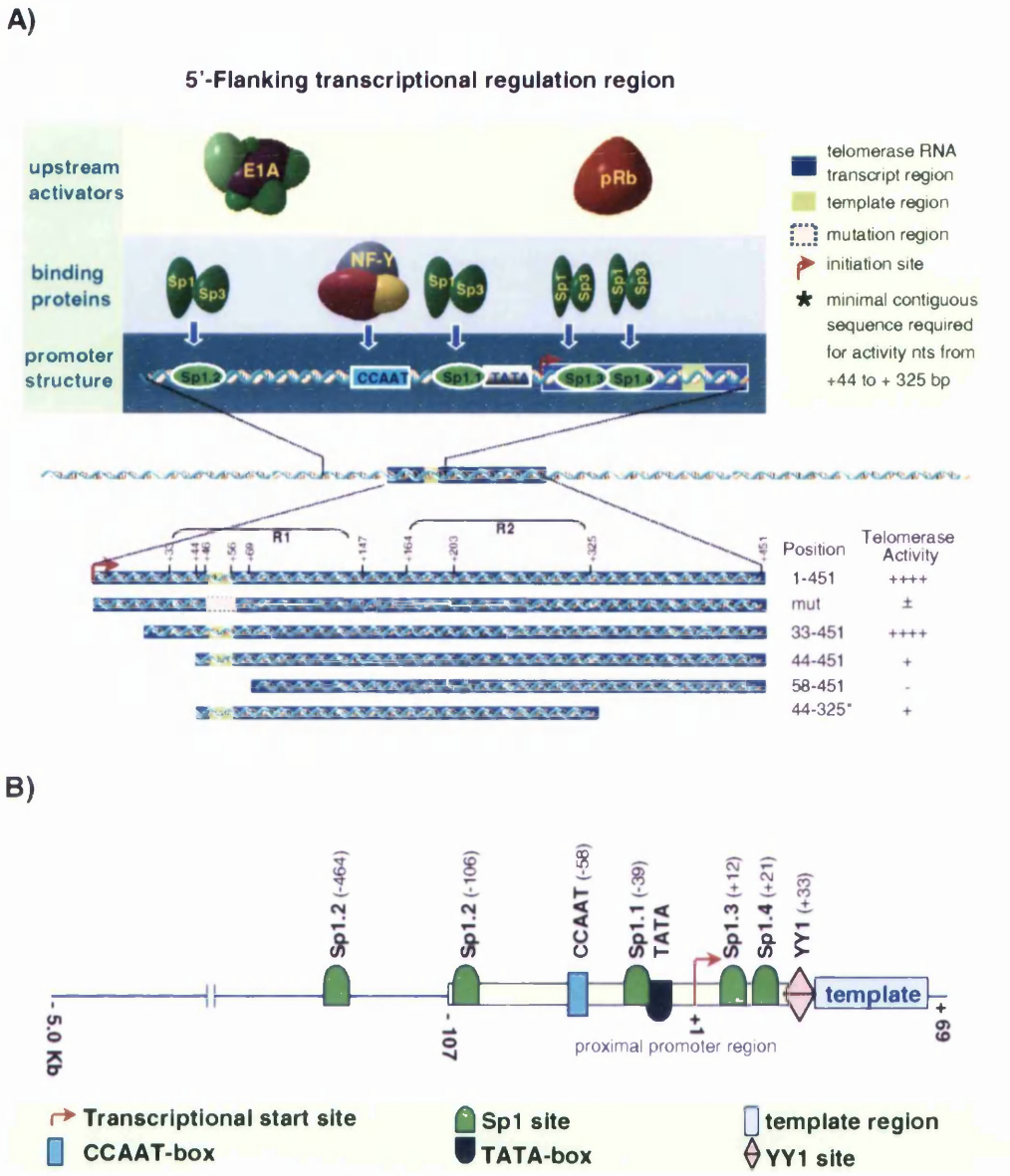


Figure 6.1 Model illustrating the human telomerase RNA gene structure

A) The genomic DNA of the h*TERC* gene is indicated in middle of diagram. Previously identified regions of functional h*TERC* RNA are shown at the bottom, and the 5'-flanking transcriptional regulatory region (5'-FTR) is shown at the top of diagram.

B) Schematic diagram of h*TERC* promoter region The identified transcriptional elements between -5.0 kb and +69 bp in the full-length h*TERC* 5'-flanking transcription regulatory region are shown. The core promoter of h*TERC* (-107/+69) are shown as yellow box. The various colours symbols or circles represent the different transcription factor binding sites and are indicated. The numbers indicate the position of DNA-binding sites related to the TSS.

6.1.2 h*TERC* transcription depends on NF-Y binding to the CCAAT-box

A major finding in this study is the h*TERC* CCAAT-box that is bound by nuclear factor NF-Y. Deletion or mutation of the CCAAT-box in the proximal promoter abolished h*TERC* promoter activity therefore demonstrating that the CCAAT-box is a key controlling element. EMSA experiments further identified the CCAAT-box motif as being specifically recognised by the transcription factor NF-Y. Furthermore, NF-Y was demonstrated to be essential for the h*TERC* gene transcription by using a dominant-negative NF-YA vector. Thus a functional CCAAT-box residing at -58 to -54 bp and nuclear factor NF-Y binding to this element appear to be essential for the transcriptional activation of h*TERC* gene.

6.1.3 Further regulation of h*TERC* transcription

h*TERC* promoter activity was investigated in several cell lines including human and mouse, the results show high transcriptional activity in several immortal cell lines in comparison to two mortal cell lines (WI38 and IMR90). Furthermore, co-transfection of the h*TERC* promoter constructs with E1A or pRb expression vectors causes up-regulation of promoter activity in immortal cells. How these gene products activate h*TERC* transcription was not further investigated in this study.

6.1.4 Mammalian TERC gene promoter comparisons

Sequence analysis has identified three conserved transcriptional elements. A typical mammalian TERC promoter contains a TATA-box located at about -25 bp, a single sense-orientated Y-box located around 50 bp upstream of the TSS, and a template sequence (Figure 5.12). Of all 21 TERC genes, only the bovine and pig TERC gene promoters are significantly close to the human in terms of overall sequence. The 5'-FTR sequence varies between different species, although most (11 of 21) contain the Sp1 site identical to h*TERC* GGGTGG motif. Only the mouse, hamster, rat and vole TERC genes differ most significantly in the 5'-FTR region. Despite conservation of sequence elements

within the promoter region, the regulation mechanism of TERC gene transcription may be different from different species. By using human, mouse and bovine promoter in transient co-transfection analyses, a functional divergence was observed; although Sp1 activates and Sp3 represses all three promoters, the human and bovine promoter are activated by pRb whereas the mouse promoter is not, and NF-Y regulation only appears to be important for the human promoter.

6.2 Final discussion

Regulation of gene expression by sequence-specific DNA binding proteins has emerged as one of the most important mechanisms governing cell differentiation, development, and homeostasis in metazoans. The population of transcription factors that are active in the cell nucleus largely dictates the transcriptional output and hence the proliferative or differentiated phenotype of the cell. Thus, it is not surprising that alteration of transcription factor function, as a result of either gain or loss of function mutations, are now been established as a frequent cause of neoplastic transformation and tumour progression in humans. Telomerase activity is easily detected in immortal cells but not in mortal cells suggesting that up-regulation of telomerase occurs in tumour progression. Recent studies have suggested that expression of telomerase is regulated at the transcription level (Zhao et al., 1998; Hinkley et al., 1998; Fujimoto et al., 2000; Kyo et al., 1999, 2000; Wick et al., 1999; Cong et al., 1999; Horikawa et al., 1999; Hargrove et al., 1999; section 1.5.2). In this study, progress has been made in identifying molecules involved in binding to and regulating the h*TERC* promoter (Figure 5.1 and 6.1). Specifically, the transcription factor NF-Y has been shown to be a major transactivator that can influence h*TERC* transcription, possibly by maintenance of the telomerase RNA gene promoter architecture through its binding to the CCAAT-box. NF-Y in combination with other factors controls h*TERC* gene expression.

There is an abundance of *cis*-acting elements identified in the h*TERC* promoter. The presence of multiple Sp1 binding sites in a promoter region is a good indication that they

play a role in regulating gene transcription. Sp1 is a member of a family of transcription factors consisting of Sp1, Sp2, Sp3 and Sp4. These proteins bind to GC-rich sequence (*e.g.* GGGCGG) found in the promoters of many cellular genes. Sp1 can activate transcription through a variety of mechanisms, functioning as a basal promoter element and as an upstream activator, depending on promoter context. In many instances over-expression of Sp3 has indicated that it may serve as an inhibitory member of the Sp family, causing repression of Sp1-mediated promoter activation (Hagen et al., 1994; Majello et al., 1994, 1995; Kumar & Butler, 1997). As discussed in section 4.3.1, Sp1 activates h*TERC* transcription through interaction with multiple Sp1 sites and Sp3 represses transcription via these sites, in addition there are unknown ways in which these factors affect h*TERC* transcription. Sp1 elements in the h*TERC* promoter may therefore serve as bifunctional promoter elements, acting as either positive or negative regulatory elements depending upon the complement of *trans-acting* factors interacting at nearby elements. Based on the present results and the current scientific literature, Sp1-mediated hTERC transcription is stimulated by the retinoblastoma gene product. One could speculate that pRb may regulate h*TERC* transcription by interaction with other nuclear factors rather than by binding directly to the DNA (Kim et al., 1992b; Udvadia et al., 1992, 1995), however this regulation appears to be subject to transcription factor binding to the CCAAT-element.

Mutation of all four Sp1 sites in the proximal promoter does not prevent efficient h*TERC* transcription, a clear indication that additional activators are indeed operating. Among upstream elements, the CCAAT-box is one of the most common, being found in 25% of eukaryotic promoters, with a strong position preference at -60 to -80 (Matovani, 1998). As described in section 5.3.1, NF-Y binding to the CCAAT-box plays a major role in transcriptional regulation of the h*TERC* gene. NF-Y is generally considered to function as a transcriptional activator and appears to increase the affinity of transcription factors for their target sequence (Kunzler et al., 1994; Muller-Immergluck et al., 1990; Gerster et al., 1990; Tanaka & Herr, 1990; Williams & Tjian, 1991; Arents & Moudrianakis, 1993; Reith et al., 1994). Cooperative interactions between NF-Y and different transcription factors

have been described for several promoters (Mantovani, 1998; Maity & deCrombrugghe, 1998). NF-Y may also be involved in the transcriptional initiation complex (Liberati et al., 1999; Bellorini et al., 1996, 1997a). The CCAAT-box located within the h*TERC* promoter is required for NF-Y inducible transcription.

As discussed in section 5.3.1, NF-Y might represent a key component of the transcriptional machinery of the h*TERC* promoter. h*TERC* transcription depends on NF-Y possibly for both physical organisation and in functional regulation. This finding may point to a potential anti-cancer therapy. Thus a molecular approach has validated the NF-Y/h*TERC* CCAAT-box interaction as a possible drug target. If a specific molecule such as polyamides, sequence specific DNA-binding small molecule (Dickinson et al., 1999; Dervan & Burli, 1999), could be designed to target and disrupt NF-Y binding to the h*TERC* CCAAT-box, h*TERC* expression could be inhibited. On the other hand, NF-Y is a pharmacological target with three known but unrelated anticancer agents inhibiting NF-Y activity. These drugs are Genistein which may operate through phosphorylation (Zhou & Lee, 1998), HMN-154 which may inhibit NF-Y complex formation by interaction with NF-YB (Tanaka et al., 1999) and the alkaloid isolated from the marine ascidian, *Ecteinascidia turbinata,* ET-743, which may interfere with the interaction of NF-YA with DNA (Minuzzo et al., 2000; Jin et al., 2000; Bonfanti et al., 1999). Although it is likely that drugs such as ET-743 will still lack specificity and may target numerous NF-Y regulated promoters, an ideal situation is to develop DNA binding drugs that target specific sequences and alter the expression of individual genes. We speculate that stable inhibition of such NF-Y binding might consequently inhibit telomerase and/or telomere elongation *in vivo* by prevention of h*TERC* gene expression.

Many transcription factors have been shown to be important in the regulation of hTERT (section 1.5.2), NF-Y can now be added to this to make a list of known regulators of telomerase as a whole. A physical and functional interaction between c-Myc and the NF-YC subunit has been reported recently (Taira et al., 1999). Since NF-Y and c-Myc appear to be important regulators of h*TERC* and hTERT transactivation respectively, this might suggest a degree of cross-transcriptional regulatory control between the h*TERC* and

hTERT. This speculation is consistent with the discussion reported by Stanta (1999) and Masutomi (2000). There may be a reciprocal control at the transcriptional level of the expression of h*TERC* and hTERT which in turn is associated with tumour progression (Stanta et al., 1999; Masutomi et al., 2000). Therefore, transcriptional regulation of the telomerase genes in certain cell-type seems to be complex issue including distinct mechanisms for each gene and possibly interactions between these.

In summary, the activity of the h*TERC* promoter was found to be controlled by a combination of the activities of the transcription factors Sp1, Sp3, and NF-Y. The interplay between Sp1, Sp3, and NF-Y within the architecture of the h*TERC* promoter, the ubiquitous nature of these *trans-acting* factors, and the action of tissue selective repressor element(s) may combine to enable a wide variety of cell types to differentially regulate h*TERC* expression through transcriptional control.

6.3 Concluding remarks

The present study has uncovered parts of the transcriptional machinery involved in the regulation of human telomerase RNA gene (h*TERC*) expression demonstrating that the Sp-family of factors, NF-Y and other regulators participate in this modulation through the h*TERC* natural promoter context. The h*TERC* promoter structure, in conjunction with the relative abundance and state of activity of the *trans-acting* factors, may provide the basis for selective cell-specific transcriptional regulation of the widely distributed telomerase RNA. Use of different context-dependent promoters to drive the synthesis of the telomerase RNA component in different species could reflect distinct pathways of telomerase gene regulation. Analyses of other *cis*-elements essential for promoter regulation as well as of proteins that interact with the NF-Y factor may provide further insights into the molecular mechanisms of telomerase RNA gene regulation in normal cells and during carcinogenesis. The understandings of telomerase regulation will not only satisfy the curiosity surrounding this complex and important enzyme but will also provide a foundation for the pursuit of telomerase-based therapeutic intervention.

6.4 Future studies

1. Several steps can be taken to achieve a more thorough picture of h*TERC* promoter structure. Firstly, DNase I footprinting and EMSA analysis are required to investigate the 3' end of h*TERC* promoter region. Mutation and deletion analysis of the h*TERC* proximal promoter region did not totally abolish the activity suggesting the involvement of other unknown machinery. Indeed, there is a potential YY1 site and another Sp1 site present in the more 3' end of promoter. It is also interesting to know whether the proteins binding to the template region regulate h*TERC* promoter activity. Secondly, further investigation of 5'-FTR function should be undertaken. It would be helpful to know whether the pRb activation effect is controlled through the 5'-FTR, and whether insertion of this region into the same position in m*Terc* would make the m*Terc* promoter responsible to pRb. This study could give an insight into the regulation mechanism for context-dependent TERC transcription. Further investigation of the upstream promoter region would also be important to identify further regulatory regions where positive or negative regulatory factors might bind and contribute to the overall regulation of h*TERC*.

2. In considering the regulation of telomerase genes, h*TERC* and hTERT, in normal development and during oncogensis, it is unclear how to h*TERC* and hTERT are co-expressed during the passage from mortal normal cells to immortal cancer cells. As with studies on almost any biological molecule, analysis of h*TERC* transcriptional regulation raises more questions than it answers. Of immediate interest is defining the role of NF-Y in transcriptional modulation in normal cells and cancer cells. It would also be of help to understand how NF-Y regulates h*TERC in vivo*, possibly by establishing a stable cell line expressing a dominant negative NF-YA. Further studies are required to address the nature of this signaling and the biological relevance of the NF-Y regulation of telomerase function.

3. Information has accumulated during the past few years to suggest that transcription is the primary level at which eukaryotic gene activity is controlled and chromatin

structure plays a vital role in this through acetylation and deacetylation of histones (Davie & Spencer, 2000). Therefore it would be interesting to investigate whether acetylation is important in the regulation of telomerase genes, and whether recruitment of chromatin remodelling proteins in general, and HAT and/or HDAC in particular, holds the key to activation or repression of telomerase gene expression. The reagents created in this study can be used to test this hypothesis.

We are like the seven blind men and the elephant in the Indian fable, each focusing on a very limited aspect of telomerase, awaiting the day when scientists can piece all the information into a complete picture.

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Appendix

Appendix I: Homo sapiens telomerase RNA (*TERC*) gene, promoter and complete sequence

AF047386 . Homo sapiens telom...[gi:3005555]

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LOCUS       AF047386 1765 bp    DNA         PRI       02-APR-1998
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            complete sequence.
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VERSION     AF047386.1  GI:3005555
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae;
            Homo.
REFERENCE   1  (bases 1 to 1765)
  AUTHORS   Zhao,J.Q., Hoare,S.F., McFarlane,R., Muir,S.,
            Parkinson,E.K., Black,D.M. and Keith,W.N.
  TITLE     Cloning and characterization of human and mouse
            telomerase RNA gene promoter sequences
  JOURNAL   Oncogene 16 (10), 1345-1350 (1998)
  MEDLINE   98206512
REFERENCE   2  (bases 1 to 1765)
  AUTHORS   Zhao,J.Q., Hoare,S.F., McFarlane,R., Muir,S.,
            Parkinson,E.K., Black,D.M. and Keith,W.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-FEB-1998) Medical Oncology, Beatson
            Institute, Switchback Rd, Glasgow G61 1BD, UK
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 121 aaaaaaaaaa tcgttacaat ttatggtgga ttactcccct ctttttacct catcaagaca
 181 cagcactact ttaaagcaaa gtcaatgatt gaaacgcctt tctttcctaa taaaagggag
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 481 aaatcttcct gtgaattcca tttttaaggt agtcgaggtg aaccgcgtct ggtctgcaga
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Sequence feature view of the RNA region: gi|3005555:799-1248

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VERSION     AF047386.1
KEYWORDS    .
SOURCE      human.
ORGANISM    Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata;
            Vertebrata; Mammalia; Eutheria; Primates;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 799 to 1248)
  AUTHORS   Zhao,J.Q., Hoare,S.F., McFarlane,R., Muir,S.,
            Parkinson,E.K., Black,D.M. and Keith,W.N.
  TITLE     Cloning and characterization of human and mouse
            telomerase RNA gene promoter sequences
  JOURNAL   Oncogene 16 (10), 1345-1350 (1998)
  MEDLINE   98206512
REFERENCE   2  (bases 799 to 1248)
  AUTHORS   Zhao,J.Q., Hoare,S.F., McFarlane,R., Muir,S.,
            Parkinson,E.K., Black,D.M. and Keith,W.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-FEB-1998) Medical Oncology, Beatson
            Institute, Switchback Rd, Glasgow G61 1BD, UK
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Appendix II: Mus musculus telomerase RNA (terc) gene, promoter and complete sequence

AF047387 . Mus musculus telom...[gi:3005556]

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            complete sequence.
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VERSION     AF047387.1  GI:3005556
KEYWORDS    .
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  ORGANISM  Mus musculus
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            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae;
            Murinae; Mus.
REFERENCE   1  (bases 1 to 4044)
  AUTHORS   Zhao,J.Q., Hoare,S.F., McFarlane,R., Muir,S.,
            Parkinson,E.K., Black,D.M. and Keith,W.N.
  TITLE     Cloning and characterization of human and mouse
            telomerase RNA gene promoter sequences
  JOURNAL   Oncogene 16 (10), 1345-1350 (1998)
  MEDLINE   98206512
REFERENCE   2  (bases 1 to 4044)
  AUTHORS   Zhao,J.Q., Hoare,S.F., McFarlane,R., Muir,S.,
            Parkinson,E.K., Black,D.M. and Keith,W.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-FEB-1998) Medical Oncology, Beatson
            Institute, Switchback Rd, Glasgow G61 1BD, UK
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3061 gccaagattc tgaaacccaa cgtcaagcct gacgagttcg agcctggcat ctctcagccg
3121 ctgctcgagc tggagatgac cacggatctc aaggcacagc tgtgggaact caacatcacc
3181 gaagccaagg aaaattgaag ttggtggtgg tcagaaggct gttataattt ttgtaccagt
3241 tcctcagctt aaatctttcc agaaaatcca agtctggcta gtttgtgaat tggagaaaaa

3301 gttcagcgga aagcacgtgt cttcattgct cagaagagga tctgtccaag ccaaccagga
3361 aaagctgtac gaaaaataag ccaaagcacc ctagaagctg caccctgaca gcagtgcatg
3421 tcttctcaag tgaaattgtg ggaaagagga tccatccgtg tgaaactgga tggcaatctg
3481 gagcaggttc atcttcctct ctggtacatc ccatgtctcc tcatctccat cctcccctct
3541 gcctctgtgt ctcatctcta aaactctcag cccatcttcc tttaccactg cccaatcaca
3601 ggctctagcc ttacctttca cctgccctca cctgcttata gacagcaatc tacatttctc
3661 ccttttgtc caattaaaag actcttttct ctcggatata aaatgagcac aactattatt
3721 accattctgt aatttataaa gtatagatag acctaacacc cagtctatca ttttgacagt
3781 taaataaagc attctgcaat cctatcctaa ctttaaaagg cttataattc tacacttgtt
3841 atgtcctggt tcagcttgta tattagaaaa ccatctcaaa ttatatatat atatatatta
3901 cacacacaca tatgtatata tacatatata tgtatacaca cacacacata tatatatgta
3961 tatgtatgta tgtatgtata tatatatact tttaatgcta aatagcctgg gttggctaag
4021 actacttcaa tcctgccaga attc

Sequence feature view of the RNA region: gi|3005556:1524-1952

```
LOCUS       AF047387      429 bp    DNA     ROD   02-APR-1998
DEFINITION  RNA from: Mus musculus telomerase RNA (terc) gene,
            promoter and complete sequence.
ACCESSION   AF047387
VERSION     AF047387.1
KEYWORDS    .
SOURCE      house mouse.
ORGANISM    Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
            Mammalia; Eutheria; Rodentia; Sciurognathi;
            Muridae; Murinae; Mus.
REFERENCE   1  (bases 1524 to 1952)
  AUTHORS   Zhao,J.Q., Hoare,S.F., McFarlane,R., Muir,S.,
            Parkinson,E.K., Black,D.M. and Keith,W.N.
  TITLE     Cloning and characterization of human and mouse
            telomerase RNA gene promoter sequences
  JOURNAL   Oncogene 16 (10), 1345-1350 (1998)
  MEDLINE   98206512
REFERENCE   2  (bases 1524 to 1952)
  AUTHORS   Zhao,J.Q., Hoare,S.F., McFarlane,R., Muir,S.,
            Parkinson,E.K., Black,D.M. and Keith,W.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-FEB-1998) Medical Oncology, Beatson
            Institute, Switchback Rd, Glasgow G61 1BD, UK
FEATURES    Location/Qualifiers
  gene            <1..429
                  /gene="terc"
  misc_RNA        1..429
                  /gene="terc"
                  /product="telomerase RNA"
BASE COUNT      63 a    141 c    145 g     80 t
ORIGIN

  1 ggggtattta aggtcgaggg cggctaggcc tcggcaccta accctgattt tcattagctg
 61 tgggttctgg tcttttgttc tccgcccgct gtttttctcg ctgacttcca gcgggccagg
121 aaagtccaga cctgcagcgg gccaccgcgc gttcccgagc ctcaaaaaca aacgtcagcg
181 caggagctcc aggttcgccg ggagctccgc ggcgccgggc cgcccagtcc cgtacccgcc
241 tacaggccgc ggccggcctg gggtcttagg actccgctgc cgccgcgaag agctcgcctc
301 tgtcagccgc ggggcgccgg gggctggggc caggccgggc gagcgccgcg aaggacagga
361 atggaactgg tccccgtgtt cggtgtctta cctgagctgt gggaagtgca cccggaactc
421 ggttctcac
```

Appendix III: The human telomerase reverse transcriptase (hTERT) gene promoter sequence from -499 to + 86 bp

DEFINITION Homo sapiens telomerase reverse transcriptase (TERT) gene, promoter.

SOURCE human.

ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 535)
Kyo et al., 2000;
Takakura et al., 1999;

FEATURES Sequences of the hTERT core promoter and consensus motifs for factor binding sites. The start site of transcription is shown by an arrow (Kyo et al., 2000; Takakura et al., 1999). The -1 indicates the first nucleotide 5' to the start site of transcription, while 1 indicates the first nucleotide of the mRNA. The initiating ATG codon is shown in capital bold. The E-box is boxed and the Sp1 consensus motifs are underlined.

BASE COUNT 535

ORIGIN

```
                                                        -449 ccctgggtct
-439 ccggatcagg ccagcggcca aagggtcgcc gcacgcacct gttcccaggg cctccacatc
-379 atggcccctc cctcgggtta ccccacagcc taggccgatt cgacctctct ccgctggggc
-319 cctcgctggc gtccctgcac cctgggagcg cgagcggcgc gcgggcgggg aagcgcggcc
                                                  WT1
-259 cagacccccg ggtccgcccg gagcagctgc gctgtcgggg ccaggccggg ctcccagtgg

-199 attcgcgggc acagacgccc aggaccgcgc ttcccacgtg gcggagggac tggggacccg
                                          E-box
-139 ggcacccgtc ctgccccttc accttccagc tccgcctcct ccgcgcggac cccgccccgt
                                        Sp1
 -79 cccgacccct cccgggtccc cggcccagcc ccctccgggc cctcccagcc cctcccttc
       Sp1                  Sp1                  Sp1
                        +1
 -19 ctttccgcgg ccccgccctC tcctcgcggc gcgagtttca ggcagcgctg cgtcctgctg
                   Sp1
 +42 cgcacgtggg aagccctggc cccggccacc cccgcgATGc cgcgc +86
       E-box
```

- The hTERT promoter region used in this study is from -449 bp to +32 bp in length.

Appendix IV: The bovine telomerase RNA gene (b*TERC*) promoter sequence

Bos taurus telomerase RNA gene, promoter region and partial sequence

```
LOCUS       AF176663  320 bp DNA             MAM       02-NOV-1999
DEFINITION  Bos taurus telomerase RNA gene, promoter region and
            partial sequence.
ACCESSION   AF176663
VERSION     AF176663.1  GI:6175865
KEYWORDS    .
SOURCE      cow.
  ORGANISM  Bos taurus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
            Euteleostomi; Mammalia; Eutheria; Cetartiodactyla;
            Ruminantia; Pecora; Bovoidea;
            Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 320)
  AUTHORS   Zhao,J.Q., Bilsland,A. and Keith,W.N.
  TITLE     Bovine telomerase RNA gene promoter
  JOURNAL   Neoplasia, 2(6); 531-539. (2000)
REFERENCE   2  (bases 1 to 320)
  AUTHORS   Zhao,J.Q., Bilsland,A. and Keith,W.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-AUG-1999) Medical Oncology, CRC Beatson
            Labs, Switchback Rd, Glasgow G61 1BD, UK
FEATURES             Location/Qualifiers
     source          1..320
                     /organism="Bos taurus"
                     /db_xref="taxon:9913"
     promoter        1..281
     misc_RNA        282..>320
                     /product="telomerase RNA"
BASE COUNT       86 a     72 c     98 g     64 t
ORIGIN

   1 ccgtacctgg cttttaagag gtggaaaaca gcgctcagaa gcactcaagt taattttcaa
  61 gtcacaaggt cagacgtcgg aagtaaaata ttcaaatctg cggataaaaa acgtgaggta
 121 gggcggcgac tatccttttg gccggaaaca gaacttttga gaaaaaaaaa aattgcgaga
 181 ccggttatcg cgagagccgc tgtggacagg ctcggccaat cagcgtaggc gccggctgct
 241 gcattcataa ggagacgcag ccagcggcgc ggcgggttgc ggagggtggg ccccgggttg
 301 gtggcagcca tttctcatct
//
```

Appendix V: The list of CCAAT-box in vertebrate *TERC* gene

Class	Gene	CCAAT-box	MAT	POS	TATA	DIS	REF
	Consensus	YYRRCCAAT(C/G)(A/G)G	-------	-----	-----	-----	Chen 1997, Matuoka 1999
	NF-Y	TTAACCAATCAG	12/12	-----	-----	-----	Vourio et al., 1990
	CBF	TTAACCAATCAC	11/12	-----	-----	-----	Lum et al., 1990
Mammalian (21)	Bovine	TCGGCCAATCAG	12/12	-58/-54	a	24	AF221936
	Pig	TCGGCCAATCGG	12/12	-58/-54	a	24	AF221920
	Horse	TCGGCCAATGGG	12/12	-57/-53	a	23	AF221925
	Ferret	CCGGCCAATCGG	12/12	-58/-54	a	24	AF221931
	Racoon	CCGGCCAATCGG	12/12	-58/-54	a	24	AF221917
	Mouse	TCGACCAATCAG	12/12	-53/-49	b	18	AF221922
	Rat	TCAGCCAATCAG	12/12	-53/-49	b	18	AF221916
	Hamster	CCGGCCAATCAG	12/12	-55/-51	b	20	AF221928
	Vole	CCGACCAATCAG	12/12	-57/-53	b	22	AF221909
	Human	TTGGCCAATCCG	11/12	-58/-54	a	24	AF221907
	Rabbit	TCGGCCAATCCG	11/12	-57/-53	a	23	AF221918
	Elephant	TTGGCCAATCCG	11/12	-56/-52	a	23	AF221932
	Armadillo	TCGGCCAATCCG	11/12	-56/-52	a	22	AF221906
	Chinchilla	TCAGCCAATCCG	11/12	-58/-54	a	24	AF221937
	GuineaPig	TCAGCCAATCCG	11/12	-57/-53	a	23	AF221929
	Manatee	TTGGCCAATCCG	11/12	-44/-40	-16/-11	24	AF221923
	Cat	TAAGCCAATCGG	11/12	-58/-54	a	24	AF221939
	Shrew	TCAGCCAATCAG	12/12	-57/-53	b	22	AF221921
	Gopher	TCAGCTAATACG	9/12	-57/-53	b	22	AF221930
	TreeShrew	TCGTCCAATCCG	10/12	-57/-53	a	23	AF221912
	Quoll	GCGACCAATGAG	11/12	-66/-62	a	32	AF221919
Aves (2)	Chicken	CCGGCCAATGGG	12/12	-65/-61	a	31	AF221938
	Macaw	CTGACCAATGGA	11/12	-64/-60	a	30	AF221924
		GCAGCCAATGCG*	11/12	-91/-87	-		
Reptilia (1)	Turtle	CTAGCCAATCCA	10/12	-65/-61	a	31	AF221911
		CGAGCCAATGGG*	11/12	-94/-90	-		
Amphibia (7)	Typhlonectes	GGAACCAATCAG	10/12	-71/-67	a	37	AF221910
		CGAACCAATCAG*	11/12	-95/-91	-		
	Xenopus	AGAACCAATCGA	9/12	-95/-91	a	61	AF221908
	Toad	ATAACCAATCAA	10/12	-68/-64	a	34	AF221913
	HornedFrog*	CCGGCCAATGCG	11/12	-12/-8	a	7	AF221926
	Bullfrog	CCGTCCAATAAA	9/12	-63/-59	a	29	AF221940
	Dermophis	CGAACCAATCAG	11/12	-83/-79	a	49	AF221934
	Herpele	CGAACCAATCAG	11/12	-73/-69	a	39	AF221927
Chondri-chthyes (4)	Stingray	TGGACCAATGGA	10/12	-71/-67	a	37	AF221914
	CownoseRay	TGGACCAATGGA	10/12	-71/-67	a	37	AF221935
	Sh. Shark	ATGGCCAATGGG	11/12	-79/-75	a	45	AF221915
	DogfishShark	CTCGCCAATGGG	11/12	-80/-76	a	46	AF221933

The name of the gene is indicated as is the CCAAT sequence in the promoter. The CCAAT-box consensus sequence (Matuoka & Chen, 1999; Chen et al., 1997; Pang et al., 1993) is shown (Y=pyrimidines and R=purines). Bold sequence indicates a proven binding site for transcription factor NF-Y. MAT is the number of base pairs matching the consensus CCAAT-box sequence and the pink colour shows a mismatch. POS is the position of the CCAAT motif with respect to the +1 transcriptional start site (TSS). TATA indicates whether the promoter has a consensus TATA signal in the -30/-25 bp region shown as "a" or in the -31/-26 bp region shown as "b". DIS indicates the distance between the CCAAT and the TATA signals. The CCAAT signal has not been reported by Chen (2000) shown as "*". REF is the reference or GeneBank accession number.

Class Mammalia includes *Homo sapiens* (human), *Trichechus manatus* (manatee), *Elephas maximus* (elephant), *Dasypus novemcinctus* (armadillo), *Oryctolagus cuniculus* (rabbit), *Tupaia glis belangeri* (tree shrew), *Chinchilla brevicaudata* (chinchilla), *Cavia porcellus* (guinea pig), *Equus caballus* (horse), *Bos taurus* (bovine), *Sus scrofa* (pig), *Felis catus* (cat), *Procyon lotor* (raccoon), *Mustela putorius furo* (ferret), *Suncus murinus* (shrew), *Geomys breviceps* (gopher), *Microtus ochrogaster* (vole), *Cricetulus griseus* (hamster), *Mus musculus* (Mouse), *Rattus norvegicus* (rat), and *Dasyurus hallucatus* (quoll).

Class Aves includes *Gallus* (Chicken) and *Anodorhynchus hyacinthinus* (Macaw). **Class Reptilia includes** *Chelydra serpentina* (Turtle). **Class Amphibia includes** *Xenopus laevis* (Xenopus), *Bombina japonica* (Toad), *Ceratophrys ornata* (HornedFrog), *Pyxicephalus adspersus* (Bullfrog), *Dermophis mexicanus* (Dermophis), *Herpele squalostoma* (Herpele), and *Typhlonectes natans* (Typhlonectes). **Class Chondrichthyes includes** *Dasyatis sabina* (Stingray), *Rhinoptera bonasus* (CownoseRay), *Rhizoprionodon porosus* (SharpnoseShark), and *Mustelus canis* (DogfishShark). (For reference see Chen et al., 2000).

Appendix VI: World wide web address

The useful web sites in this study:

Telomere and Telomerase	-http://resolution.colorado.edu/~nakamut/telomere/ telomere.html
Sequence Interpretation Tools	-http://www.genome.ad.jp/SIT/SIT.html-
TFSEARCH	-http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html-
TESS	-http://agave.humgen.upenn.edu/tess/-
Gene recognition & assembly	-http://compbio.ornl.gov/ Grail-1.3/.-
Gene Cards	-http://bioinformatics.weizmann.ac.il/cards/-
Sequence motif search	-http://www.motif.genome.ad.jp/-
NCBI	-http://www.ncbi.nlm.nih.gov/ -
TRANSFAC	-http://transfac.gbf-braunschweig.de/TRANSFAC/index.html-
ExPASy	-http://www.expasy.ch/-
InforMax	-http://www.informaxinc.com/-
doubletwist	-http://doubletwist.com/-
genestream	-http://vega.crbm.cnrs-mop.fr/-
Chromatin structure & function	-http://www.mdanderson.org/%7Egenedev/Bone/ chrom.html-
adenovirus 5 homepage	-http://www.mdanderson.org/%7Egenedev/Bone/ chrom.html-
NF-Y	-http://users.unimi.it/~mantor/-
Top10 link	-http://www.med.rug.nl/mdl/toplink.htm
Online Journals & Impact Factors	-http://www.med.rug.nl/mdl/journal.htm
Oncogene	-http://www.stockton-press.co.uk/onc/
Neoplasia	-http://www.nature.com/neo