Human Telomerase Determinants of Processivity and Fidelity

by

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to Thérèse, Aline, Octave, Karine, Julie

ABSTRACT

Telomeres are dynamic nucleoprotein complexes that protect the fragile termini of chromosomes. Without telomeres, chromosome ends are recognized as DNA breaks and inclined to nucleolytic degradation and end-to-end fusions. Telomeres consist of DNA repeats that are synthesized by telomerase, a specialized reverse transcriptase (RT) enzyme minimally composed of a catalytic protein subunit (TERT) and an essential RNA component (TR). Although the functional aspects of telomerase are not well elucidated, this ribonucleoprotein enzyme clearly regulates cellular life-span through its ability to maintain or elongate telomeres. Human TERT (hTERT) has a central region containing conserved (RT)-like motifs, and N- and C-terminal regions that are unique to the TERT family. We analysed the precise role of the C-terminus of hTERT by introducing small deletions and amino acid substitutions throughout the C-terminal region, and demonstrated that the hTERT C-terminal region is essential for enzyme catalysis *in vitro*. We reported that hTERT multimerization requires the presence of catalytically essential C-terminal amino acid residues and intact RT-like motifs on the same hTERT molecule. We also examined DNA synthesis at nucleotide resolution with a direct primer extension assay, and found that the hTERT C-terminus modulates telomerase processivity. In addition, we reported that human telomerase reconstituted in rabbit reticulocyte lysate (RRL) and partially purified telomerase from human cells catalyze the cleavage of DNA substrates prior to their elongation. Finally, we determined the experimental conditions for human telomerase expression in insect cells in order to obtain an abundant source of catalytically active recombinant telomerase.

RÉSUMÉ

Les télomères sont des complexes nucléoprotéiques situés à l'extrémité des chromosomes. Ils protègent les fragiles extrémités des chromosomes contre l'action de certains processus cellulaires qui engendrent leurs dégradations ou leurs fusions. Les télomères sont constitués de courtes séquences d'ADN répétées en tandem qui sont synthétisées par la télomérase, une transcriptase inverse (RT) spécialisée qui utilise une composante protéique (TERT) et une composante ARN (TR) comme molécule matrice lors de la synthèse de l'ADN télomérique. Malgré une connaissance incomplète des aspects fonctionnels de cette enzyme, la télomérase régule la longévité des cellules par sa capacité de maintenir la longueur des télomères ou de les allonger. La composante protéique de la télomérase humaine (hTERT) est constituée d'une région centrale arborant des motifs RT qui sont conservés parmi les membres de la famille des transcriptases inverses, et d'une région N- et C-terminale qui sont uniques aux membres de la famille TERT. Nous avons analysé le rôle précis de la région C-terminale de hTERT en introduisant dans ce domaine de petites délétions ou de simples substitutions d'acides aminés, et nous avons démontré que la région C-terminale de hTERT est essentielle pour générer une enzyme active. De plus, la multimérisation de hTERT requiert que les acides aminés de la région C-terminale essentiels pour l'activité enzymatique de la télomérase doivent être situés sur la même molécule que les motifs RT. Par une analyse fonctionnelle de la synthèse d'ADN, nous avons établi que la région C-terminale de hTERT régule la processivity de l'enzyme. Nous avons aussi démontré que la télomérase humaine, reconstituée dans un lysat de réticulocyte de lapin ou partiellement purifiée à partir d'extraits protéiques de cellules humaines, catalyse la

coupure de substrats d'ADN avant leur élongation. Finalement, nous avons déterminé les paramètres optimaux d'expression de la télomérase humaine dans des cellules d'insecte dans le but d'obtenir une source abondante d'enzyme.

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PREFACE

This Ph.D. thesis is written in accordance with the guidelines for thesis preparation of the Faculty of Graduate and Postdoctoral Studies of McGill University. I have exercised the option of writing the thesis as a manuscript-based thesis. As chapters of this thesis, I have included original manuscripts that have been published (chapter 2 and 3). Each chapter contains its own abstract, introduction, materials and methods, results, discussion, acknowledgements and references sections. A preface is also included at the beginning of each chapter in order to introduce the manuscript and to ensure that the thesis is a cohesive unit with a logical progression from one chapter to the next. Currently unpublished experimental results are described in chapter 4. A comprehensive review of the literature and a final discussion are presented in chapter 1 and chapter 5, respectively. The references appear at the end of the thesis.

Published manuscripts included in this thesis:

Chapter 2:

> **Huard, S.**, Moriarty, T.J., Autexier, C. **(2003)**. *The C terminus of the human telomerase reverse transcriptase is a determinant of enzyme processivity.* Nucleic Acids Res., **31**, 4059-4070.

Chapter 3:

> **Huard, S.**, Autexier, C. **(2004)**. *Human telomerase catalyzes nucleolytic primer cleavage.* Nucleic Acids Res., **32**, 2171-2180.

In addition to the published manuscripts included in this thesis, the candidate has contributed to the following studies, which have also been published:

> **Huard, S.** and Autexier, C. **(2002)**. *Targeting human telomerase in cancer therapy.* Curr. Med. Chem. – Anti. Canc. Agents **2**, 577-587.
>
> Moriarty, T.J., **Huard, S.**, Dupuis, S., Autexier, C. **(2002)**. *Functional multimerization of human telomerase requires an RNA interaction domain in the N terminus of the catalytic subunit.* Mol. Cell. Biol. **22**, 1253-1256.

CONTRIBUTIONS OF AUTHORS

The candidate performed most of the work presented in this thesis and wrote all of the included manuscripts with support from Dr. Chantal Autexier. The contribution of other authors to this work is described below. In chapter 2, the analysis of the processivity data was performed with the collaboration of Tara J. Moriarty, who is the second author on the published manuscript. In chapter 4, pFastBac-HTc(hTERT) and pFastBac-HTb(hTERT-hTR *cis*) plasmids as well as recombinant hTERT and hTERT-hTR *cis* baculoviruses derived from the previous plasmids were generated by Dr. Chantal Autexier and Dr. François Bachand, respectively. All the studies were conducted under the supervision of Dr. Autexier.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The work presented in this thesis focus on the biochemical and functional aspects of the human telomerase ribonucleoprotein enzyme. Investigations on the mechanism of action of telomerase have been performed and published in peer-reviewed journals. The major contributions of this work to original knowledge are summarized below:

1. The establishment of a direct extension primer assay using RRL-reconstituted human telomerase or native enzyme from human cells that monitors at nucleotide resolution DNA synthesis catalyzed by telomerase.

2. The finding that the C-terminal region of hTERT is a determinant of telomerase processivity and essential for enzyme catalysis.

3. The identification of a telomerase-dependent nucleolytic cleavage catalyzed by RRL-reconstituted human telomerase or partially purified endogenous enzyme from human cells, as a determinant of enzyme fidelity.

4. The determination of experimental conditions for human telomerase expression in insect cells in order to obtain an abundant source of catalytically active recombinant telomerase.

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CHAPTER 1

Literature review

1.1 *Introduction*

When a cell is subjected to genetic alterations that impair the stability of the genome, cancer may result. Cancer is characterized by a deregulation of the cell cycle leading to an uncontrolled proliferation of the cells. Over the years, intensive investigations of telomere biology have answered important questions about the mechanisms that maintain telomere homeostasis and the role of telomeres and telomerase in cancer development. Telomerase is activated in approximately 85% of human cancer tissues and is viewed as a useful marker in cancer diagnosis (Kim *et al.*, 1994). Only few tumour cells maintain telomeres by alternative mechanisms relying on recombination (Bryan *et al.*, 1995). However, our understanding of the relationship between telomeres, telomerase and cancer remains incomplete.

Telomeres are essential nucleoprotein structures located at the ends of chromosomes that are continuously shortened at each cell division (reviewed in Greider, 1996). Telomere attrition eventually results in a cell growth arrest and limits the number of cell divisions. In contrast, telomere lengths of immortalized cells and tumour cells are stable (Counter *et al.*, 1992). The primary mechanism by which human cells maintain telomeres involves the activation of an enzyme called telomerase, a specialized reverse transcriptase that synthesizes telomeric DNA directly onto the ends of chromosomes (reviewed in Blackburn, 2001). Most normal human somatic cells do not exhibit telomerase activity and undergo telomere shortening with consecutive cell divisions (Kim *et al.*, 1994). However, highly proliferating germline cells, hematopoietic stem cells and various types of cancer cells display telomerase activity that prevents telomere shortening

and enables indefinite proliferation of the cells (Kim *et al.*, 1994; Broccoli *et al.*, 1995; Wright *et al.*, 1995).

The importance of telomeres and telomerase in cellular immortalization has been demonstrated by different experimental approaches. The ectopic expression of telomerase in telomerase-negative human primary cells extends their life-span (Bodnar *et al.*, 1998), whereas the inhibition of telomerase in established telomerase-positive tumour cell lines induces telomere shortening and eventually cell growth arrest (Hahn *et al.*, 1999; Herbert *et al.*, 1999; Zhang *et al.*, 1999). Consequently, telomerase is an attractive target for anti-cancer therapeutic strategies (reviewed in Shay and Wright, 2002). Telomerase is a ribonucleoprotein complex that minimally consists of a protein subunit and an RNA component (Greider and Blackburn, 1985; Greider and Blackburn, 1989). A short sequence within the RNA is used as a template for the synthesis of telomeric DNA repeats onto the ends of chromosomes (Greider and Blackburn, 1989).

The work presented in this thesis focuses mainly on the biochemical and the functional aspects of the human telomerase ribonucleoprotein. Considerable experimental investigations have highlighted mechanistic questions that need to be answered in order to understand the function of each component of the telomerase enzyme and to facilitate the design of telomerase-specific anti-cancer molecules. In this literature review, I will draw together the genetic and biochemical results that have provided insights regarding the molecular properties of telomeres and telomerase. First, I will describe the functional organization of telomeres at the ends of chromosomes followed by an explanation of the mechanism of action of the telomerase enzyme.

Finally, I will explain the role played by telomeres and telomerase in replicative senescence, cellular immortalization and cancer.

1.2 *Telomeres*

Telomeres are particular chromatin structures found at the ends of chromosomes. This DNA-protein complex caps the chromosome termini and protects them against nuclease degradation, aberrant recombination and end-to-end fusions (reviewed in Blackburn, 2001). Chromosome ends that are uncapped by different types of molecular changes in telomeres are recognized as DNA breaks that induce a DNA damage response, resulting in an inappropriate processing of chromosome ends by DNA repair enzymes characterized by DNA fragmentation, chromosomal translocations and fusions. The presence of abnormal chromosome end structures can trigger cell cycle arrest and apoptosis. Thus the integrity of the telomere cap is essential to stabilize the genome against potential inducers of cell death. In most organisms, telomeres are composed of a long stretch of DNA repeats rich in guanine residues (reviewed in Neidle and Parkinson, 2003). The DNA strand oriented 5' to 3' towards the chromosome ends is referred to as the G-rich strand. The G-rich strand extends beyond the double-stranded DNA repeats to form a single-stranded G-rich overhang referred to as the G-tail (Wright *et al.*, 1997).

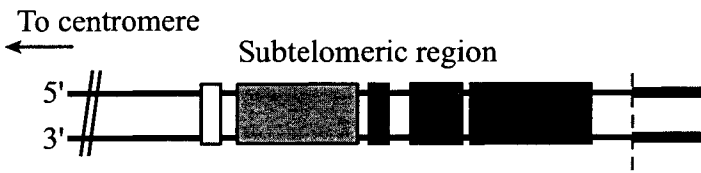
Sequence-specific telomere-associated proteins have been identified. These proteins physically protect chromosome ends and regulate telomere length and structure (reviewed in Kanoh and Ishikawa, 2003). These proteins can recruit additional partners important for maintaining the overall structure and preserving the appropriate functions of telomeres. In the following section, I will describe the functional

organization of telomeres which is conserved in eukaryotes. Telomeres can be divided into distinct regions: (1) subtelomeres; (2) double-stranded telomeric DNA repeats; and (3) G-tail (Fig. 1.1).

1.2.1 *Subtelomeres*

Human subtelomeres form a transitional DNA region of 10 to 300 kb between chromosome-unique sequences and telomeric DNA repeats (Fig. 1.1) (Flint *et al.*, 1997). Fluorescence *in situ* hybridization (FISH) analyses of the human subtelomeric regions provided evidence of duplicated DNA sequences in tandem at these sites (Trask *et al.*, 1998), obtained by frequent homologous recombination or gene conversion events (reviewed in Mefford and Trask, 2002). Yeast and vertebrate subtelomeres are structurally similar, but the primary sequence of these repeated elements diverges greatly among chromosomes and different organisms (reviewed in Mefford and Trask, 2002). The variable nature of subtelomeric sequences in different species has made it difficult to assess a specific function of these regions. Mutational analyses revealed that the subtelomeres may serve biological roles other than simply to operate as an intermediary zone. They are specialized regions of the chromosomes implicated in the transcriptional silencing of genes near the telomeres (reviewed in Tham and Zakian, 2002). However, the functional significance of their variability is still unknown.

In several organisms, genes in close proximity to telomeres are transcriptionally silenced, a process termed telomere position effect (TPE). TPE has been documented in yeast and humans by measuring the expression of a subtelomeric reporter gene (Gottschling *et al.*, 1990; Baur *et al.*, 2001; de Bruin *et al.*, 2001; Koering *et al.*, 2002). The level of gene transcription is not only dependent on its position near the



	Subtelomeric duplicated sequences	ds telomeric repeats	ss G-tail	Telomeric G-rich repeats
Budding yeasts	X, Y' elements	200-300 bp	12-14 nt; S phase >25 nt	
S. cerevisiae				$(TG)_{1\text{-}6}TG_{2\text{-}3}$
K. lactis				$ACG_2AT_3GAT_2AG_2TATGTG_2TGT$
Ciliates	Various	14-16 bp	14-16 nt	
T. thermophila				T_2G_4
O. nova, E. crassus				T_4G_4
Mammals	Various		>100 nt	T_2AG_3
H. sapiens		5-15 kb		
M. musculus		up to 100 kb		
Plants	Various	Variable, >50 kb in some cases	Either >20-30 nt or <12 nt	T_3AG_3
A. thaliana				

Figure 1.1 Organization of telomeric DNA in different eukaryotes.
Based on Chakhparonian and Wellinger, 2003.

telomeres, but also on the regulation of telomere length by telomere-associated proteins (Kyrion *et al.*, 1993; Sprung *et al.*, 1996; Baur *et al.*, 2001; Koering *et al.*, 2002). Longer telomeres are more effective silencers than short telomeres. The ability to induce TPE correlates well with the heterochromatic nature of telomeres which is dependent on histone deacetylation. The use of specific inhibitors of histone deacetylases can transiently increase the expression of telomere-proximal genes (Gottschling, 1992; de Bruin *et al.*, 2000; Baur *et al.*, 2001; Koering *et al.*, 2002).

In future studies, the most intriguing question to be addressed is whether the different patterns of gene expression during the life-span of a cell are a consequence of TPE. TPE may provide a mechanism to modify gene expression as a function of age (reviewed in Tham and Zakian, 2002). In this scenario, as telomeres shorten during each cell division, some genes would be expressed at higher levels in aged cells, correlating with the length-dependent silencing effect.

1.2.2 *Double-stranded telomeric DNA repeats*

Ciliated protozoa were the first organisms used for detailed studies of telomere biology. *De novo* telomere addition occurs after massive fragmentation and amplification of the micronuclear genome into thousands of linear DNA molecules that ultimately constitute the macronuclear genome. The first telomeric DNA sequence was identified in the unicellular protozoan *Tetrahymena thermophila*. It has been possible to directly sequence these DNA repeats because of their short length and their abundance during the developmentally-regulated genome reorganization of the ciliate (Klobutcher *et al.*, 1981). The telomeric tract of the eukaryotic chromosome is double-stranded for most of its length and composed of a long array of G-rich repeats (Fig. 1.1). The length of this

array varies greatly among species, ranging from 14-16 bp in certain ciliates to 100 kb in mammals. Usually, the G-rich unit is short, between 6 to 8 nucleotides. The telomeric DNA is composed of T_2G_4 repeats in *Tetrahymena thermophila*, T_2AG_3 in mammals and T_3AG_3 in many plants (Klobutcher *et al.*, 1981; Moyzis *et al.*, 1988; Richards and Ausubel, 1988). In contrast, the G-rich unit is 25 nucleotides in the yeast *Kluyveromyces lactis* (Fulton and Blackburn, 1998) and has a more complex pattern, $(TG)_{1\text{-}6}TG_{2\text{-}3}$, in the yeast *Saccharomyces cerevisiae* (Kramer and Haber, 1993). Point mutations in the telomeric arrays are not tolerated *in vivo* and lead ultimately to a decrease in cell viability, indicating stringent sequence requirements for the telomeric DNA repeats (Yu *et al.*, 1990; McEachern and Blackburn, 1995; Kirk *et al.*, 1997; Marušic *et al.*, 1997; McEachern *et al.*, 2000; Guiducci *et al.*, 2001; Kim *et al.*, 2001; Lin *et al.*, 2004).

Recently, the molecular mechanism underlying the sequence diversity of the G-rich repeats in budding yeast has been elucidated. Different intrinsic biochemical properties of the yeast telomerase enzyme contribute to the synthesis of divergent telomeric DNA repeats *in vivo* (Förstemann and Lingner, 2001). This investigation determined that the repeat diversity in *Saccharomyces cerevisiae* results from several alignment possibilities of the same DNA substrate with the telomerase RNA template and frequent incomplete reverse transcription of the template by telomerase (Förstemann and Lingner, 2001). Another study explained the specific preponderance of G-rich repeats in *Saccharomyces cerevisiae* telomeres (Förstemann *et al.*, 2003). A randomly mutagenized DNA library encoding template mutants of the yeast telomerase RNA was screened for telomerase activity complementation in a strain lacking the telomerase RNA component. This analysis revealed that telomerase RNA templates containing adenine and cytidine

residues were preferentially reverse transcribed (Förstemann *et al.*, 2003). Consistent with this observation, yeast telomerase can incorporate human telomeric DNA repeats into yeast chromosome ends when adenine/cytidine-rich humanized yeast telomerase RNA template is used (Henning *et al.*, 1998; Bah *et al.*, 2004).

Some organisms such as the well studied fruit fly *Drosophila melanogaster* do not have G-rich repeats and do not express the telomerase enzyme, but they utilize different mechanisms for telomere maintenance. In *Drosophila melanogaster*, it is now clear that the progressive shortening of telomeres is counteracted by repeated retrotransposition of two non- long terminal repeat (LTR) elements, *HeT-A* and *TART* (reviewed in Pardue and DeBaryshe, 2003). These two telomere-specific transposable elements generate the telomeric arrays. Other insects such as the midge *Chironomus pallidivittatus* and the mosquito *Anopheles gambiae* are also species without telomerase components; they maintain their telomeres by recombination rather than reverse transcription or retrotransposition (López *et al.*, 1996; Roth *et al.*, 1997).

1.2.3 *G-tails*

The G-tail is a single-stranded DNA that extends the G-rich strand 3'-end of the telomeric repeat arrays (Fig. 1.1). The length of the G-tail is variable, ranging from 12-14 nucleotides in yeast to more than 100 nucleotides in mammals. *In vivo*, G-tails of the ciliated *Oxytricha nova* and *Euplotes crassus* were first reported to be present on both chromosome ends (Klobutcher *et al.*, 1981). A combination of electron microscopy and oligonucleotide hybridization/primer extension experiments has permitted analysis of the G-tail of *Tetrahymena thermophila* at nucleotide resolution (Jacob *et al.*, 2001; Muñoz-Jordán *et al.*, 2001). These experiments led to the consensus

that most eukaryotic chromosomes have a G-tail on both ends, but they can differ in length (Jacob *et al.*, 2001). An analysis of linear minichromosomes in yeast also concluded that both ends of linear DNA molecules possess a G-tail (Wellinger *et al.*, 1996). Moreover, the length of the G-tail changes little during the cell cycle of ciliates (Jacob *et al.*, 2001) as opposed to their variation during the S phase in yeasts (Wellinger *et al.*, 1993; Wellinger *et al.*, 1996).

In human cells, conflicting results have been reported regarding the similarity of the G-tail length at both chromosome ends. One study concluded that the two ends of a single chromosome have relatively long G-tails (Makarov *et al.*, 1997). Another study argued that a single chromosome has one telomere with a long G-tail and one with a short G-tail or a blunt end (Wright *et al.*, 1997). These experiments have been interpreted later as indicating that G-tails are present on both chromosome ends, but they have an asymmetrical length which probably results from the DNA synthesis machinery trying to resolve the end replication problem at the chromosome ends (McElligott and Wellinger, 1997; Wright *et al.*, 1999; Huffman *et al.*, 2000; Riha *et al.*, 2000; Ohki *et al.*, 2001).

1.3 *Telomere-associated proteins and substrate access*

Several effective mechanisms regulate the subtle balance between telomere lengthening and shortening. Mechanisms controlling telomere length requires not only a functional telomerase enzyme, but also a complex network of telomere-associated proteins that may restrict access of other DNA-interacting factors to the chromosome ends (reviewed in Blackburn, 2001). Telomere-associated proteins with overlapping features and functions have been found in yeast, ciliates and vertebrates.

They create a protective cap that is imperative for the maintenance of genomic stability and promote a similar organization at the chromosome ends suggesting that telomere integrity mechanisms have been conserved through evolution (reviewed in Wei and Price, 2003). A large number of *in vivo* experiments, in which telomeric proteins mutated in their DNA-binding site were overexpressed, show that changes in the composition of telomeric complex lead to telomere dysfunction (reviewed in de Lange, 2002).

Based on these experimental observations, Elizabeth H. Blackburn and coworkers suggested recently that telomeres switch between two structural configurations: an "open" and a "closed" complex (reviewed in Blackburn, 2001). The "open" complex mimics a free DNA end that is available to the action of telomerase or other enzymes. In contrast, the "closed" complex prevents the extremity of chromosomes to be accessible to enzymes that could elicit permissive and nonregulated biochemical activities leading to DNA damage. Although direct experimental evidence for the existence of both states is lacking, the transient architecture of the chromosome ends probably involves the formation of diverse protein networks, for instance through the interactions of DNA-binding proteins with different partners (Smith *et al.*, 2003; Teixeira *et al.*, 2004). Numerous studies are now exploring the components of the telomere cap and their elaborate mechanisms of control. In the following section, I will describe the main telomere-associated proteins, giving a selective picture of the available information to date (Table I).

1.3.1 *Double-stranded DNA-binding proteins*

Very few proteins that bind to the double-stranded telomeric DNA repeats have been identified. A main player in telomere homeostasis of *Saccharomyces*

Table I Telomere-associated proteins in budding yeasts and humans*

Factors	Budding yeast	Humans	Functions and interactions
Telomerase catalytic core	TLC1	hTR	RNA subunit
	Est2	hTERT	Reverse transcriptase subunit
Telomerase-associated proteins	Est1, Est3	hEST1A, hEST1B	Associates with telomerase (Sc, Hs) Binds TLC1 RNA (Sc)
G-tail-binding proteins	Cdc13	POT1	Binds TFR1 (Hs) Binds Est1p (Sc)
Double-stranded DNA-binding proteins	Rap1	TRF1	Binds telomeres (Sc, Hs) Telomere length regulator (Sc, Hs)
		TRF2	Binds telomeres; role in T-loops (Hs)
Proteins recruited to telomeres by protein-protein interactions	Rif1, Rif2		Recruited by RAP1 (Sc)
		hRAP1	Recruited by TRF2 (Hs)
		TANK1	Binds TRF1; PARP activity (Hs)
		TIN2	Binds TRF1 and TANK1 (Hs)
	Stn1, Ten1		Recruited by Cdc13; end protection (Sc)
Others	Ku heterodimer (yKu70/yKu80)	Ku heterodimer (hKu70/hKu80)	Localizes to telomeres (Sc, Hs) Binds TLC1 RNA (Sc) Associates with telomerase (Hs) End protection (Sc, Hs)

*See text for references. Based on Vega et *al.*, 2003.
Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae.*

cerevisiae is the Rap1 complex, composed of the duplex telomeric repeat binding factor Rap1 (repressor/activator protein 1), and its associated proteins Rif1 and Rif2 (Rap1-interacting factors 1 and 2) (Conrad *et al.*, 1990; Hardy *et al.*, 1992; Wotton and Shore, 1997). In humans, the telomeric binding protein TRF1 (telomeric repeat binding factor 1) and its interacting factors tankyrase 1 and TIN2 (TRF1-interacting nuclear protein 2), form the major telomere length regulatory complex (Bianchi *et al.*, 1997; Smith *et al.*, 1998; Kim *et al.*, 1999).

In its central region, Rap1p bears two conserved DNA-binding domains of the Myb/homeodomain family that are common DNA-recognition motifs found in many transcription regulators (König *et al.*, 1998). Rap1p has the ability to directly bind to the G-rich motifs of the double-stranded telomeric DNA as a monomer and regulates telomere length by recruiting Rif1p and Rif2p (Conrad *et al.*, 1990; Hardy *et al.*, 1992; Kyrion *et al.*, 1992; Kyrion *et al.*, 1993; Wotton and Shore, 1997). Rif1 and Rif2 proteins have been identified to interact with the C-terminus of Rap1p in yeast two-hybrid assay (Hardy *et al.*, 1992; Wotton and Shore, 1997). The deletion of the genes encoding either Rif1p or Rif2p results in telomere lengthening (Hardy *et al.*, 1992; Wotton and Shore, 1997), similarly to the phenotype observed in cells expressing Rap1p with C-terminal truncations (Kyrion *et al.*, 1992). These studies suggested a negative role for Rap1p in telomere length regulation.

Recent reports have proposed a model for the regulation of telomere length by telomere-associated proteins (Smith *et al.*, 2003; Teixeira *et al.*, 2004). The group of Joachim Lingner developed an *in vivo* method to measure elongation of single telomeres in *Saccharomyces cerevisiae* (Teixeira *et al.*, 2004). They found that the telomerase

enzyme preferentially elongates short telomeres. This study also suggests that Rif1p and Rif2p may regulate the access of telomerase to G-tails by promoting the formation of a nonextendible telomeric state when telomeres are long (Teixeira *et al.*, 2004). On the other hand, the group of Elizabeth H. Blackburn investigated the ability of Rap1p/Rif1p/Rif2p complexes to interact with telomeric DNA during the cell cycle (Smith *et al.*, 2003). They observed that the association properties of Rif1p, Rif2p and Rap1p with telomeric DNA change during the cell cycle. They proposed a model suggesting that Rif2, strongly associated with telomeric DNA in G1 phase, may inhibit premature telomere elongation. The progressive displacement of Rif2p from telomeres during the S phase allows the access of telomerase. Once telomere replication is completed, the binding of Rap1p/Rif1p/Rif2p complex to telomeric DNA in G2/M phases prevent overelongation of telomeres by again restricting the productive association of telomerase (Smith *et al.*, 2003). Taken together, these two studies suggest, at least in yeast, a dynamic remodelling of telomeres between extendible and nonextendible states (Smith *et al.*, 2003; Teixeira *et al.*, 2004).

In human cells, TRF1 and TRF2 are two homologues of yeast Rap1p (Bianchi *et al.*, 1997; Broccoli *et al.*, 1997). They have a single Myb-like DNA-binding domain (Bianchi *et al.*, 1997; Broccoli *et al.*, 1997) and a TRF domain permitting their homodimerization at telomeres (Bianchi *et al.*, 1997; Broccoli *et al.*, 1997; Fairall *et al.*, 2001). TRF1 and TRF2 directly bind to the duplex telomeric DNA (Bianchi *et al.*, 1997; Broccoli *et al.*, 1997), but they have distinct roles in the cell. TRF1 seems to be the functional homologue of the yeast Rap1p (Bianchi *et al.*, 1997). It is a negative regulator of telomere length because its overexpression shortens telomeres, and the overexpression

of a dimerization-competent dominant-negative TRF1 mutant lacking the DNA-binding domain elongates telomeres (van Steensel and de Lange, 1997; Smogorzewska *et al.*, 2000). Like Rap1p, TRF1 recruits several proteins to telomeres such as tankyrase 1 (TANK1), a member of the telomeric poly(adenosine diphosphate-ribose) polymerase (PARP) family, and TIN2 that have been found to colocalize with TRF1 at human telomeres (Smith *et al.*, 1998; Kim *et al.*, 1999).

TANK1 has a PARP activity that inhibits the binding of TRF1 to telomeres. The ankyrin domain of TANK1 is required for its interaction with TRF1 (Seimiya *et al.*, 2004). Overexpression of TANK1 elongates telomeres by inducing the release of TRF1, suggesting that TANK1 positively regulates telomere length (Smith and de Lange, 2000; Cook *et al.*, 2002). In contrast, TIN2 is a negative mediator of telomere length because its overexpression slightly shortens telomeres, whereas the overexpression of TIN2 N-terminal truncation mutants that retain their ability to interact with TRF1 elongates telomeres (Kim *et al.*, 1999; Kim *et al.*, 2003b). TIN2 also modulates PARP activity of TANK1 and may form a ternary complex with TRF1 and TANK1, thereby preventing the premature release of TRF1 by TANK1 from telomeres (Ye and de Lange, 2004).

Similarly to the phenotype observed in cells overexpressing TRF1, expression of TRF2 results in a gradual decrease of telomere length (van Steensel *et al.*, 1998; Smogorzewska *et al.*, 2000). However, the expression of a dimerization-competent dominant-negative TRF2 mutant that is unable to bind to DNA, and also containing an N-terminal deletion, results in loss of G-tails, severe chromosomal end-to-end fusions and cell cycle arrest accompanied by senescence and apoptosis (van Steensel *et al.*, 1998;

Karlseder *et al.*, 1999; Smogorzewska *et al.*, 2000; Bailey *et al.*, 2001; Smogorzewska *et al.*, 2002). Thus, TRF2 is thought to protect chromosome ends by masking G-tails from nonregulated cellular activities leading to DNA damage (van Steensel *et al.*, 1998; Smogorzewska *et al.*, 2000).

Titia de Lange and coworkers have proposed that the rapid loss of telomere function following TRF2 inhibition results from an immediate dysfunction of the telomere capping (reviewed in de Lange, 2004). The protective activity of TRF2 may be related to its role in the formation of a specific structure referred to as T-loop at the chromosome ends (Griffith *et al.*, 1999). Visualized by electron microscopy, this high-order chromatin structure at the telomeres is created when the G-tails loop back and invade the double-stranded telomeric DNA. The T-loop may sequester the telomere terminus from degradative and/or DNA repair activities. The T-loop stability depends in large part on the preferential binding of TRF2 to the loop's duplex-tail junction (Griffith *et al.*, 1999; Stansel *et al.*, 2001). Complementary studies also revealed that TRF2 can bind to very short telomeres and delay the senescence setpoint, suggesting an additional way for TRF2 to protect chromosome ends (Karlseder *et al.*, 2002). Taken together, these data support a structural solution to protect chromosome ends against inappropriate exposure to DNA damage checkpoints that can initiate apoptotic pathways.

The absence of G-tails has also been proposed as an altered telomere state inducing cell growth arrest (Li *et al.*, 2003; Stewart *et al.*, 2003). As explained previously, the loss of G-tails is a direct consequence of the inhibition of TRF2 that may favour the opening of the T-loop structure (van Steensel *et al.*, 1998; Karlseder *et al.*, 1999). The formation of the T-loop at the chromosome ends is sufficient to protect G-

tails against nuclease degradation or aberrant telomere fusions (Karlseder *et al.*, 1999; Stansel *et al.*, 2001). The loss of G-tails could be due to T-loop remodelling when TRF2 function is impaired or telomeres are very short (Karlseder *et al.*, 2002). Thus, the telomere state of chromosome ends seems to be a critical parameter for the initiation of a DNA damage response.

TRF2 also interacts with several proteins that mediate telomere length homeostasis. hRAP1 has been identified as a TRF2-interacting protein (Li *et al.*, 2000). Unlike yeast Rap1p, hRAP1 does not have a DNA-binding domain, but it localizes to telomeres in the presence of functional TRF2 (Li *et al.*, 2000). hRAP1 interacts with TRF2 through its C-terminus (Li and de Lange, 2003), and overexpression of hRAP1 promotes telomere elongation indicating that hRAP1 plays a negative role in telomere length regulation (Li and de Lange, 2003).

The mechanisms that allow the physical association of telomere-associated proteins with the double-stranded telomeric DNA are well conserved among eukaryotes, although the human and yeast proteins lack significant sequence similarity. First, sequence-specific DNA-binding proteins recognize and bind to the G-rich motifs. Second, telomere regulators are recruited to telomeres. These observations led to a "protein counting" model in which the number of DNA-binding sites determines the overall telomere length in yeast (Marcand *et al.*, 1997). As the telomeres become elongated, negative regulators accumulate along the duplex telomeric DNA array, and their presence at the chromosome ends results in an increasing inhibition of telomerase. Short telomeres are more prone to elongation than long telomeres (Marcand *et al.*, 1997; Smogorzewska *et al.*, 2000; Hemann *et al.*, 2001; Teixeira *et al.*, 2004).

1.3.2 *G-tail-binding proteins*

In the absence of G-tails, the level of chromosomal end-to-end fusions is elevated (Smogorzewska *et al.*, 2002). Telomere-associated proteins that directly bind to the G-tails are thought to play an essential role in telomere length homeostasis and chromosomal stability (Table I). The Cdc13 protein is the most characterized G-tail-binding protein in *Saccharomyces cerevisiae* (Lin and Zakian, 1996; Nugent *et al.*, 1996). The deletion of the *CDC13* gene causes gradual telomere shortening and replicative senescence (Nugent *et al.*, 1996). Cdc13p has been implicated in several distinct roles at telomeres which include positive and negative regulation of telomerase accessibility (Evans and Lundblad, 2002; Taggart *et al.*, 2002).

Telomere length homeostasis is negatively regulated by Cdc13p through interactions with Stn1 and Ten1 (Grandin *et al.*, 1997; Grandin *et al.*, 2001; Pennock *et al.*, 2001). All Stn1p and Ten1p mutants exhibit abnormally elongated telomeres (Grandin *et al.*, 1997; Grandin *et al.*, 2001), thereby favouring a model where Cdc13p/Stn1p/Ten1p complexes protect the chromosome ends by binding to the G-tails, making them less available for elongation by telomerase (Grandin *et al.*, 2001; Pennock *et al.*, 2001). Interactions of Cdc13p with Est1p also positively regulate telomere length by recruiting telomerase to the G-tails (Qi and Zakian, 2000). *EST* (ever shorter telomeres) genes were identified by a genetic screen for mutants with telomere replication defects (Lendvay *et al.*, 1996). Est1p is also a telomerase RNA-binding protein (Zhou *et al.*, 2000; Livengoog *et al.*, 2002; Seto *et al.*, 2002). All mutations that impair Cdc13p/Est1p interactions alter telomere elongation, suggesting that Est1p bridges the interaction between telomerase and Cdc13p (Taggart *et al.*, 2002). It is thought that

Est1p competes with Stn1p for overlapping binding sites on Cdc13p. The Cdc13p recruitment model is further supported by the analysis of a series of fusion proteins that couple the catalytic protein subunit of telomerase with the DNA-binding domain of Cdc13p. In the presence of these telomerase fusion proteins, Est1p and Cdc13p becomes dispensable for telomere length maintenance (Evans and Lundblad, 1999; Evans and Lundblad, 2002; Taggart *et al.*, 2002).

In human cells, hPOT1 (protection of telomeres) binds to single-stranded telomeric DNA (Baumann and Cech, 2001; Baumann *et al.*, 2002). A study demonstrated that the number of bound hPOT1 molecules correlates with the length of the single-stranded DNA (Loayza and de Lange, 2003). The same study showed that hPOT1 specifically interacts with TRF1 (Loayza and de Lange, 2003). Expression of hPOT1 mutant lacking its DNA-binding domain, but retaining its ability to interact with TRF1, promotes telomere elongation, indicating that hPOT1 is a negative regulator of overall telomere length (Loayza and de Lange, 2003). In accordance with the "protein counting" model of telomere length homeostasis, the group of Titia de Lange proposed that telomere length is governed by the ability of TRF1 complex to recruit hPOT1 (Loayza and de Lange, 2003). The control of telomere length is accomplished by the loading of TRF1 directly on the double-stranded DNA. As the number of TRF1 molecule increases, the recruitment of hPOT1 on the single-stranded DNA increases as well, limiting telomere elongation (Loayza and de Lange, 2003).

1.3.3 *Double-stranded DNA breaks and telomeres*

Telomeres and double-stranded breaks (DSB) share the common feature of being physical ends of chromosomes (reviewed in Vega *et al.*, 2003). DSBs are

substrates for homologous recombination (HR) or nonhomologous end-joining (NHEJ) events. Telomeres are normally protected against NHEJ and therefore do not fuse with other telomeres (reviewed in de Lange, 2002). Thus the telomeres must be hidden from the checkpoint recognition and DNA repair machinery. In yeast and humans, the conserved Ku heterodimer binds with high affinity to both DSBs and telomeres (Table I) (reviewed in Vega *et al.*, 2003).

In yeast, Ku (yKu70/yKu80) has been shown to bind to telomeres (Gravel *et al.*, 1998). Ku can also interact specifically with a segment of the yeast telomerase RNA (Peterson *et al.*, 2001; Stellwagen *et al.*, 2003). Ku mutations that alter its interaction with telomeres or the telomerase RNA result in telomere shortening (Baumann and Cech, 2000; Peterson *et al.*, 2001). These experimental data suggest that Ku is a good candidate to recruit telomerase to telomeres. Ku could promote the access of telomerase to telomeres by its ability to bind to the telomerase RNA (Peterson *et al.*, 2001; Stellwagen *et al.*, 2003). The human Ku heterodimer (hKu70/hKu80) also interacts with telomeres and the telomerase complex (Hsu *et al.*, 1999; Chai *et al.*, 2002). However, the function of Ku at human telomeres remains to be determined. In mice, cells lacking a Ku subunit are subjected to chromosomal end-to-end fusions and apoptosis, which are consistent with the role of mammalian Ku in telomere capping (Espejel *et al.*, 2002).

Ku mediates opposing roles at DSBs and telomeres. At DSBs, Ku promotes chromosomal end-to-end fusions. At telomeres, Ku specifically protects against DNA end-joining events. A model has been proposed to explain the different activities performed by Ku at the two classes of DNA ends (Bertuch and Lundblad,

2003). When Ku is associated with the telomeric ends, specific telomere-associated proteins can modulate cellular activities performed by the heterodimer that are not allowed at DSB ends (Bertuch and Lundblad, 2003). This model implies that Ku can execute functionally distinct activities depending on the identity of its interacting partners.

A picture starts to emerge from the studies concerning the capping function of telomeres and the regulation of telomere length homeostasis. Telomeres are a site of multiple DNA-protein interactions involving different molecular components. Telomeres must first unfold or open, such that the free DNA end is accessible to the appropriate enzymatic machinery. Second, telomerase must be recruited to telomeres during DNA synthesis, and thereafter, telomeres must refold into a protective structure. Finally, altering the function of one molecular component is sufficient to compromise telomere length homeostasis and promote severe telomere dysfunction.

1.4 *End replication problem*

The extreme ends of linear chromosomes create a unique problem for the eukaryotic DNA replication machinery (reviewed in Vega *et al.*, 2003). The properties of DNA polymerases prevent the complete replication of chromosome ends. At the replication fork, DNA replication is a bidirectional process in which the lagging strand and the leading strand are replicated by a different strategy. The leading strand synthesis occurs in the same direction of the replication fork movement. Consequently, it results in a continuous synthesis towards the chromosome ends. On the other hand, the lagging strand synthesis occurs in the opposite direction of the replication fork movement. It requires the hybridization of short RNA primers that are used as substrates

to generate Okazaki fragments. These DNA fragments are ligated to form a continuous DNA strand.

Consistent with the model of semiconservative DNA replication, the leading strand synthesis is expected to generate blunt ends, whereas the lagging strand synthesis would create short unreplicated G-tails after the removal of the most 5' terminal RNA primer. This end replication problem was recently recapitulated *in vitro* by monitoring the DNA replication of linear DNA molecules (Ohki *et al.*, 2001). The unreplicated DNA contributes to the loss of genetic materials at each DNA replication cycle. This incomplete DNA replication can result in progressive telomere shortening at each cell division of primary human cells (Harley *et al.*, 1990). Short telomeres in human cells can cause irreversible growth arrest, cell death or genomic instability. At some point, the ends of chromosomes will be too short to continue to provide the capping function. Finally, telomere shortening due to the end replication problem can be balanced by the action of telomerase.

However, the requirement for G-tails as the presumed substrate for telomerase raises a problem during the leading strand synthesis. As explained earlier, numerous studies reported G-tails simultaneously on both chromosome ends (Wellinger *et al.*, 1996; Makarov *et al.*, 1997; Wright *et al.*, 1999; Huffman *et al.*, 2000; Jacob *et al.*, 2001). In yeast and mammals, the generation of G-tails on the leading strand requires a form of DNA processing that occurs by a telomerase-independent mechanism (Wellinger *et al.*, 1996; Makarov *et al.*, 1997). A more recent work with *Tetrahymena thermophila* indicates that the formation of G-tails involves at least two separate DNA cleavage steps (Jacob *et al.*, 2003). It may involve the combined action of a nuclease acting on the C-

rich strand, and telomerase, which extends the G-rich strand (Wellinger *et al.*, 1996; Jacob *et al.*, 2003).

1.5 *Telomerase ribonucleoprotein complex*

The human telomerase complex is known to be functional in most immortal cells, whereas telomerase activity is absent in most normal somatic cells. Thus telomerase seems to be an important diagnostic marker of human cancers and an attractive target for the design of anti-cancer agents. Telomerase is a ribonucleoprotein (RNP) that minimally consists of an RNA component (TR) and a catalytic protein subunit (TERT) (Greider and Blackburn, 1985; Greider and Blackburn, 1989). Telomerase is a specialized reverse transcriptase that was first detected in cellular extracts made from *Tetrahymena thermophila* (Greider and Blackburn, 1985). It is an RNA-dependent DNA polymerase that uses an internal segment of its RNA as a template for the synthesis of telomeric DNA repeats onto the ends of chromosomes (Greider and Blackburn, 1989).

Several features distinguish the telomerase enzyme from other conventional reverse transcriptases (RTs). Most significant is the stable association of TERT with an RNA molecule, which is an intrinsic part of the RNP. A small segment within the telomerase RNA is delineated as a template with strictly defined boundaries during DNA synthesis. Telomerase is a specialized enzyme that processively adds telomeric DNA repeats onto the chromosome ends by reverse transcribing the same RNA segment repeatedly. Repeat addition processivity refers to the ability of telomerase to add multiple DNA repeats to a single bound substrate before its dissociation from the DNA products. Repeat addition processivity is unique to telomerase and distinct from

nucleotide addition processivity, which refers to the successive nucleotide additions within each single DNA repeat.

For experimental purposes, it has been possible to reconstitute an active telomerase complex *in vitro* and to perform functional and biochemical studies related to its mechanism of action. The rabbit reticulocyte lysate (RRL) *in vitro* transcription/translation expression system allows the synthesis of TERT in the presence of an *in vitro*-transcribed TR (Weinrich *et al.*, 1997; Beattie *et al.*, 1998; Collins and Gandhi, 1998; Bryan *et al.*, 2000a). This recombinant telomerase enzyme accurately defines its template boundaries, repeatedly reverse transcribes the same template, and demonstrates similar features to the endogenous enzyme. The activity of the endogenous and the recombinant enzymes is detected *in vitro* by direct addition of radiolabelled deoxynucleotides (dNTPs) onto an oligonucleotide substrate that mimics the single-stranded G-tail (direct primer extension assay) (Greider and Blackburn, 1985; Morin, 1989; Sun *et al.*, 1998; Huard *et al.*, 2003). Radiolabelled PCR amplification of the telomerase elongation products generated by the enzyme is also used as an alternative assay (TRAP assay: telomeric repeat amplification protocol) (Kim *et al.*, 1994; Kim and Wu, 1997).

Several studies have used the RRL-reconstituted telomerase enzyme to investigate the contribution of TERT amino acid residues or TR sequences to catalytic activity. TERT and TR mutations can alter specific features of the enzyme such as the template definition, the nucleotide selectivity, processivity and the level of activity. In the following section, I will describe in detail the biochemical and functional aspects of the telomerase enzyme. I will focus on the mechanism of action of telomerase and will

emphasize the mechanistic questions that are essential to understand the function of each component of the enzyme.

1.5.1 *The telomerase RNA*

The telomerase RNA isolated from the ciliated *Tetrahymena thermophila* was the first telomerase subunit identified (Greider and Blackburn, 1989). Subsequently, the genes encoding other telomerase RNAs were cloned from other ciliates, yeast and vertebrates (Shippen-Lentz and Blackburn, 1990; Romero and Blackburn, 1991; Lingner *et al.*, 1994; Singer and Gottschling, 1994; Blasco *et al.*, 1995; Feng *et al.*, 1995; McCormick-Graham and Romero, 1996; Chen *et al.*, 2000). The sizes and the sequences vary dramatically among ciliates (150-200 nt), vertebrates (300-500 nt) and yeast (~1300 nt).

Independent phylogenetic-based sequence comparative analyses established a common core secondary structure for the ciliate and vertebrate telomerase RNAs (Fig. 1.2) (Romero and Blackburn, 1991; Bhattacharyya and Blackburn, 1994; Chen *et al.*, 2000). Although there is no obvious sequence homology between ciliate and vertebrate telomerase RNAs, their structures display significant architectural similarities, suggesting strongly conserved evolutionary functions (Romero and Blackburn, 1991; Bhattacharyya and Blackburn, 1994; Lingner *et al.*, 1994; McCormick-Graham and Romero, 1996; Chen *et al.*, 2000). Conformational analyses of the telomerase RNA have been a prerequisite for functional studies of the enzyme. Important extrapolations have been made concerning which regions are likely to be essential for telomerase activity and assembly.

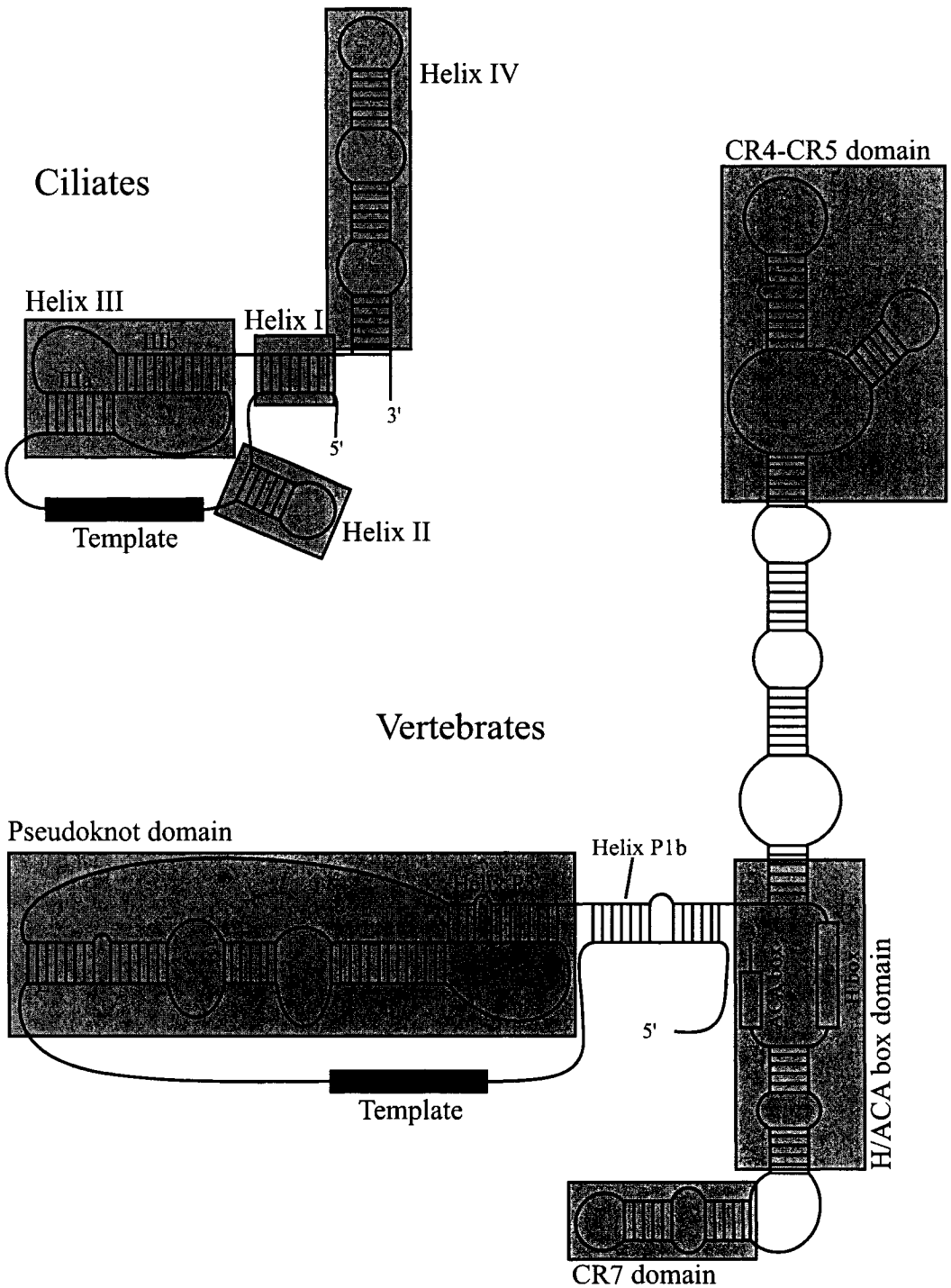


Figure 1.2 Comparison of ciliate and vertebrate telomerase RNA secondary structures. Based on Chen *et al.*, 2000.

Most ciliate telomerase RNAs fold into four helices labelled as I to IV (Fig. 1.2). Helices II, III and IV are all stem-loop structures, each having distinct attributes. The secondary structure predicts a single-stranded region located between helices II and III that contains the template. The folding of helix III (helices IIIa and IIIb) generates a pseudoknot region. The 5'-end and the 3'-end of the RNA are brought together by the conserved helices I and IV. The stem-loop IV is an extension of helix I. Each structural domain of the ciliate telomerase RNAs shows distinct functions. The template sequence that varies between different organisms specifies the addition of the appropriate deoxynucleotide during DNA synthesis (Greider and Blackburn, 1989; Yu *et al.*, 1990). Mutations in the template sequence create specific changes in the enzymatic properties of telomerase, but they are tolerated *in vitro* (Autexier and Greider, 1994; Ware *et al.*, 2000). However, cell viability is reduced *in vivo*, because they alter the telomeric sequences (Yu *et al.*, 1990; Gilley and Blackburn, 1996; Kirk *et al.*, 1997; Ware *et al.*, 2000).

Mutational analyses have been used to investigate the contribution of the sequences located outside the template to enzymatic properties. Helix I and II are required for telomerase activity (Autexier and Greider, 1998; Licht and Collins, 1999; Mason *et al.*, 2003). Helix II also binds with high affinity to the catalytic protein subunit (Lai *et al.*, 2001; Mason *et al.*, 2003). Substitutions in the pseudoknot domain alter telomerase activity *in vitro* (Autexier and Greider, 1998; Licht and Collins, 1999; Lai *et al.*, 2003; Mason *et al.*, 2003), but restoring the base-pairing by compensatory mutations reestablishes the structural motif as well as telomerase activity (Gilley and Blackburn, 1999; Mason *et al.*, 2003). Thus the specific base-pairing topology within the

pseudoknot is necessary to promote the *in vivo* assembly of a catalytically active telomerase (Gilley and Blackburn, 1999). Finally, the deletion of helix IV abolishes telomerase activity, but it does not alter the binding of TR to TERT (Autexier and Greider, 1998; Licht and Collins, 1999; Sperger and Cech, 2001; Lai *et al.*, 2003; Mason *et al.*, 2003).

The secondary structure of the ciliate telomerase RNAs originally predicted by phylogenetic-based sequence comparisons (Romero and Blackburn, 1991) was later validated in many respects by chemical and enzymatic analyses (Bhattacharyya and Blackburn, 1994; Lingner *et al.*, 1994). A similar core secondary structure to the ciliate telomerase RNAs was proposed for the vertebrate telomerase RNAs (Chen *et al.*, 2000), and was corroborated by nuclease, chemical and mutational mapping experiments (Antal *et al.*, 2002; Chen *et al.*, 2002; Ly *et al.*, 2003a). The vertebrate telomerase RNA folds into four conserved structural domains referred to as the pseudoknot domain, the CR4-CR5 domain (conserved regions 4 and 5), the H/ACA box and the CR7 domain (conserved region 7) (Fig.1.2). Similarly to the ciliate telomerase RNAs, each structural domain shows distinct functions.

The minimal RNA sequences required for a fully active human telomerase have been identified using *in vitro*-reconstituted enzyme (Autexier *et al.*, 1996; Tesmer *et al.*, 1999; Beattie *et al.*, 2000; Bachand and Autexier, 2001). The motif corresponding to the pseudoknot is a common feature shared by ciliate and vertebrate telomerase RNAs. The pseudoknot is required for telomerase activity, but it is not essential for TERT binding (Autexier *et al.*, 1996; Bachand and Autexier, 2001; Martín-Rivera and Blasco, 2001; Ly *et al.*, 2003a). The CR4-CR5 domain, that is not present in ciliate telomerase

RNAs, is indispensable for telomerase activity and for the binding to TERT (Mitchell and Collins, 2000; Bachand and Autexier, 2001; Chen *et al.*, 2002). The CR4-CR5 domain corresponds functionally to helix II; both interact with TERT and are major sites of RNP assembly. The H/ACA box and CR7 domain are two additional elements that are not found in ciliate telomerase RNAs. They are dispensable for telomerase activity *in vitro* (Autexier *et al.*, 1996; Tesmer *et al.*, 1999; Beattie *et al.*, 2000; Mitchell and Collins, 2000; Bachand and Autexier, 2001). The H/ACA box shares homology with a small nucleolar RNA-like box and may direct the *in vivo* 3'-end processing of precursor telomerase RNAs to mature RNAs (Mitchell *et al.*, 1999a). This domain seems to be essential for the stability of the mature telomerase RNA *in vivo* (Mitchell and Collins, 2000; Martín-Rivera and Blasco, 2001; Fu and Collins, 2003).

The secondary structure of the yeast telomerase RNAs has recently been predicted by phylogenetic-based sequence comparisons (Dandjinou *et al.*, 2004; Zappulla and Cech, 2004). In addition to the template, several stem-loop structures have been identified in *Saccharomyces cerevisiae* and *Kluyveromyces lactis* telomerase RNAs (Roy *et al.*, 1998; Tzfati *et al.*, 2003; Dandjinou *et al.*, 2004). The disruption of some potential base-pairing predicted to be elements of the putative pseudoknot dramatically alters cell growth, telomere maintenance and telomerase activity (Tzfati *et al.*, 2003). Separate stem-loop structures seem to bind to telomerase-associated Est1p, yeast TERT (Est2p), Ku protein and Sm protein (Seto *et al.*, 1999; Peterson *et al.*, 2001; Livengoog *et al.*, 2002; Seto *et al.*, 2002; Stellwagen *et al.*, 2003).

1.5.2 *The catalytic protein subunit*

The TERT subunit was first purified from *Euplotes aediculatus*, as p123, by a biochemical fractionation approach (Lingner and Cech, 1996). p123 was found to be homologous to Est2p, the catalytic protein subunit of *Saccharomyces cerevisiae* (Counter *et al.*, 1997; Lingner *et al.*, 1997). *EST2* was originally identified by a genetic screen as a gene required for telomere maintenance in yeast (Lendvay *et al.*, 1996). Both p123 and Est2 proteins contain RT-like motifs. Subsequently, the genes encoding other telomerase catalytic protein subunits were cloned from other yeast (Nakamura *et al.*, 1997), human (Harrington *et al.*, 1997a; Kilian *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997), ciliates (Bryan *et al.*, 1998; Collins and Gandhi, 1998) and mouse (Greenberg *et al.*, 1998; Martín-Rivera *et al.*, 1998). In contrast to the lack of sequence homology between telomerase RNA subunits of different organisms, the amino acid sequences of TERT are relatively well conserved through evolution.

Human TERT (hTERT) has a modular organization that can be delineated into three major regions (Fig. 1.3): an N-terminal region that includes highly conserved telomerase-specific motifs, a central region that corresponds to the active site and contains RT-like motifs, and a C-terminal region that has no substantial sequence conservation among members of the TERT family. Most of the functional information reported so far has been obtained by RRL-reconstituted telomerase. Functional reconstitution has also been accomplished *in vivo* by the expression of the human telomerase subunits in human cells (Bodnar *et al.*, 1998) or in *Saccharomyces cerevisiae* (Bachand and Autexier, 1999), and by the addition of *in vitro*-transcribed hTR to partially purified recombinant hTERT synthesized in insect cells (Masutomi *et al.*, 2000).

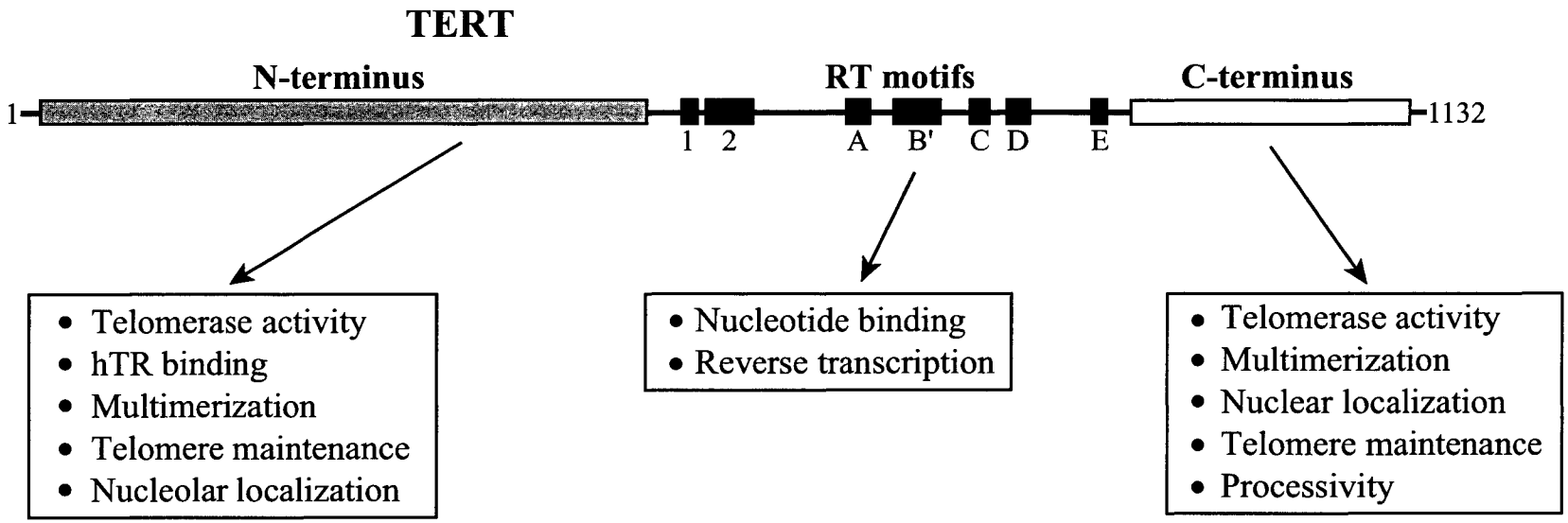


Figure 1.3 Structural and functional organization of TERT.
Based on Kelleher *et al.*, 2002.

The RT domain of TERT defines the active site of the enzyme that catalyzes the addition of deoxynucleotides during DNA synthesis (Harrington *et al.*, 1997b; Lingner *et al.*, 1997; Nakamura *et al.*, 1997). The catalytic site harbours seven RT-like motifs (motifs 1, 2, A, B', C, D, E) that are conserved in all members of the RT family (reviewed in O'Reilly *et al.*, 1999). TERT contains three invariant aspartate amino acid RT residues involved directly in catalysis (Weinrich *et al.*, 1997). Mutagenesis analyses confirmed the importance of the RT-like motifs *in vitro* and *in vivo* for telomere maintenance (Counter *et al.*, 1997; Harrington *et al.*, 1997a; Lingner *et al.*, 1997; Weinrich *et al.*, 1997; Beattie *et al.*, 1998; Collins and Gandhi, 1998; Haering *et al.*, 2000), and supported evidence that TERT is mechanistically similar to the conventional RTs. The conservation of both sequences and functions suggests that the three-dimensional architecture of the RT-like motifs is similar as well (reviewed in O'Reilly *et al.*, 1999).

The crystal structure of TERT is not available yet, but the crystal structure of HIV-1 RT provides a structural framework for TERT. The catalytic subunit of HIV-1 RT is reminiscent of the shape of a right hand, consisting of fingers, palm and thumb domains (Huang *et al.*, 1998). The RT-like motifs are located in the fingers and palm domains. Important TERT-specific amino acid residues in the RT domain modulate dNTP selectivity, template use, processivity and telomere maintenance (Bryan *et al.*, 2000a; Miller *et al.*, 2000; Bosoy and Lue, 2001; Peng *et al.*, 2001). Recently, a TERT-specific domain (IFD: insertion in fingers domain) located between the RT-like motifs A and B' of yeast telomerase was reported to optimize substrate interactions and to mediate the synthesis of multiple DNA telomeric repeats (Lue *et al.*, 2003).

Although the amino acid sequence of various TERT's N-terminal regions is significantly less conserved than that of the RT-like motifs, sequence alignments have identified several conserved regions, including the telomerase-specific T motif and RNA interaction domains (RID1 and RID2) (Friedman and Cech, 1999; Xia *et al.*, 2000; Moriarty *et al.*, 2002). Functional mapping has allowed several roles to be assigned to the N-terminal region of TERT: TR-TERT interactions (Friedman and Cech, 1999; Beattie *et al.*, 2000; Bryan *et al.*, 2000b; Armbruster *et al.*, 2001; Bachand *et al.*, 2001; Lai *et al.*, 2001; Moriarty *et al.*, 2002; Bosoy *et al.*, 2003), telomere maintenance (Xia *et al.*, 2000; Armbruster *et al.*, 2001; Bosoy *et al.*, 2003), TERT multimerization (Beattie *et al.*, 2001; Arai *et al.*, 2002; Moriarty *et al.*, 2002) and nucleolar localization of TERT (Etheridge *et al.*, 2002; Yang *et al.*, 2002). In contrast to the N-terminal region, the catalytic site contributes negligibly to TR-TERT binding (Mitchell and Collins, 2000; Bachand *et al.*, 2001). Reverse transcription and TR-TERT binding are two separable activities since the impairment of telomerase activity does not necessarily alter TR-TERT interactions.

The comparison of yeast, ciliate and vertebrate TERT C-termini reveals no obvious sequence conservation or functional similarity among families, suggesting that the C-terminal domain is a highly divergent region (Peng *et al.*, 2001). In yeast, the TERT C-terminal region is not required for cell survival, suggesting that other cellular components might rescue the deficiency imposed by the *in vivo* C-terminal truncation (Friedman and Cech, 1999; Peng *et al.*, 2001). However, TERT C-terminal deletions or alterations abolish telomerase activity, but they do not compromise TR-TERT binding (Beattie *et al.*, 2000; Bachand *et al.*, 2001; Lai *et al.*, 2001; Hossain *et al.*, 2002; Huard *et*

al., 2003). They also impair processivity of yeast and human telomerase (Hossain *et al.*, 2002; Huard *et al.*, 2003), suggesting that the mechanistic similarity between TERT members may be greater than the apparent sequence identity anticipates. In yeast, processivity mediated by the TERT C-terminal region is a determinant of telomere length homeostasis *in vivo* (Peng *et al.*, 2001). These results raise the possibility that the C-terminal region of TERT members might constitute the thumb domain, according to the structural context and functions of HIV-1 RT (Huang *et al.*, 1998). hTERT C-terminal region contains binding sites for the 14-3-3 and CRM1 proteins, which regulate the intracellular localization of telomerase (Seimiya *et al.*, 2000). It also mediates hTERT multimerization (Beattie *et al.*, 2001; Arai *et al.*, 2002) by interacting with RID1 (Moriarty *et al.*, 2004).

Specific mutations in the C-terminal and N-terminal regions of hTERT are implicated in the dissociation of telomerase functions (DAT: dissociates activities of telomerase). hTERT mutated in the DAT regions and expressed in cells generates catalytically active enzymes *in vitro*, but these enzymes are defective in telomere length maintenance (Armbruster *et al.*, 2001; Banik *et al.*, 2002). Mutations in the N-terminal region of Est2p as well as the addition of a hemagglutinin epitope tag to the hTERT C-terminal end give rise to a similar phenotype (Counter *et al.*, 1998; Friedman and Cech, 1999; Ouellette *et al.*, 1999; Kim *et al.*, 2003a). These mutants are still able to localize to the nucleus, suggesting that they are defective in telomere elongation once a catalytically functional enzyme is assembled in the nucleus. A targeting approach in human cells involving the fusion of a DAT mutant with a telomere-associated protein (TRF2 or hPOT1) induces a progressive telomere elongation (Armbruster *et al.*, 2003; Armbruster

et al., 2004). Rescue of the telomere shortening DAT phenotype may be due to the efficient recruitment of the DAT mutants by TFR2 and hPOT1 at telomeres. Telomere length defect may also reflect an altered interaction between the DAT mutants and telomeres that compromises telomere elongation (Lee *et al.*, 2003).

1.6 *Biochemistry of the telomerase ribonucleoprotein complex*

1.6.1 *Synthesis of telomeric DNA repeats*

In vitro-reconstituted telomerase has been very useful to further characterize the mechanism of DNA polymerization performed by the enzyme. Telomerase is processive, which implies its ability to repeatedly reverse transcribe the same segment of its RNA into DNA before its dissociation from the DNA products (Greider, 1991). The predicted model of DNA synthesis involves a cycle of multiple steps (Fig. 1.4): (1) the telomerase enzyme must hybridize its template with the 3'-end of a potential DNA substrate. A single-stranded oligonucleotide can be used *in vitro* as an alternative to the telomeric G-tails; (2) DNA extension of the substrate occurs soon after its annealing; (3) translocation of the DNA substrate takes place when the farthest 5' residue of the RNA template has been reverse transcribed into DNA. Translocation moves the 3'-end of the substrate at the 3'-end of the template; (4) telomerase performs another cycle of DNA extension synthesizing more telomeric DNA repeats (reviewed in Greider, 1996). Thus telomerase initiates the synthesis of telomeric DNA repeats onto primers and can add hundred of nucleotides before its dissociation from the DNA products (Greider, 1991). The efficiency of the polymerization process is achieved by the appropriate alignment of the substrate along the template during each round of elongation (Autexier and Greider, 1995; Gilley *et al.*, 1995; Gilley and Blackburn, 1996).

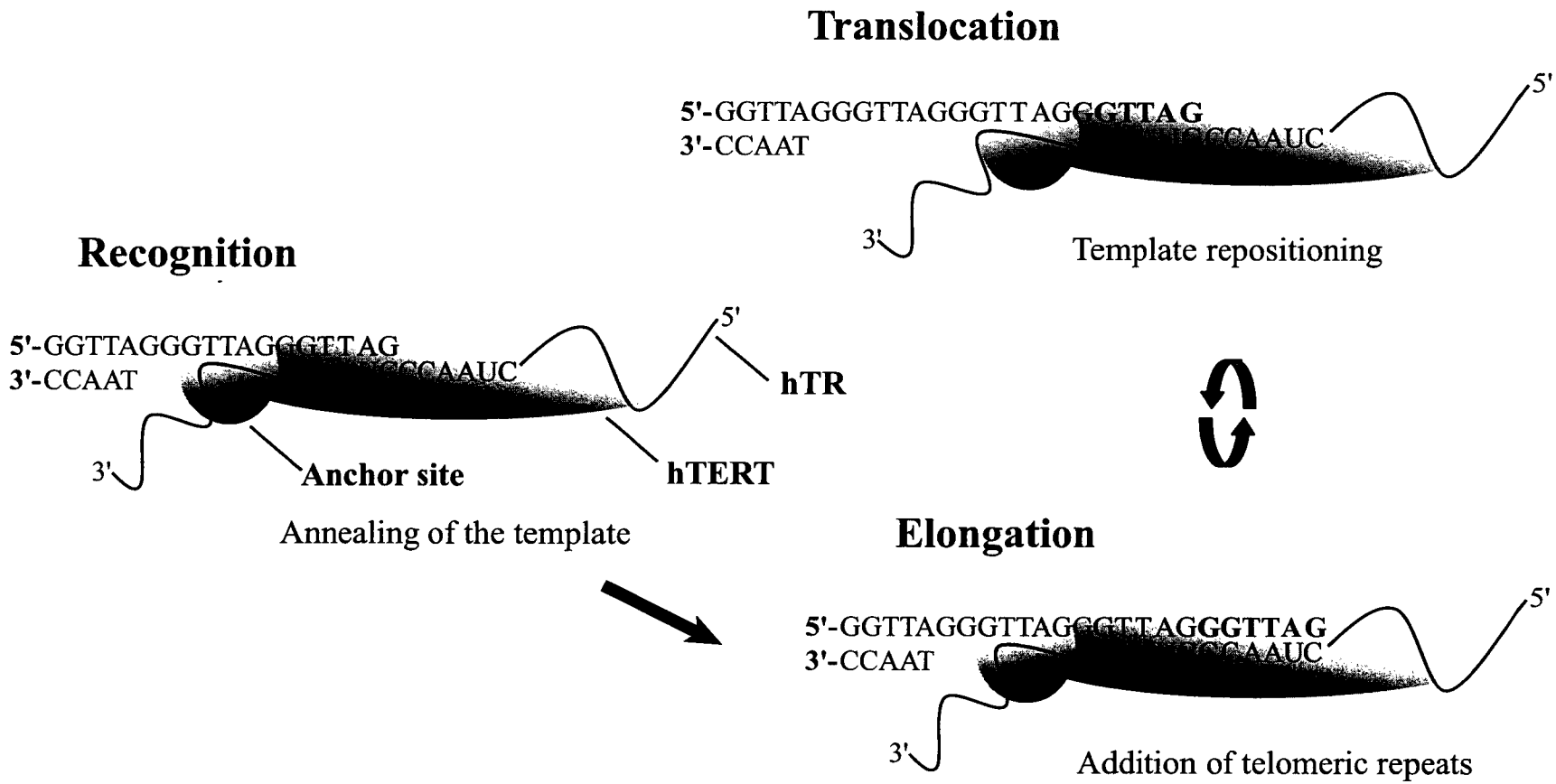


Figure 1.4 Synthesis of telomeric DNA repeats by telomerase.
Based on Greider, 1996.

DNA synthesis involves transient interactions between the substrate and the template. The correct template region must be delineated within the telomerase RNA prior to its hybridization with the DNA substrate. In *Tetrahymena thermophila*, two fragments of the telomerase RNA (tTR), corresponding to the template sequence alone and a truncated tTR without the template region, have been mixed together with the tTERT subunit in order to reconstitute telomerase in RRL (*trans*-template reconstitution). This recombinant telomerase selects the appropriate template sequence by binding to a sequence-specific RNA motif adjacent to the 3'-end of the template, referred to as template recognition element (TRE) (Miller and Collins, 2002). The deletion or substitution of TRE dramatically inhibits telomerase activity in the *trans*-template reconstitution experiments (Miller and Collins, 2002).

1.6.2 *Template and anchor site*

Minimal base-pairing between the template and the substrate determines the efficiency of the DNA polymerization performed by telomerase (Morin, 1989). The transient template/primer complex directs the initial starting position of DNA extension once the substrate is annealed. Subtle variations in the primer sequence can alter their binding properties and compromise their elongation. The template region of telomerase is functionally divided into two distinct domains known as the alignment and elongation domains (Autexier and Greider, 1994; Gavory *et al.*, 2002). The elongation domain encodes the telomeric repeats and the alignment domain serves to align the substrate or subsequent elongation products at the 3'-end of the template.

G-rich primers are more efficiently elongated by telomerase *in vitro* (Morin, 1989; Harrington and Greider, 1991; Morin, 1991; Melek *et al.*, 1996; Lue and

Peng, 1998; Wallweber *et al.*, 2003). Elongation of G-rich primers predominantly generates long DNA products (Morin, 1989; Morin, 1991). Thus processive elongation is believed to require interactions between a 5' region of the DNA substrate and a second site (anchor site) in the TERT subunit (Greider, 1991; Harrington and Greider, 1991; Morin, 1991; Collins and Greider, 1993; Hammond *et al.*, 1997; Lue and Peng, 1998). Arrays of guanine residues preferentially bind to the anchor site, helping to establish a stable template/primer binary complex (Collins and Greider, 1993; Hammond *et al.*, 1997; Lue and Peng, 1998). Elongation of short primers without such a 5' region generates mostly partial telomeric repeats (Morin, 1989; Collins and Greider, 1993; Baran *et al.*, 2002). Therefore, long telomeric primers may interact with an anchor site, preventing their dissociation during the repositioning of the primer 3'-end and facilitating processive synthesis of telomeric DNA repeats.

Although nontelomeric oligonucleotides are poor substrate of telomerase elongation, *de novo* addition of DNA repeats onto nontelomeric DNA, referred to as chromosome healing, has been reported in several organisms (Harrington and Greider, 1991; Bednenko *et al.*, 1997; Wang and Blackburn, 1997). The mechanisms allowing telomerase to heal chromosomes are poorly understood. Minimal base-pairing between the DNA substrate and the telomerase RNA seems to be sufficient to initiate DNA elongation (Harrington and Greider, 1991; Morin, 1991; Wang and Blackburn, 1997). The formation of the template/primer complex is a prerequisite for DNA polymerization, but mismatches between the template and the primer can destabilize the binary complex and abolish the initiation of DNA synthesis. In the context of chromosome healing, the negative impact of mismatches may be dependent on the number of contiguous

complementary base-pairing that a DNA substrate can form with the template, and the position of the mismatches along the binary complex (Wang *et al.*, 1998). The anchor site may also contribute to stabilize the interactions between telomerase and the DNA substrate (Wallweber *et al.*, 2003).

1.6.3 *Template boundary*

Accurate DNA repeat synthesis requires a precise delineation of the template boundaries. The disruption of the boundary by reverse transcription of RNA residues beyond the template may reduce cellular viability and increase apoptosis as a result of the incorporation of nontelomeric DNA repeats onto the chromosome ends (Yu *et al.*, 1990; McEachern and Blackburn, 1995; Kirk *et al.*, 1997; Marušic *et al.*, 1997; McEachern *et al.*, 2000; Guiducci *et al.*, 2001; Kim *et al.*, 2001; Lin *et al.*, 2004). A boundary defect can also prevent the repositioning of the substrate at the next round of DNA synthesis. The 3' template boundary is determined in part by the hybridization of the DNA substrate with the 3'-end of the template through base-pairing. The 5' template boundary needs to be highly regulated in order to prevent the reverse transcription of RNA residues outside the template.

In ciliates, the 5' template boundary seems to be defined by a short RNA sequence located upstream of the template (Autexier and Greider, 1995). Studies performed in *Tetrahymena thermophila* have reported that the previously identified RNA sequence (residues within helix II) interacts with a specific RNA-binding domain of tTERT (Miller *et al.*, 2000; Lai *et al.*, 2001). tTR and tTERT mutants that impair tTR-tTERT binding alter the template boundary definition (Lai *et al.*, 2002). The establishment of a proper template boundary likely engages RNA-protein interactions

that sterically hinder the use of nontemplate RNA sequences for DNA synthesis (Lai *et al.*, 2002).

In budding yeast, the 5' template boundary is dependent on intramolecular RNA interactions that form a base-paired structure adjacent to the template (Tzfati *et al.*, 2000; Seto *et al.*, 2003). Substitutions disrupting the base-paired region result in altered telomere sequences and cellular growth defects (Tzfati *et al.*, 2000; Seto *et al.*, 2003). Restoring the base-pairing disruption by compensatory mutations alleviates this defect, suggesting that the base-paired region physically restricts the single-stranded region adjacent to the template to be accessible to telomerase (Tzfati *et al.*, 2000). Similar experiments reveal that helix P1b of hTR is essential to establish the 5' template boundary of human telomerase (Chen and Greider, 2003b). In contrast to *Tetrahymena thermophila*, helix P1b is not essential for hTR-hTERT binding (Bachand and Autexier, 2001; Ly *et al.*, 2003a). A defined length of the single-stranded linker, which connects the RNA template and helix P1b, is also required for the template boundary definition, suggesting that the linker may constrain the movement of the RNA template within the catalytic active site when hTR is anchored to hTERT (Chen and Greider, 2003b).

1.6.4 *Nucleolytic cleavage*

In addition to DNA polymerization activity, telomerase from yeast, ciliates and humans can catalyze a DNA nucleolytic cleavage *in vitro* which removes nucleotides from a primer prior to DNA synthesis (Collins and Greider, 1993; Melek *et al.*, 1996; Prescott and Blackburn, 1997; Greene *et al.*, 1998; Niu *et al.*, 2000; Huard and Autexier, 2004; Oulton and Harrington, 2004). Telomerase is able to cleave DNA substrates containing mismatches against the telomerase RNA template, suggesting a proofreading

activity that retains only residues complementary to the template (Collins and Greider, 1993; Melek *et al.*, 1996; Prescott and Blackburn, 1997; Greene *et al.*, 1998; Niu *et al.*, 2000; Huard and Autexier, 2004; Oulton and Harrington, 2004). However, the cleavage activity does not seem to follow the classical rules of proofreading since fully complementary telomeric primers are also cleaved, suggesting that telomerase may convert poor elongation substrates to more efficient substrates, which may increase their affinity with the template (Collins and Greider, 1993; Melek *et al.*, 1996; Huard and Autexier, 2004). In yeast and ciliate, the cleavage activity seems to be regulated by an endonucleolytic mechanism (Melek *et al.*, 1996; Niu *et al.*, 2000). In humans, the mechanism of cleavage is still unclear (Huard and Autexier, 2004; Oulton and Harrington, 2004). Moreover, divergences concerning the *in vitro* properties of DNA nucleolytic cleavage have been reported between RRL-reconstituted telomerase and endogenous telomerase extracted from human cells (Huard and Autexier, 2004). It seems that endogenous telomerase regulates more stringently the nucleolytic cleavage activity, probably with several associated partners (Huard and Autexier, 2004).

1.6.5 *Multimerization*

Most reverse transcriptases dimerize to form a catalytically active enzyme. Human immunodeficiency virus type 1 (HIV-1) RT is a functional heterodimer that consists of two polypeptides known as p66 and p51 (Huang *et al.*, 1998). Thus protein-protein interactions involving TERT subunits may be important for the enzymatic properties of telomerase. Over the years, numerous studies have investigated the potential multimerization state of telomerase. In yeast, the telomerase complex seems to contain two functional telomerase RNA (TLC1) molecules (Prescott and Blackburn,

1997). Telomerase, isolated from a diploid strain carrying one wild-type allele (*TLC1*) and one mutated allele (*tlc1-476GUG*) coding for a telomerase RNA template mutant, can synthesize both intact and altered telomeric DNA repeats, suggesting at least two active sites in the same RNP complex (Prescott and Blackburn, 1997). However, telomerase 476GUG template mutant isolated from *tlc1-476GUG* haploid cells is inactive. The same telomerase template mutant is also inactive when it is complemented with active wild-type telomerase extracted from *TLC1* haploid cells (Prescott and Blackburn, 1997). Taken together, telomerase with the mutated TLC1 is only functional when it is preassembled *in vivo* in the presence of wild-type TLC1, suggesting that some components are stabilized by the presence of wild-type TLC1 RNA (Prescott and Blackburn, 1997).

Telomerase isolated from the ciliated *Euplotes aediculatus* and *Euplotes crassus* may exist as either a monomer or a dimer (Lingner and Cech, 1996; Wang *et al.*, 2002; Aigner *et al.*, 2003), whereas catalytically active recombinant telomerase from *Tetrahymena thermophila* is a monomer (Bryan *et al.*, 2003). In humans, the apparent molecular mass of the telomerase complex after purification is consistent with its multimerization (Schnapp *et al.*, 1998; Wenz *et al.*, 2001). Affinity purification against the telomerase RNA subunit confirmed the existence of recombinant human telomerase as a dimer (Wenz *et al.*, 2001). Every RNP complex seems to contain two hTR molecules cooperating together during elongation of an oligonucleotide substrate (Wenz *et al.*, 2001).

Other evidence demonstrated that the multimerization state of telomerase is mediated by TERT interactions. Differentially tagged hTERT molecules can be

coimmunoprecipitated (Armbruster *et al.*, 2001; Beattie *et al.*, 2001; Moriarty *et al.*, 2002) while two independent catalytically inactive hTERT subunits can complement each other *in trans* to reconstitute an active complex *in vitro* (Beattie *et al.*, 2001; Moriarty *et al.*, 2002). However, at least one hTERT molecule must maintain hTR binding activity (Beattie *et al.*, 2001; Moriarty *et al.*, 2002). Investigations delineating regions of hTERT involved in the telomerase multimerization localize them to the N-terminal and C-terminal regions of hTERT (Arai *et al.*, 2002).

Similarly to protein-protein interactions, RNA-RNA interactions may be important for the assembly of the telomerase complex. A sophisticated method of detection based on tagged RNA with fluorescent residues was designed to detect interactions between labelled molecules in solution (Ren *et al.*, 2003). This study found that the loop connecting the H/ACA box and the CR7 domain mediates homodimerization of hTR, which may have structural implications for hTR stability (Ren *et al.*, 2003). Two independent studies revealed that the pseudoknot region of hTR may mediate RNA-RNA interactions in a functional homodimer of human telomerase (Ly *et al.*, 2003b; Keppler and Jarstfer, 2004). One study demonstrated that two hTR molecules having their pseudoknot disrupted (helix P3) by mutations restore telomerase activity by hTR-hTR interactions in RRL-reconstituted telomerase (*trans*-pseudoknot reconstitution) (Ly *et al.*, 2003b). The other study also concluded that intermolecular P3 base-pairing promotes homodimerization of hTR when an oligonucleotide hybridizing helix P3 inhibits the formation of the homodimer (Keppler and Jarstfer, 2004).

Although the role of telomerase multimerization at telomeres remains unknown, three speculative models for telomere elongation can be proposed based on the

experimental evidence that inactive telomerase subunits cooperate together for reconstituting telomerase activity (reviewed in Kelleher *et al.*, 2002). In the anchor site model, one template stabilizes the interaction with telomeres, while the other template is used for the reverse transcription. In the template switching model, processive elongation of telomeres is accomplished by using two templates alternately. In the parallel extension model, two chromosome ends are simultaneously elongated (reviewed in Kelleher *et al.*, 2002).

1.6.6 *Processivity*

Important questions regarding the modulation of processivity and catalytic activity of telomerase have been addressed. Telomerase modulates processive DNA elongation *in vitro* as long as it forms a stable complex with the DNA products. Telomerase exhibits two types of processivity: (1) repeat addition processivity which refers to the addition of multiple telomeric repeats onto the same primer; (2) nucleotide addition processivity which corresponds to the addition of nucleotides within a single telomeric repeat. Processivity is an intrinsic property of the enzyme, relying on its ability to continuously synthesize DNA without being interrupted. TERT-specific amino acid residues in the RT domain modulating the binding of primers and dNTPs have been shown to regulate repeat and nucleotide addition processivity, respectively (Bryan *et al.*, 2000a; Bosoy and Lue, 2001). Moreover, the hTERT C-terminus and RID1 domain are determinants of enzyme processivity (Huard *et al.*, 2003; Moriarty *et al.*, 2004). RID1 seems to be a major accessory domain of hTERT that interacts with the hTR pseudoknot-template domain and hTERT C-terminus, contributing all together to repeat addition processivity (Moriarty *et al.*, 2004). TR-specific nucleotide residues also mediate

telomerase processivity (Chen and Greider, 2003a; Lai *et al.*, 2003; Mason *et al.*, 2003; Moriarty *et al.*, 2004).

Besides the mutations in both telomerase subunits, changing parameters such as temperature, primer sequence and length, dNTP concentration and salt concentration may alter telomerase processivity *in vitro* (Maine *et al.*, 1999; Sun *et al.*, 1999). More specifically, it has been suggested that the increase of dGTP concentration results in an increase of repeat addition processivity due to the stimulation of primer translocation for another round of DNA repeat synthesis (Hammond and Cech, 1997; Hardy *et al.*, 2001). Several studies reported that endogenous telomerase extracted from cells is more processive than RRL-reconstituted telomerase (Collins and Gandhi, 1998; Bryan *et al.*, 2000a). This difference may be due to the additional stabilization of the endogenous enzyme/primer complexes provided by chaperones or other accessory proteins which contribute to the assembly of telomerase (Collins and Gandhi, 1998; Licht and Collins, 1999; Bryan *et al.*, 2000a). The RRL-reconstituted telomerase complex may also be assembled in a different conformation which can differ from the native enzyme (Collins and Gandhi, 1998; Licht and Collins, 1999; Bryan *et al.*, 2000a).

1.7 *Telomerase associated proteins*

The telomerase complex often contains additional proteins that are not required for enzyme catalysis. Telomerase-associated proteins have been identified in several organisms including yeast, ciliates and humans, but these accessory factors are very diverse making it difficult to find a conserved family of proteins that seems to be associated with telomerase in all organisms (reviewed in Harrington, 2003). In the

following section, I will describe briefly the major telomerase-associated proteins and their possible functions.

Several telomerase-associated proteins interact with the telomerase RNA subunit. Recently, dyskerin, mammalian Staufen and L22 ribosomal proteins were implicated in hTR processing and telomerase assembly (Mitchell *et al.*, 1999b; Le *et al.*, 2000). Dyskerin is a putative pseudouridine synthase involved in the ribosomal processing and binds to H/ACA box motifs of small nucleolar RNA (snoRNA). hTR contains also a dyskerin H/ACA box binding site that is only present in vertebrate telomerase RNA (Mitchell *et al.*, 1999a; Mitchell *et al.*, 1999b; Chen *et al.*, 2000). The product of the survival motor neuron (*SMN*) gene was also identified as a binding partner of telomerase, and may have a role in telomerase RNP biogenesis (Bachand *et al.*, 2002). Several other proteins involved in RNP assembly and RNA splicing have been identified as interacting with telomerase RNA (reviewed in Harrington, 2003). However, further analysis is required to determine the role of all these proteins for telomerase RNA functions *in vivo*.

Telomerase-associated proteins have been also identified to bind to the TERT subunit. The hTERT C-terminus contains binding sites for the 14-3-3 and CRM1 proteins, both involved in the localization of the enzyme in the nucleus (Seimiya *et al.*, 2000). Chaperone proteins, p23 and Hsp90, may promote the folding of the enzyme (Holt *et al.*, 1999). Recently, two independent investigators identified hEST1A and hEST1B as human homologues of *Saccharomyces cerevisiae* Est1p (Reichenbach *et al.*, 2003; Snow *et al.*, 2003). They interact with hTERT and are involved in the capping

function of telomeres, suggesting functional conservation between yeast and humans (Reichenbach *et al.*, 2003; Snow *et al.*, 2003).

1.8 *Senescence, immortalization and tumorigenesis*

In all organisms, the genome is continually damaged by cellular stresses. In dividing cells, the major risk of genomic damage is due to mutations generated by unsuccessful repair after DNA replication. Mutations that confer a growth advantage to the cells may increase their susceptibility to malignant transformation or tumorigenesis. In the following section, I will describe the concept of replicative senescence and immortalization, and the roles played by telomeres and telomerase in the process of malignant transformation. Understanding the cellular context and the mechanisms by which telomere maintenance and telomerase activation contribute to tumorigenesis will facilitate the development of novel therapeutic approaches for cancer therapy.

1.8.1 *Replicative senescence and immortalization of cells*

Most mammalian cells have a finite replicative potential *in vitro* when they are serially passaged in culture (Hayflick limit). After approximately 50 to 70 divisions, cells stop dividing and enter in irreversible growth arrest referred to as replicative senescence (Fig. 1.5) (reviewed in Reddel, 1998). Morphologically, senescent cells are flattened and enlarged, and show senescence-associated β-galactosidase enzyme activity. Several other physiological offences, such as oxidative stress, DNA damage, oncogene activation or cell culture conditions can induce a senescence-like growth arrest of cultured mammalian cells (Kiyono *et al.*, 1998; Hemann *et al.*, 2001; Ramirez *et al.*, 2001; Espejel *et al.*, 2002; Liu *et al.*, 2003).

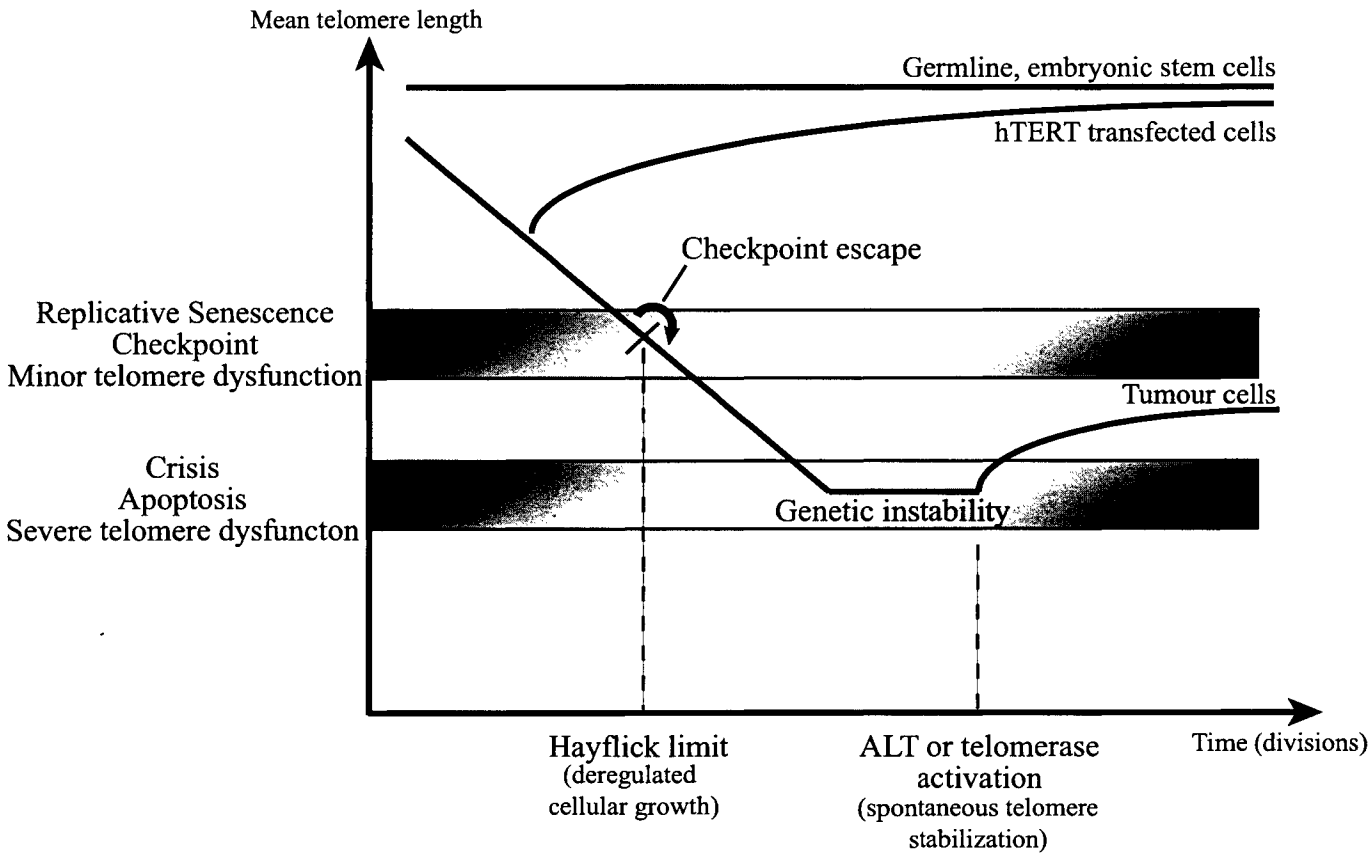


Figure 1.5 Telomere hypothesis of cellular aging and immortalization.
Based on Harley, 2002.

Replicative senescence is a nonproliferative state characterized by checkpoint arrest likely triggered by a DNA damage response due to critical telomere loss. However, expression of viral oncogenes, such as simian virus 40 (SV40) large T antigen (LTAg) or human papillomavirus (HPV) E6 and E7 oncoproteins, allows the cells to escape from senescence due to the inactivation of p53 and Rb tumour suppressor pathways (Fig. 1.5) (reviewed in Harley, 2002). Consequently, cells continue to proliferate until they reach a second proliferative barrier referred to as crisis, which is characterized by massive cell death and extremely short and dysfunctional telomeres (Fig. 1.5). Immortal cells might emerge spontaneously at very low frequency from this population (reviewed in Reddel, 1998). Therefore, replicative senescence and crisis represent two obstacles to malignant transformation of mammalian cells.

1.8.2 *Consequences of telomere dysfunction*

Genomic instability is necessary to initiate tumorigenesis or to promote the acquisition of additional genetic alterations contributing to tumour development (Gisselsson *et al.*, 2001). Telomere dysfunction is one mechanism that favours the initiation of chromosomal aberrations and eventually malignant transformation. On the other hand, critically short and dysfunctional telomeres limit the cell life-span. Numerous investigations raise the evidence of a dual role of telomere dysfunction either promoting tumorigenesis or inhibiting tumour development (Fig. 1.6) (reviewed in Hackett and Greider, 2002).

In response to telomere dysfunction, cells may activate a senescence response. Consistent with the telomere hypothesis of cellular aging and immortalization, critically short telomeres promote genomic instability which likely triggers cellular

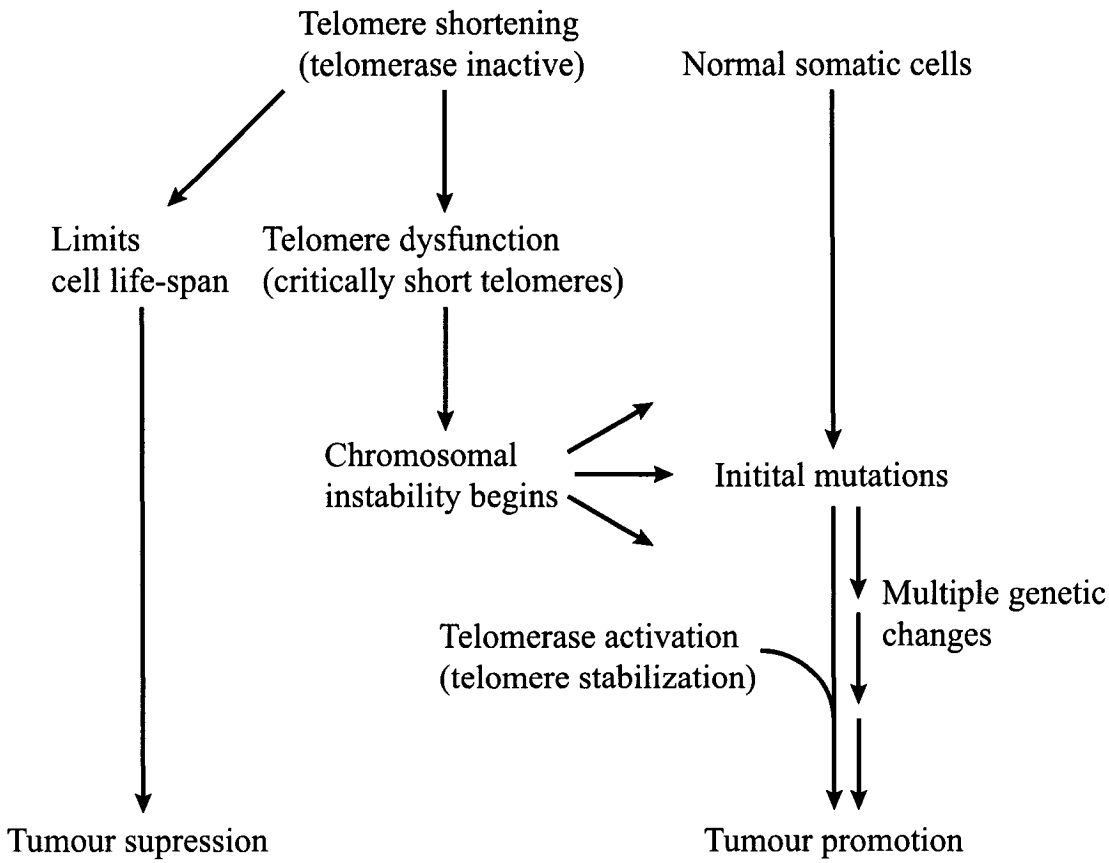


Figure 1.6 The dual role of telomeres and telomerase in cancer development.
Based on Feldser *et al.*, 2003.

senescence (Fig. 1.5). As demonstrated in yeast, short telomeres in a telomerase-deficient strain induce a DNA damage response which initiates senescence and eventually promotes cell death (Hackett *et al.*, 2001; Ijpma and Greider, 2003). Chromosomal end-to-end fusions have also been shown when critically short and dysfunctional telomeres elicit a cellular response (Counter *et al.*, 1992; Hackett *et al.*, 2001; Hemann *et al.*, 2001; Hackett and Greider, 2003). The senescence response requires cell cycle and DNA damage checkpoints controlled by pRB and p53 tumour suppressors. Genetically telomerase-deficient mice with short telomeres display severe cellular proliferative defects and, in the presence of p53, they are less susceptible to develop tumours (Chin *et al.*, 1999; González-Suárez *et al.*, 2000). Thus telomere shortening has been proposed to be a tumour suppressor mechanism by inducing senescence (Fig. 1.6). Cellular senescence prevents cell proliferation, thereby preventing tumorigenesis.

If senescence checkpoints are compromised, cells may survive and become serious candidates for malignant transformation. There is now strong evidence that dysfunctional telomeres can promote the development of cancers (Fig. 1.6) (Chin *et al.*, 1999; Artandi *et al.*, 2000; Gisselsson *et al.*, 2001; Rudolph *et al.*, 2001). Telomerase and p53-deficient mice display the development of epithelial tumours with an elevated frequency of chromosomal aberrations due to their critically short telomeres (Chin *et al.*, 1999; Artandi *et al.*, 2000). Therefore, simultaneous absence of p53 and telomerase may favour survival of cancer cells harbouring several chromosomal dysfunctions. Although there is much evidence that short telomeres contribute to tumour initiation in mouse, the evidence that a similar effect promotes human tumour formation is only correlative. Human cells must acquire mechanisms to stabilize and restore telomere functions in order

to survive (Kim *et al.*, 1994; Hahn *et al.*, 1999; Dunham *et al.*, 2000). The primary mechanism of telomere maintenance is the expression of telomerase. Telomerase is catalytically active in most immortal cells, whereas most somatic cells lack detectable levels of telomerase activity (Kim *et al.*, 1994). Telomerase expression promotes immortalization and allows cancer cells to proliferate indefinitely by stabilizing their telomeres (reviewed in Serrano and Blasco, 2001).

It is possible now to imagine a scenario where critically short telomeres initiate genomic instability through a loss of telomere protective function. Short telomeres may promote tumour suppression by limiting cell life-span as well as malignant transformation by restraining cells at multiple genetic changes that are required for cancer formation. Genomic instability may force the cells to set a mechanism maintaining the stability of short telomeres. Telomerase seems to have a protective effect on very short telomeres. The expression of telomerase may stabilize further genomic changes induced by short telomeres and facilitate cell proliferation after mutations in important genes generated by chromosomal end-to-end fusions or chromosome breaks (Rudolph *et al.*, 2001; Samper *et al.*, 2001; Masutomi *et al.*, 2003; Erdmann *et al.*, 2004; Teixeira *et al.*, 2004). The stabilization of telomere length allows immortalization and makes possible further progress of malignant transformation. In this model, critically short telomeres can initiate chromosomal instability, leading to tumorigenesis as other mutations arise (reviewed in Feldser *et al.*, 2003). The dual role of telomeres and telomerase operates both to suppress and facilitate cancer formation (Fig. 1.6).

1.8.3 *Alternative lengthening of telomeres*

Most of the time, telomere maintenance is accomplished by telomerase. In some occasions, a process referred to as alternative lengthening of telomeres (ALT) can maintain telomere length probably through a DNA recombination mechanism (reviewed in Reddel, 2003). The available evidence concerning the mechanism of ALT is consistent with a model based on homologous recombination by which a DNA strand from one telomere anneals with the complementary strand of another telomere, thereby priming DNA synthesis using the complementary strand as a template (reviewed in Henson *et al.*, 2002).

Recombination-based mechanisms for telomere maintenance have been first identified in yeast. Genetically telomerase-deficient yeast give rise to two types of survivors depending on the mechanism of homologous recombination that operates to maintain telomeres (Teng and Zakian, 1999; Chen *et al.*, 2001). Type I survivors have short telomeres that are maintained by recombination involving subtelomeric sequences (Y' elements), while type II survivors have long and very heterogeneous length telomeres that are maintained by inter-telomeric recombination (Lundblad and Blackburn, 1993; Louis *et al.*, 1994; Teng and Zakian, 1999; Chen *et al.*, 2001). The repetitive nature of subtelomeres makes them a preferential target for recombination or translocation events.

Human ALT cells are reminiscent of yeast type II survivors. They have very long and heterogeneous length telomeres and have nuclear structures referred to as ALT-associated promyelocytic leukemia (PML) bodies (APBs). APBs contain telomeric DNA, telomere-associated proteins TRF1 and TRF2, and proteins that are involved in DNA replication and recombination. They have been found only in ALT cells and their

function is unknown (reviewed in Henson *et al.*, 2002). The homologous recombination in ALT cells may involve the telomeric regions of the chromosomes (Dunham *et al.*, 2000). If human telomeres are tagged with a marker that can be monitored by FISH, the tag is copied to untagged telomeres after several cell divisions (Dunham *et al.*, 2000). However, the same experiment performed with tagged subtelomeres did not disperse the marker to other chromosomes. The authors concluded that the telomeric regions mediate recombination in human ALT cells (Dunham *et al.*, 2000). A number of recent studies have also examined the consequences of reconstitution of telomerase activity in human ALT cells as a result of ectopic expression of the telomerase subunits (Cerone *et al.*, 2001; Grobelny *et al.*, 2001; Perrem *et al.*, 2001). The authors concluded that short telomeres are preferentially elongated by telomerase, but the ALT pathway is not repressed. Taken together, these results indicate that biologically active telomerase and ALT can coexist in human cells and may cooperate in telomere maintenance.

1.8.4 *Telomere maintenance and disease*

Defects in genes other than those encoding the telomerase subunits can result in premature telomere loss. Distinct phenotypes can arise dependent on which tissues are subjected to the higher rates of cellular turnover and which cell types have the least telomerase-dependent compensation for proliferation (reviewed in Wong and Collins, 2003). Dyskeratosis congenita (DC) is a rare X-linked inherited disorder caused by point mutations in the *DKC1* gene that encodes dyskerin (Heiss *et al.*, 1998). An autosomal dominant form of DC also exists and is correlated with mutations in the human telomerase RNA (Vulliamy *et al.*, 2001). DC is characterized by a progressive bone marrow failure and an increased susceptibility to tumour formation (reviewed in Mason,

2003). DC is an example of the direct impact of telomerase deficiency or insufficiency. It was demonstrated recently that lymphocytes and fibroblasts isolated from X-linked DC patients have a defect in hTR expression level and stability, leading to lower levels of telomerase activity, and have shorter telomeres than age-matched control cells (Mitchell *et al.*, 1999b).

Other experiments have also revealed that some proteins implicated in the cellular response due to damaged DNA play an indispensable role in the control of telomere length regulation and functionality. In particular, a link has been made between ataxia telangiectasia (AT) disease and telomere regulation (Metcalfe *et al.*, 1996). AT is an autosomal recessive disorder characterized by point mutations in the *ATM* gene that encodes a protein kinase (ATM) involved in the signalling response to DNA damage (Savitsky *et al.*, 1995). AT patients have an increased predisposition to lymphoid malignant diseases. Cells from these patients have shorter telomeres than age-matched control cells, consistent with a greater rate of cellular turnover due to unrepaired genome damage (Metcalfe *et al.*, 1996). Constitutive expression of hTERT in cells from AT patients increases telomere length and prevents senescence, but does not fully rescue telomere dysfunction, suggesting that ATM may have a direct role at the telomere as well (Wood *et al.*, 2001). Several other diseases involve telomere dysfunction, but the molecular mechanisms that underlie telomere length determination and regulation are not yet fully understood.

1.9 *Objectives*

The goal of the present thesis was to characterize particular aspects of the mechanism of action of the minimal human telomerase ribonucleoprotein complex (hTERT and hTR). We were interested to understand the biochemical properties of human telomerase at the chromosome ends. To identify human telomerase determinants of processivity and fidelity, we first dissected the role of the C-terminal domain of hTERT *in vitro* by reconstituting the telomerase complex in RRL (Chapter 2). In addition to its DNA polymerase function, we characterized a telomerase-dependent nucleolytic cleavage activity performed by *in vitro*-reconstituted telomerase and endogenous telomerase from human cells (Chapter 3). Finally, since detailed structural and functional analyses of the human telomerase are currently limited by the lack of an abundant recombinant source of active enzyme, we set up experimental conditions to express recombinant telomerase in insect cells (Chapter 4).

CHAPTER 2

The C terminus of the Human Telomerase Reverse Transcriptase is a Determinant of Enzyme Processivity

A version of this chapter is published in *Nucleic Acids Res.* (2003), **31**, 4059-4070

2.1 *Preface*

The catalytic protein subunit of telomerase (TERT) is divided in three functional domains: a central region that is identified as the active site, and N- and C-terminal regions that are implicated in a number of telomerase-specific functions. The role played by the C-terminal region of human TERT (hTERT) *in vivo* has recently started to be investigated, but amino acid residues mediating these functions remain largely uncharacterized for hTERT. In this study, we delineated functional domains of the hTERT C-terminal region that are required for enzyme catalysis and hTERT multimerization. Furthermore, we analyzed the processivity of human telomerase by monitoring DNA synthesis with a direct primer extension assay performed with RRL-reconstituted enzyme.

2.2 *Abstract*

The catalytic subunit of telomerase (TERT) contains conserved reverse transcriptase-like motifs but N- and C-terminal regions unique to telomerases. Despite weak sequence conservation, the C terminus of TERTs from various organisms has been implicated in telomerase-specific functions, including telomerase activity, functional multimerization with other TERT molecules, enzyme processivity and telomere length maintenance. We studied hTERT proteins containing small C-terminal deletions or substitutions to identify and characterize hTERT domains mediating telomerase activity, hTERT multimerization and processivity. Using sequence alignment of five vertebrate TERTs and *Arabidopsis thaliana* TERT, we identified blocks of highly conserved amino acids that were required for human telomerase activity and functional hTERT complementation. We adapted the non-PCR-based telomerase elongation assay to characterize telomerase expressed and reconstituted in the *in vitro* transcription/translation rabbit reticulocyte lysate system. Using this assay, we found that the hTERT C terminus, like the C terminus of *Saccharomyces cerevisiae* TERT, contributes to successive nucleotide addition within a single 6-base telomeric repeat (type I processivity). Certain mutations in the hTERT C terminus also reduced the repetitive addition of multiple telomeric repeats (type II processivity). Our results suggest a functionally conserved role for the TERT C terminus in telomerase enzyme processivity.

2.3 *Introduction*

Telomeres are unique DNA-protein structures that cap the ends of linear chromosomes (reviewed in Blackburn, 2001). Telomere integrity is essential for chromosome stability, and the maintenance of telomeric DNA is required for the long-term proliferation of eukaryotic cells (reviewed in Harrington and Robinson, 2002). Several proteins have been identified that associate with telomeres and regulate various aspects of telomere structure, organization and length (reviewed in Blackburn, 2001). In vertebrates, telomeres are composed of G-rich repeats synthesized by the ribonucleoprotein complex telomerase (reviewed in Blackburn, 1999).

The human telomerase enzyme is minimally composed of a protein catalytic subunit, the telomerase reverse transcriptase (hTERT), and an RNA molecule, hTR. A short sequence within hTR serves as a template for *de novo* addition of telomere sequences (Feng *et al.*, 1995). Comparative analysis of the amino acid sequence of TERT from different organisms revealed several conserved domains in TERT (Nakamura *et al.*, 1997; reviewed in O'Reilly *et al.*, 1999). The central region of TERT contains reverse transcriptase (RT)-like motifs and a telomerase-specific (T) motif essential for telomerase function *in vitro* and *in vivo* (Counter *et al.*, 1997; Harrington *et al.*, 1997; Lingner *et al.*, 1997; Weinrich *et al.*, 1997). TERT-specific motifs in the N terminus are important for enzymatic activity and are implicated in telomerase RNA binding and RNA template boundary definition (Friedman and Cech, 1999; Beattie *et al.*, 2000; Miller *et al.*, 2000; Xia *et al.*, 2000; Bachand and Autexier, 2001; Lai *et al.*, 2001). Recently, hTERT N-terminal amino acid residues that interact with hTR were defined (Moriarty *et al.*, 2002b). A DAT (dissociates activities of telomerase) region essential for telomere

maintenance *in vivo* was mapped to the hTERT N terminus, and may be involved in the recruitment of the telomerase enzyme to telomeres (Armbruster *et al.*, 2001). The N terminus of hTERT is also involved in functional and physical multimerization with other hTERT molecules, in telomerase ribonucleoprotein (RNP) formation *in vivo*, and in hTERT nucleolar localization (Beattie *et al.*, 2000; Beattie *et al.*, 2001; Arai *et al.*, 2002; Etheridge *et al.*, 2002; Moriarty *et al.*, 2002b; Yang *et al.*, 2002; Bosoy *et al.*, 2003).

The C terminus of TERT is not well conserved within the TERT family (Peng *et al.*, 2001). However, the C-terminal domain is essential for human telomerase function *in vivo*. The addition of a hemagglutinin (HA) epitope tag at the C-terminal end impairs hTERT's ability to maintain telomere length, increase the cellular life span of primary cells and immortalize transformed human cells (Counter *et al.*, 1998; Ouellette *et al.*, 1999). The expression of a hTERT C-terminal polypeptide in HeLa cells leads to telomere dysfunction, characterized by chromosome end-to-end fusions, and induces a senescence-like phenotype and apoptosis in the absence of telomere shortening (Huang *et al.*, 2002). These studies suggest a role for the C-terminal domain in maintaining telomere length, integrity and structure (Counter *et al.*, 1998; Ouellette *et al.*, 1999; Huang *et al.*, 2002). Recently, a DAT region essential for telomere maintenance *in vivo* was mapped to the hTERT C terminus (Banik *et al.*, 2002). The *Saccharomyces cerevisiae* TERT C terminus also contributes to telomere length regulation, but is not required for cell survival (Friedman and Cech, 1999). In addition, the C terminus of hTERT contains binding sites for the 14-3-3 and CRM1 proteins, which regulate the intracellular localization of telomerase (Seimiya *et al.*, 2000).

The C terminus of the TERTs is also essential for telomerase catalytic function. Analysis of human and *Tetrahymena* TERT C-terminal truncations revealed that the C-terminal domain is required for *in vitro* enzymatic activity but probably not for binding to the telomerase RNA (Beattie *et al.*, 2000; Bachand and Autexier, 2001; Lai *et al.*, 2001). Sequences at the junction of the RT motif E and the C terminus of hTERT play a role in functional and physical multimerization with other hTERT molecules (Beattie *et al.*, 2001; Arai *et al.*, 2002). The processivity of human immunodeficiency virus type 1 (HIV-1) RT is mediated in part by residues in the C-terminal extension (Jacobo-Molina *et al.*, 1993; Bebenek *et al.*, 1995; Peng *et al.*, 2001). Similarly, amino acids in the C-terminal domain of Est2p (*S. cerevisiae* TERT) contribute to telomerase activity, enzyme processivity and telomere length maintenance (Peng *et al.*, 2001; Hossain *et al.*, 2002). These results reveal mechanistic similarities between HIV-1 RT and yeast telomerase, and support a role for enzyme processivity in telomere maintenance (Peng *et al.*, 2001; Hossain *et al.*, 2002).

The TERT C terminus has been implicated in a number of telomerase-specific functions. However, the TERT amino acid residues and mechanisms mediating these functions have not been completely characterized for human telomerase. In the present study, human telomerases containing small deletions and substitutions were expressed in an *in vitro* transcription/translation system to investigate the regions of the hTERT C terminus involved in telomerase activity, multimerization with other hTERT molecules and enzyme processivity.

2.4 *Materials and methods*

2.4.1 *Plasmid constructions*

The construction of pET28b-hTERT, pET28a-GST-hTERT and pET28a-GST-hTERT D868N expression plasmids have been described previously (Bachand and Autexier, 1999; Moriarty *et al.*, 2002b). Internal C-terminal deletions and amino acid substitutions were generated using the Quick-Change site-directed mutagenesis protocol (Stratagene), the template pET28b-hTERT and appropriate pairs of primers. All C-terminal derivatives of hTERT were confirmed by restriction enzyme digestion and/or sequencing. The full-length hTR expression plasmid, phTR+1, has been described previously (Autexier *et al.*, 1996).

2.4.2 *Sequence comparison*

All TERT sequences used in the comparative analysis (NCBI BLAST: Altschul *et al.*, 1997) were obtained from GenBank. The *Rattus norvegicus* TERT C-terminal amino acid sequence is a conceptual translation of the coding sequence available in the NCBI protein database.

2.4.3 *In vitro transcription/translation*

The T7-coupled transcription/translation rabbit reticulocyte lysate (RRL) system (Promega) was used as described previously (Moriarty *et al.*, 2002b). Full-length and C-terminal derivatives of hTERT were synthesized in RRL in the presence of purified hTR and [^{35}S]methionine. hTR was synthesized and purified from *Fsp*I-linearized phTR+1 plasmid as previously described (Autexier *et al.*, 1996).

2.4.4 *Telomerase activity assays*

Telomerase activity was measured by two methods. First, a PCR-based telomerase repeat amplification protocol (TRAP) assay was performed on equal amounts of protein as described (Moriarty *et al.*, 2002a). 0.5 µl to 1 µl of RRL expressing hTERT or C-terminal variants in the presence of hTR was assayed for telomerase activity.

Second, a non-PCR-based telomerase elongation (also known as a standard, direct or conventional) assay was performed (Sun *et al.*, 1999) with modifications. Equal amounts of hTERT or C-terminal variant proteins expressed in RRL in the presence of hTR (approximately 20 µl of RRL sample) were assayed for telomerase activity in a 40 µl final volume reaction using a gel-purified 5'-biotinylated $(T_2AG_3)_4$ as telomeric primer (Operon). Standard reaction conditions were 50 mM Tris-HCl pH 8.3, 50 mM KOAc, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM spermidine, 1 µM telomere primer, 1.25 µM [α-^{32}P]dGTP (800 Ci/mmol; NEN Life Science Products), 1 mM dATP and 1 mM dTTP. The reaction mix was incubated at 30°C for 2 hours. The elongation step was terminated by adding 50 µl of RNase stop buffer (10 mM EDTA, 2 M NaCl, 0.1 mg/ml RNase A) and incubated at 37°C for 15 min. After addition of 50 µl of proteinase K solution (10 mM Tris-HCl pH 7.5, 0.3 mg/ml proteinase K) and a 15 min incubation at 37°C, a pre-washed Streptavidin MagnaSphere® Paramagnetic bead suspension (Promega) in 10 mM Tris-HCl pH 7.5, 1 M NaCl, 0.5 mM EDTA was added. The elongation products were immobilized on magnetic beads at room temperature for 30 min. Bead-elongation product complexes were washed three times with buffer A (10 mM Tris-HCl pH 7.5, 1 M NaCl), once with buffer B (10 mM Tris-HCl pH 7.5), resuspended in 10 µl of formamide dye (95% deionized formamide, 10 mM

EDTA) and boiled for 30 min. Telomerase reaction products were analyzed by 8% polyacrylamide-urea gel electrophoresis. Gels were dried and exposed to X-ray film. Standard reaction assays were also performed using native telomerase extracted from telomerase positive HL-60 cells (Mayeda and Krainer, 1999) or with RRL and a 5'-end labeled primer $(T_2AG_3)_4$.

2.4.5 *Multimerization assay*

Nonradiolabeled GST-hTERT proteins synthesized in RRL were immunopurified with a GST antibody (Amersham Pharmacia) pre-bound to protein A-Sepharose (Sigma) (Moriarty *et al.*, 2002b), and [^{35}S]methionine-labeled His_6-tagged hTERT and C-terminal variants were immunopurified using Talon resin (Clontech) as described by the manufacturer. Equal amounts of partially purified [^{35}S]-labeled His_6-tagged hTERT or C-terminal variants were added to the immobilized GST-hTERT proteins and coimmunoprecipitation was performed as previously described (Moriarty *et al.*, 2002b). Immunoprecipitated GST-hTERT and coprecipitated hTERT were resolved on SDS-7.5% PAGE gel and detected by Western blot analysis and by visualization of [^{35}S]-labeled proteins, respectively.

Signals corresponding to GST-hTERT and [^{35}S]-labeled proteins were quantified by densitometric analysis of the autoradiographs (ImageQuant software, Molecular Dynamics). The efficiencies of coimmunoprecipitation of hTERT and C-terminal variant proteins were calculated by expressing the signal obtained from the coprecipitated [^{35}S]-labeled proteins as a percentage of the input signal. This coimmunoprecipitation value was then normalized to the levels of nonradiolabeled GST-hTERT detected in Western blots, and the resulting values for each C-terminal variant

were expressed as a percentage of the value obtained for wild-type hTERT. Data for individual mutants were statistically compared with the data for wild-type proteins using a paired two-tailed Student t-test.

2.4.6 *Quantification of processivity*

The signal of each telomerase elongation product was quantified by densitometric analysis of the autoradiographs or by phosphorimager analysis (ImageQuant software, Molecular Dynamics). Results of quantification using both methods were similar. The total counts for each signal were normalized to the amount of transcript by dividing each elongation product signal by the number of labeled dGTPs it contains.

The processivity (P_i) at each position within a single base pair repeat (type I) was calculated using the formula $P_i = \text{sum}(T_{i+1} + T_{i+2} + \ldots + T_6)/\text{sum}(T_i + T_{i+1} + \ldots + T_6)$ described in Peng *et al.* (2001) (Peng *et al.*, 2001). T corresponds to the normalized signal at a given position, designated by i. The values obtained in independent experiments for equivalent positions within the first and second telomerase repeats (e.g. +1 is equivalent to +7) were averaged and statistically compared with average wild-type values using a paired two-tailed Student t-test. +1 and +7 refer to the second G in the human telomeric sequence TTAGGG. Similar processivity profiles were obtained for hTERT and C-terminal variants when equivalent telomerase elongation products within the first and second repeat were compared.

Repeat addition processivity (type II) was calculated using the normalized signals of the +6, +12, +18 and +24 telomerase elongation products (where products were detectable and quantifiable), and by applying the formula $P_i = (T_{i+6})/\text{sum}(T_i + T_{i+6})$

described in Hardy *et al.* (2001) (Hardy *et al.*, 2001). For example, the normalized intensity of the second repeat addition products is divided by the sum of signals from the first and second repeat addition products. We obtained repeat processivity values for each pair of repeats separately. Repeat processivity values for each of the +6, +12, +18, and +24 elongation products from independent experiments were multiplied by 100, and then averaged. Data were statistically analyzed using a paired two-tailed Student t-test. Products included in this analysis were within the linear range of signal detection. Similar values for repeat addition processivity were obtained when first and second repeat addition products, second and third repeat addition products, or third and fourth repeat addition products were compared. As pulse-chase experiments were not performed, we cannot distinguish if long elongation products result from the continuous addition of multiple repeats onto a given telomeric primer, or from dissociation and subsequent rebinding and elongation.

2.5 *Results*

2.5.1 *BLAST alignment and mutagenesis*

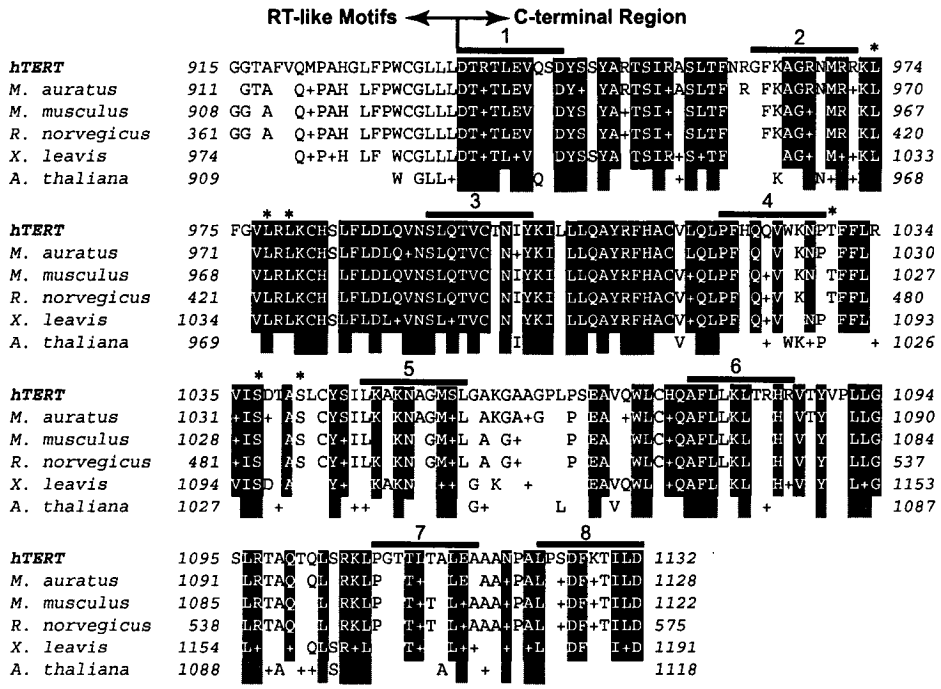
Alignment of C-terminal TERT sequences from diverse organisms indicates that this domain is not well conserved within the TERT family (data not shown; Peng *et al.*, 2001). We aligned five vertebrate TERT sequences and *Arabidopsis thaliana* TERT (Harrington *et al.*, 1997; Kilian *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997; Greenberg *et al.*, 1998; Martín-Rivera *et al.*, 1998; Fitzgerald *et al.*, 1999; Guo *et al.*, 2001; Kuramoto *et al.*, 2001) using the NCBI BLAST program (Altschul *et al.*, 1997) to determine if portions of the C terminus were conserved among vertebrate and *A. thaliana* TERTs (Fig. 2.1A). The C terminus is defined in this report as beginning at hTERT residue 936. In contrast to the low degree of C terminus sequence homology among members of the entire TERT family, the C terminus of vertebrate and *A. thaliana* TERTs contained blocks of highly conserved residues. These residues are not conserved between hTERT and Est2p or between the TERTs and HIV-1 RT (data not shown).

We generated a series of recombinant telomerases containing single amino acid substitutions or 10 amino acid deletions (Fig. 2.1B) to characterize the roles of the hTERT C terminus in telomerase activity, multimerization with other hTERT molecules and enzyme processivity. Deletions were regularly spaced at approximately 20 amino acid intervals. Single amino acid substitutions of the leucines to alanines at positions 974, 978 and 980 (L974A, L978A, L980A) in the CRM1-binding site (nuclear export signal) were generated (Seimiya *et al.*, 2000). Single amino acid substitutions of the threonine to alanine at position 1030 and the serines to alanines at positions 1037 and 1041 (T1030A, S1037A, S1041A) in the 14-3-3-binding site were also generated

Figure 2.1

A

BLAST Alignment of the C-terminus of Vertebrate and *Arabidopsis thaliana* TERTs



B

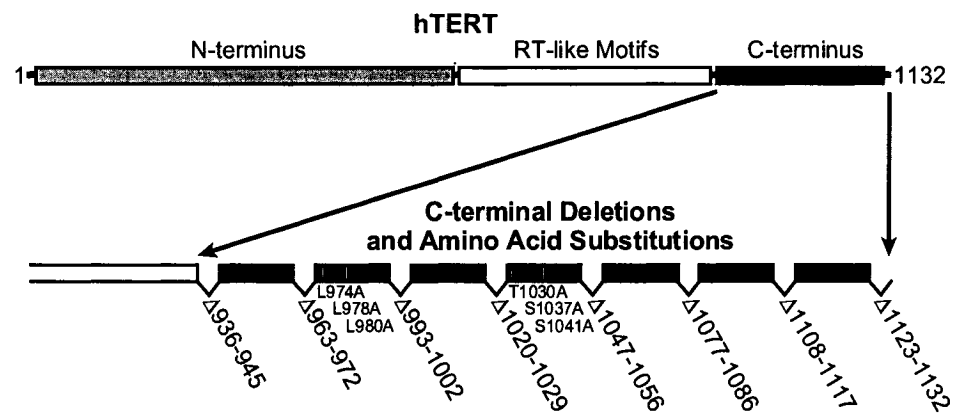


Figure 2.1 Map of the hTERT C terminus, location of C-terminal mutations, and sequence alignment of the C termini of vertebrate and *Arabidopsis thaliana* TERTs. **(A)** Alignment of the C-terminal amino acid sequences of five vertebrate TERTs and *A. thaliana* TERT. Alignment of human, *M. musculus*, *M. auratus*, *R. norvegicus*, *X. laevis* and *A. thaliana* TERT sequences (Harrington *et al.*, 1997; Kilian *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997; Greenberg *et al.*, 1998; Martín-Rivera *et al.*, 1998; Fitzgerald *et al.*, 1999; Guo *et al.*, 2001; Kuramoto *et al.*, 2001) was performed using the BLAST program. The symbol "+" indicates nonidentical conserved residues. Residues conserved in all vertebrate sequences are boxed in black. Residues boxed in gray are *A. thaliana* residues conserved with vertebrate sequences. Thick black lines above the protein sequence indicate the amino acids that are deleted in the hTERT variants. 1=Δ936-945, 2=Δ963-972, 3=Δ993-1002, 4=Δ1020-1029, 5=Δ1047-1056, 6=Δ1077-1086, 7=Δ1108-1117, 8=Δ1123-1132. The single amino acid substitutions, L974A, L978A, L980A, T1030A, S1037A and S1041 are indicated by an asterisk. **(B)** A schematic illustration of hTERT. Mutations characterized in this study are indicated as broken (deletions) or white bars (substitutions) on the linear map of the hTERT C terminus. The numbering for each C-terminal variant indicates the amino acid positions of the deleted or substituted residues.

(Seimiya *et al.*, 2000). All C-terminal deletions and single amino acid substitutions affected highly conserved residues (the deletions removed between 4 to 8 highly conserved residues). Thus, we identified vertebrate-conserved elements in the hTERT C terminus that may mediate functions specific to vertebrate telomerases. This mutagenic approach has been successfully employed to define various functions of hTERT N-terminal amino acid residues (Moriarty *et al.*, 2002b). We first addressed whether C-terminal vertebrate-conserved regions are implicated in human telomerase catalytic function.

2.5.2 *Telomerase activity of hTERT C-terminal mutants detected by TRAP or by a conventional telomerase assay*

Wild-type hTERT or hTERT C-terminal mutants were expressed *in vitro* in RRL in the presence of *in vitro*-transcribed hTR. Wild type hTERT and all mutant proteins were stably expressed in RRL (Fig. 2.2, bottom panel). The catalytic activities of reconstituted telomerases were assayed by the TRAP technique. The relative telomerase activities for mutant enzymes reconstituted in RRL are shown in figure 2.2 (top panel). The Δ936-945, Δ963-972, Δ993-1002, Δ1020-1029 and Δ1077-1086 C-terminal mutants were inactive. The Δ1047-1056, Δ1108-1117, Δ1123-1132, L974A, L978A, L980A, T1030A, S1037A and S1041A hTERT mutants reconstituted similar, or slightly reduced levels of activity compared with those reconstituted by wild-type hTERT. A previous study also reported that a triple alanine substitution T1030A, S1037A, S1041A in the 14-3-3-binding site does not alter telomerase activity, supporting the results we obtain with the variants containing single amino acid mutations at these positions (Seimiya *et al.*, 2000). In addition, our results show that residues in the nuclear

Figure 2.2

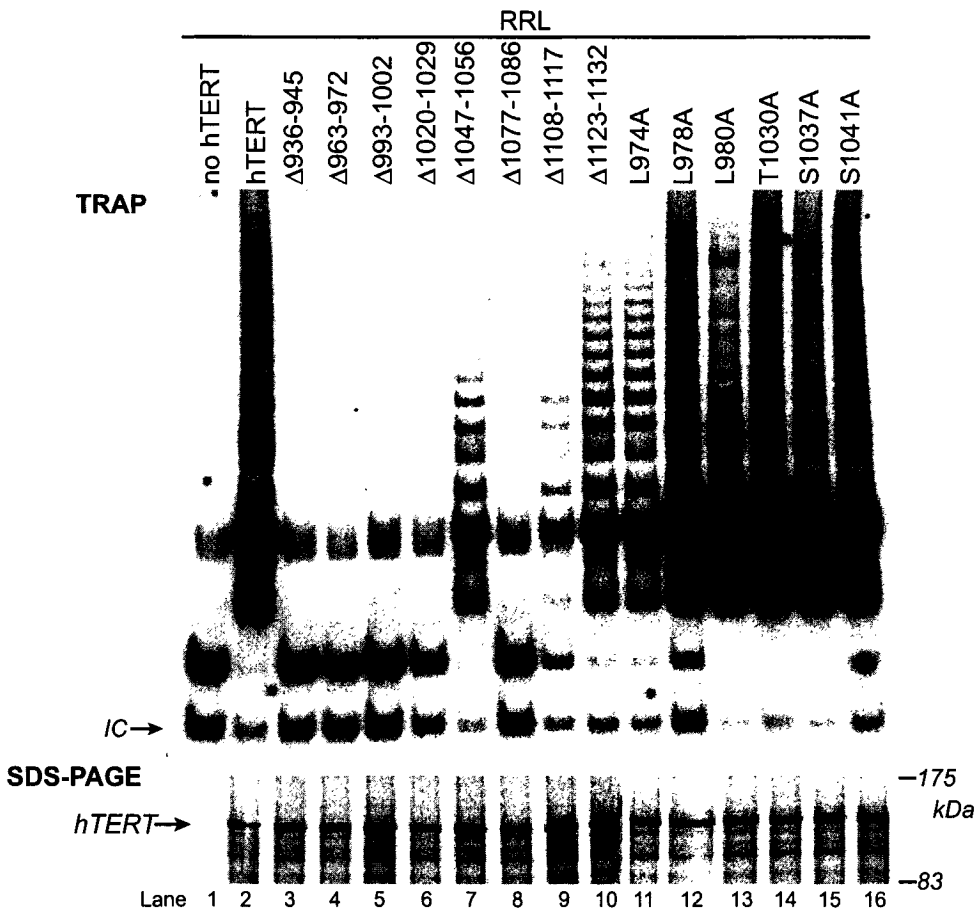

RRL
no hTERT
hTERT
Δ936-945
Δ963-972
Δ993-1002
Δ1020-1029
Δ1047-1056
Δ1077-1086
Δ1108-1117
Δ1123-1132
L974A
L978A
L980A
T1030A
S1037A
S1041A
TRAP
IC
SDS-PAGE
hTERT
175
kDa
83
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 2.2 Reconstitution of recombinant telomerases in an *in vitro* transcription/translation system and detection of telomerase activity using TRAP. Telomerase activity of C-terminal variants of hTERT expressed in RRL in the presence of hTR was detected using the PCR-based TRAP assay. Top panel: reconstituted telomerase activity of hTERT C-terminal mutants (Fig. 2.1). A PCR amplification control is indicated (IC). Bottom panel: expression of hTERT C-terminal mutants synthesized in RRL in the presence of [^{35}S]methionine was detected by SDS-PAGE.

export signal-like (CRM1) motif in hTERT are not required for telomerase activity detected by TRAP. The loss of catalytic activity of certain of the C-terminal variants was not due to the inability of these mutants to interact with hTR (data not shown). Thus we identified multiple regions in the hTERT C terminus that are critical to the reconstitution of telomerase activity *in vitro*.

The C-terminal domain of Est2p has been implicated in enzyme processivity (Peng *et al.*, 2001; Hossain *et al.*, 2002). To date, there have been no reports characterizing the role of hTERT in human telomerase processivity. We adapted the non-PCR-based telomerase elongation (also known as a standard, direct or conventional) assay (Sun *et al.*, 1999) to characterize the processivity of human telomerases reconstituted in RRL. Wild-type telomerase reconstituted in RRL extended a telomeric $(T_2AG_3)_4$ oligonucleotide *in vitro*, generating a ladder of primer extension products with the six nucleotide pausing pattern characteristic of human telomerase (Morin, 1989) (Fig. 2.3A, compare lanes labeled hTERT and HL-60). The generation of these products was hTERT and hTR dependent, and RNase and proteinase K sensitive (data not shown). The telomerase activities of all the deletion mutants, including those characterized as inactive by the TRAP technique, were weakly but reproducibly detected using the conventional assay (Fig 2.3A). However, in contrast to the results obtained using the TRAP technique, no deletion mutant reconstituted telomerase activity as efficiently as wild-type hTERT (compare Figs 2.2 and 2.3A; hTERT, Δ1047-1056, Δ1108-1117, Δ1123-1132). In addition, many deletion mutants did not exhibit the six nucleotide pausing pattern characteristic of wild-type human telomerase (Fig. 2.3A). Levels of telomerase activity were similar for wild-type, T1030A and S1037A hTERT mutants, whether detected by

Figure 2.3

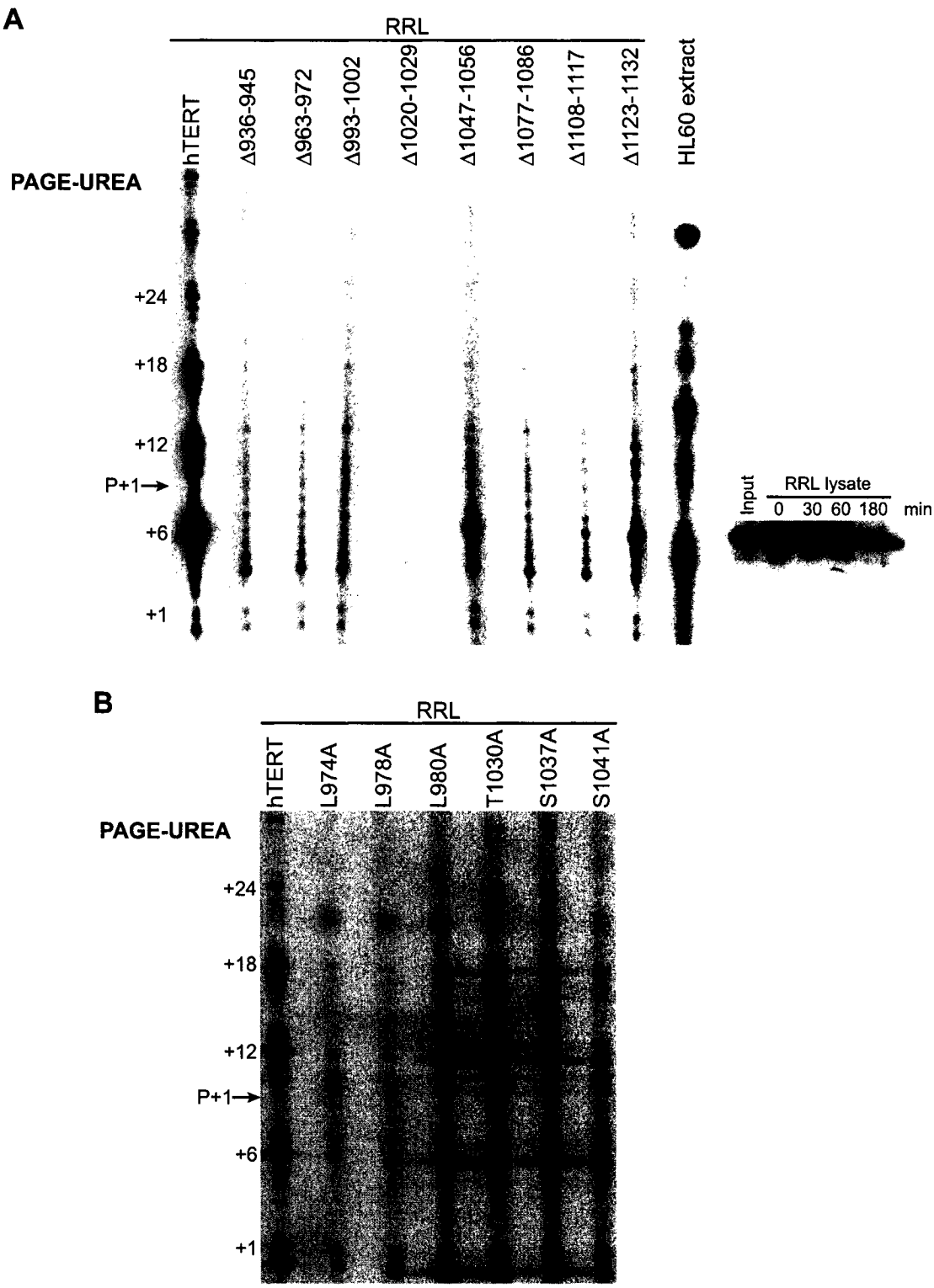

A
RRL
hTERT
Δ936-945
Δ963-972
Δ993-1002
Δ1020-1029
Δ1047-1056
Δ1077-1086
Δ1108-1117
Δ1123-1132
HL60 extract
PAGE-UREA
+24
+18
+12
P+1
+6
+1
Input
RRL lysate
0 30 60 180 min
B
RRL
hTERT
L974A
L978A
L980A
T1030A
S1037A
S1041A
PAGE-UREA
+24
+18
+12
P+1
+6
+1

Figure 2.3 Detection of the catalytic activity of *in vitro*-reconstituted human telomerases using the direct primer extension telomerase assay. The telomerase activity of hTERT C-terminal variants expressed in RRL was detected using a non-PCR-based conventional assay. +6, +12, +18, +24 refer to the first G in the telomeric sequence TTAGGG. The 3'-end labeled biotinylated primer $(T_2AG_3)_4$ migrates as a 25-mer [primer (P)+1], at the indicated position. **(A)** Upper panel: catalytic activity of RRL-expressed C-terminal deletion mutants and wild-type enzyme, and of native telomerase extracted from telomerase-positive HL-60 cells. Lower panel: a standard reaction assay was also performed with an [α-^{32}P]-labeled $(T_2AG_3)_4$ primer added to the RRL for different periods of time. **(B)** Catalytic activity of C-terminal substitution mutants and wild-type enzyme.

the conventional assay or by the TRAP technique (compare Figs 2.2 and 2.3B). However, the L980A and S1041A mutants reconstituted lower levels of activity than wild-type hTERT, whereas the L974A and L978A mutants reconstituted only weak levels of telomerase activity as detected by the conventional assay (Fig. 2.3B). Therefore, the conventional telomerase assay revealed catalytic defects that cannot be detected by the TRAP technique. Our results indicate that most vertebrate-conserved residues in the hTERT C-terminus are required for catalytic activity, and that many of these residues are implicated in regulating overall levels of DNA synthesis.

2.5.3 *Processivity defects of hTERT C-terminal mutants*

Using the conventional telomerase elongation assay, short telomerase elongation products, representing the addition of the first few telomeric repeats onto the DNA substrate were detectable for all mutant telomerases (Fig. 2.3). However, the deletion mutants reconstituted lower levels of activity than wild-type hTERT. The primer extension products generated by wild-type hTERT or C-terminal hTERT variants were quantified to determine if elongation defects were due to reduced enzyme processivity in addition to decreased levels of DNA synthesis. Telomerase processivity refers to the ability of the enzyme to (i) catalyze successive nucleotide additions within a single 6-base repeat (type I) and (ii) copy the RNA template repetitively, resulting in multiple 6-base repeats (type II). Type I processivity of yeast telomerase has been characterized, and is regulated by elements of the Est2p RT-like motifs and the C terminus (Peng *et al.*, 2001). Type II processivity, or repeat addition processivity, is a feature of human and ciliate telomerases which can add hundreds of nucleotides to a DNA substrate *in vitro* (Morin, 1989; Greider, 1991; Hardy *et al.*, 2001). We quantified all elongation products

generated by the reconstituted telomerases including products that migrated below the input primer $(T_2AG_3)_4$. Products that migrate lower than the primer substrate may be generated by the addition of nucleotides onto input primer that has been cleaved by an endonuclease activity such as that previously characterized for ciliate and yeast telomerases (Collins and Greider, 1993; Niu *et al.*, 2000). These lower products are not detected when [α–^{32}P]-labeled primer is added to RRL suggesting that they are not generated by random nucleases in RRL (Fig. 2.3A). +1 and +7 refer to the second G in the human telomeric sequence TTAGGG.

Quantification of the primer extension products (specifically, +6, +12, +18 and +24 telomerase elongation products (where applicable), see Materials and methods) generated by all mutants revealed that deletion of many of the residues between amino acids 963 and 1117 caused defects in type II processivity (Fig. 2.3 and Table I: Δ963-972, Δ1047-1056, Δ1077-1086, Δ1108-1117). The repeat addition processivity of these mutants ranged from 23% to 66% relative to wild-type, and is comparable with the repeat addition processivity of recombinant *Tetrahymena* telomerases reconstituted with mutant telomerase RNA subunits that cause defects in enzyme processivity (18 to 73% relative to wild-type) (Hardy *et al.*, 2001). Therefore, the results of these primer extension assays identify a possible type II processivity modulating region in the C terminus of hTERT.

Analysis of type I processivity revealed that certain small deletions in the hTERT C terminus affected processivity at some positions within the telomeric repeat (Fig. 2.4). Processivity at position +3 was significantly reduced by 18 to 34% for the Δ963-972, Δ993-1002, Δ1047-1056, Δ1077-1086 and Δ1108-1117 mutants compared with wild-type. Processivity was reduced by 25 to 37% at position +4, and by 41 to 53%

Table I. Type II processivity of C-terminal hTERT mutants

Mutant	Average repeat addition processivity	Repeat addition processivity relative to wild-type
hTERT	29.85+/-7.67	100
Δ936-945	33.22+/-8.24	111
Δ963-972	*14.05+/-4.32	47
Δ993-1002	25.19+/-9.99	84
Δ1020-1029	n/a	n/a
Δ1047-1056	6.86	23
Δ1077-1086	19.60+/-9.12	66
Δ1108-1117	*12.81+/-3.93	43
Δ1123-1132	26.71+/-12.54	89
L974A	23.41+/-21.28	78
L978A	23.60+/-8.96	83
L980A	21.65+/-20.74	79
T1030A	25.12+/-7.56	84
S1037A	23.81+/-10.19	79
S1041A	29.94+/-10.11	100

Processivity values were calculated as described in Materials and methods. Type II processivity-defective mutants are indicated by bold typeface. Mutants with average processivity values that are significantly different compared with wild-type values ($p<0.05$) are indicated by *. n/a indicates second-repeat products whose signal is too weak for quantification of type II processivity. Second-repeat products for mutant Δ1047-1056 were quantifiable only once, thus an average processivity value was not calculated, nor could statistical significance be calculated, despite the greatly reduced processivity.

Figure 2.4

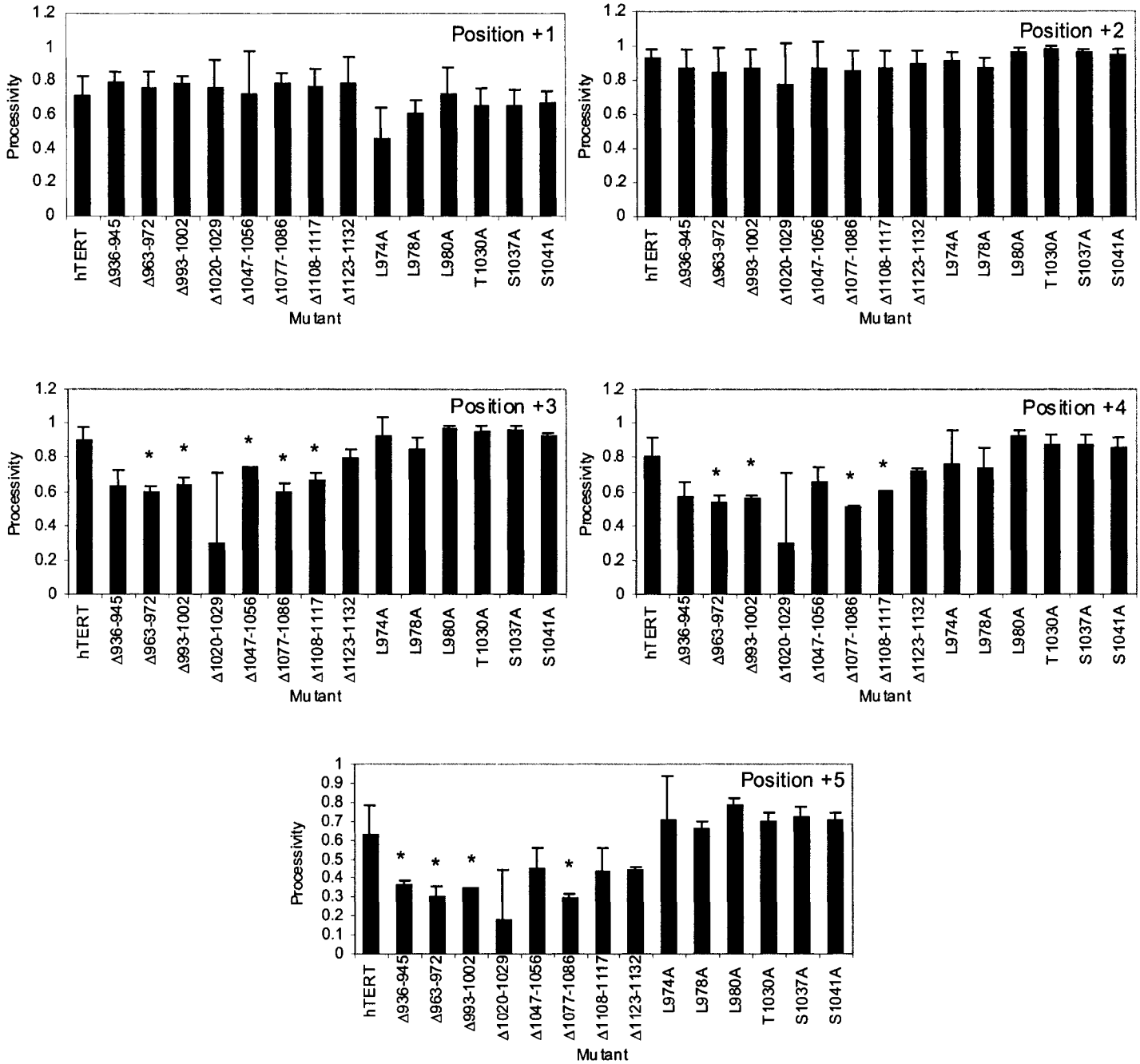

Position +1
Position +2
Position +3
Position +4
Position +5
Processivity
Mutant
hTERT
Δ936-945
Δ963-972
Δ993-1002
Δ1020-1029
Δ1047-1056
Δ1077-1086
Δ1108-1117
Δ1123-1132
L974A
L978A
L980A
T1030A
S1037A
S1041A

Figure 2.4 Average type I processivity values for wild-type and C-terminal mutant telomerases reconstituted in RRL. Processivity values were calculated as described in Materials and methods. Each plot depicts the processivity of wild-type and mutant enzymes at a specific position within the telomerase repeat product. +1 refers to the second G in the telomeric repeat TTAGGG, +2 to the third G, +3 to the first T, +4 to the second T and +5 to the A. Mutants with significantly different processivity values compared to wild-type ($p<0.01$) are indicated by *.

at position +5 for deletion mutants that had statistically significant enzyme processivity defects. The reduction in processivity observed for the C-terminal mutants is similar to the decreases reported for Est2p mutants defective in enzyme processivity (Peng *et al.*, 2001). Deletion of the last 10 C-terminal amino acids, and substitution of residues in the 14-3-3- and CRM1- binding motifs did not affect type I processivity (Fig. 2.4). These results suggest that certain C-terminal regions characterized in this study contribute to both type I and type II enzyme processivity.

2.5.4 *C-terminal mutations do not prevent the physical association of hTERT proteins*

Previous studies indicate that human telomerase is a multimer and that an RT region adjacent to the C terminus of hTERT is implicated in multimerization (Armbruster *et al.*, 2001; Beattie *et al.*, 2001; Wenz *et al.*, 2001; Arai *et al.*, 2002; Moriarty *et al.*, 2002b). We and others have previously demonstrated that the physical association of hTERT molecules is hTR-independent (Armbruster *et al.*, 2001; Arai *et al.*, 2002; Moriarty *et al.*, 2002b). Moreover, N-terminal hTERT mutations, including those that inactivate telomerase and abolish RNA binding, do not prevent the physical association of hTERT proteins (Moriarty *et al.*, 2002b). In an effort to determine the role of the C terminus in multimerization, we mixed immunopurified GST-hTERT protein with partially purified [^{35}S]methionine-labeled hTERT or C-terminal mutants, and immunoprecipitated hTERT/GST-hTERT complexes. Levels of immunoprecipitated GST-hTERT were examined by Western analysis, and coprecipitated [^{35}S]methionine-labeled hTERTs were detected by SDS-PAGE and autoradiography (Fig. 2.5). All C-terminal mutant proteins coprecipitated with GST-hTERT. The association was specific,

Figure 2.5

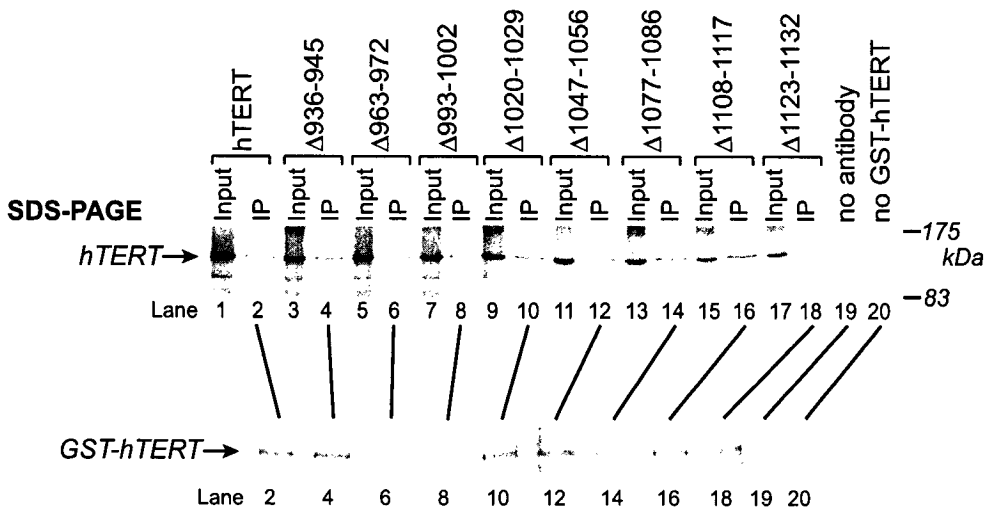

hTERT
Δ936-945
Δ963-972
Δ993-1002
Δ1020-1029
Δ1047-1056
Δ1077-1086
Δ1108-1117
Δ1123-1132
no antibody
no GST-hTERT
Input
IP
SDS-PAGE
hTERT→
175
kDa
83
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
GST-hTERT→
Lane 2 4 6 8 10 12 14 16 18 19 20

Figure 2.5 Physical association of C-terminal hTERT mutants with GST-hTERT *in vitro*. GST-hTERT and C-terminal mutants were independently synthesized in RRL in the absence of hTR. Immunopurified GST-hTERT was mixed with partially purified [^{35}S]methionine-labeled hTERT or C-terminal mutants. Coprecipitated [^{35}S]-labeled hTERT and immunoprecipitated GST-hTERT proteins were detected by SDS-PAGE/autoradiography (top panel) and by Western blot analysis (bottom panel), respectively. 5% of input proteins and 50% of immunoprecipitated proteins were loaded (top panel). Control reactions were performed in the absence of antibody (lane 19) or GST-hTERT (lane 20).

because [^{35}S]-labeled proteins were not precipitated in the absence of GST-hTERT or GST antibody (Fig. 2.5, lanes 19, 20). When compared with wild-type hTERT, none of the mutant proteins exhibited defects in hTERT-hTERT protein interactions (Fig. 2.5). Quantification of GST-hTERT/hTERT interactions indicated that all mutant proteins coprecipitated with GST-hTERT more efficiently than did wild-type hTERT (data not shown). However, only the Δ936-945 mutant exhibited a statistically significant ($p<0.01$) increased interaction with GST-hTERT (approximately 3 fold). In conclusion, none of the C-terminal mutants characterized in this study impaired the physical association of hTERT proteins.

2.5.5 *Inactive C-terminal mutants cannot functionally complement an inactive RT domain mutant*

Previous studies indicate that separately inactive hTERT fragments can functionally multimerize *in vitro* and *in vivo* to reconstitute telomerase activity (Beattie *et al.*, 2000; Beattie *et al.*, 2001; Moriarty *et al.*, 2002b). Though N-terminal mutations do not prevent the physical association of hTERT proteins, some inactive N-terminal mutants fail to complement an inactive RT domain mutant to reconstitute activity (Moriarty *et al.*, 2002b). Therefore, we determined if inactive C-terminal mutants could complement an inactive RT domain mutant. The catalytically inactive point mutant GST-hTERT D868N (Moriarty *et al.*, 2002b) and C-terminal mutants were cosynthesized in RRL in the presence of hTR, and reactions were examined for telomerase activity and the expression of C-terminal mutants and GST-hTERT D868N (Fig. 2.6). Though all C-terminal mutants were expressed equally (Fig. 2.6, bottom panel), none of the inactive C-terminal mutants functionally complemented GST-hTERT D868N to reconstitute

Figure 2.6

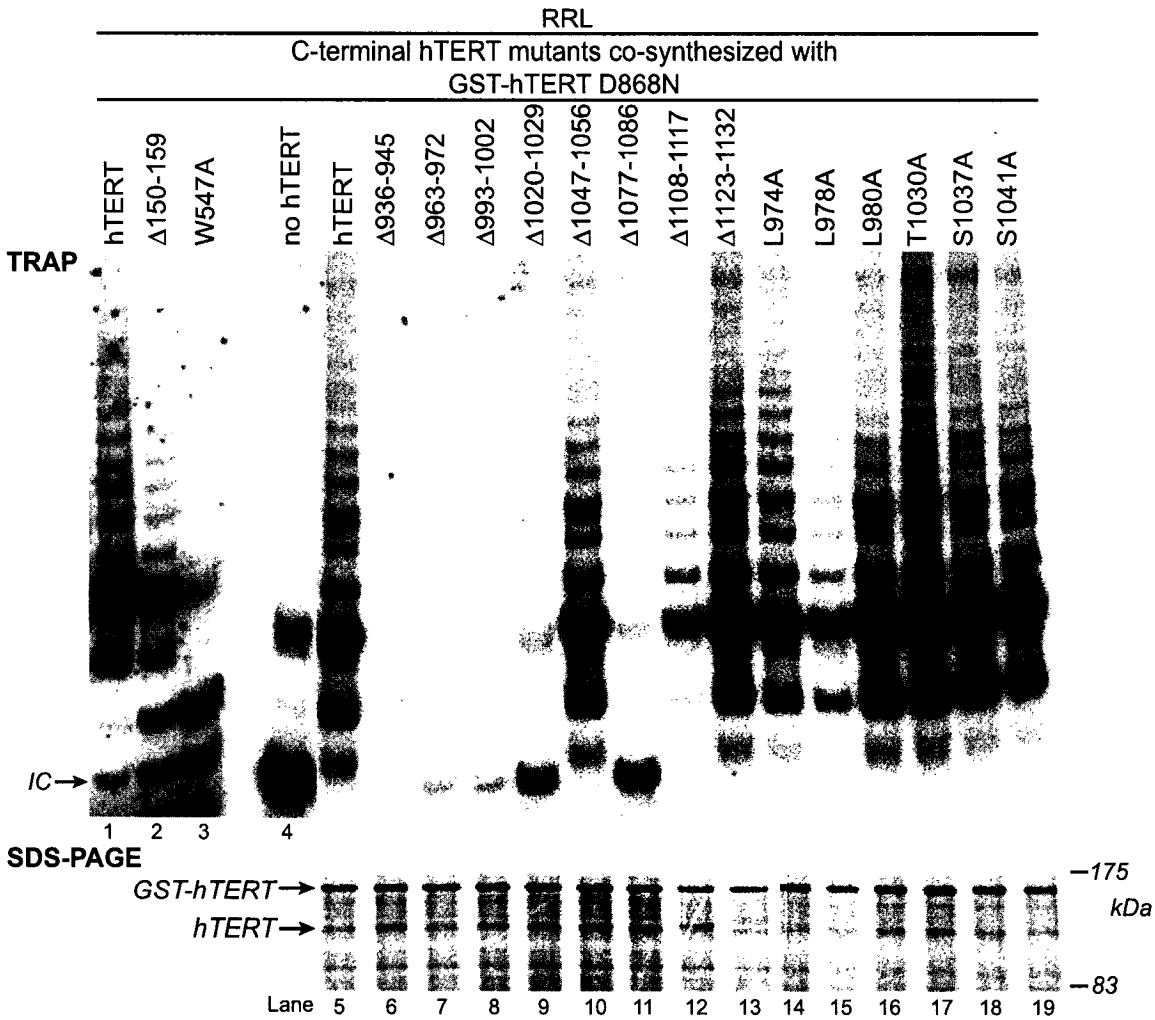

RRL
C-terminal hTERT mutants co-synthesized with
GST-hTERT D868N
hTERT
Δ150-159
W547A
no hTERT
hTERT
Δ936-945
Δ963-972
Δ993-1002
Δ1020-1029
Δ1047-1056
Δ1077-1086
Δ1108-1117
Δ1123-1132
L974A
L978A
L980A
T1030A
S1037A
S1041A
TRAP
IC
1 2 3 4
SDS-PAGE
GST-hTERT
hTERT
175
kDa
83
Lane 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 2.6 Inactive C-terminal mutants cannot functionally complement an inactive RT domain mutant to reconstitute telomerase activity. GST-hTERT D868N and C-terminal mutants of hTERT were cosynthesized in RRL in the presence of hTR and [^{35}S]methionine. Telomerase activity was measured by TRAP assay (top panel). The Δ150-159 mutant (lane 2) is an inactive N-terminal hTERT mutant that functionally complements GST-hTERT D868N to reconstitute telomerase activity (Moriarty *et al.*, 2002b). The W547A mutant is an inactive N-terminal hTERT mutant (lane 3) that does not functionally complement GST-hTERT D868N to reconstitute telomerase activity (Moriarty *et al.*, 2002b). GST-hTERT D868N and hTERT mutants were detected by SDS-PAGE/autoradiography (bottom panel).

telomerase activity (Fig. 2.6, top panel). As previously reported, the inactive N-terminal hTERT mutant Δ150-159 functionally complemented GST-hTERT D868N to reconstitute telomerase activity (Fig. 2.6, lane 2), whereas the inactive N-terminal mutant W547A did not functionally complement GST-hTERT D868N (Moriarty *et al.*, 2002b). Our results indicate that functional complementation requires the presence of intact RT motifs and catalytically essential C-terminal residues on the same hTERT molecule, supporting previously published results derived from the analysis of hTERT C-terminal truncations (Beattie *et al.*, 2001).

2.6 *Discussion*

In vitro and *in vivo* reconstitution of human telomerase has been used to map functions mediated by the N- and C-terminal extensions of hTERT (Beattie *et al.*, 2000; Armbruster *et al.*, 2001; Bachand and Autexier, 2001; Beattie *et al.*, 2001; Arai *et al.*, 2002; Banik *et al.*, 2002; Moriarty *et al.*, 2002b). Our results obtained using the TRAP technique demonstrate an *in vitro* catalytic role for many vertebrate-conserved C-terminal residues. hTERT C-terminal mutations that altered the largest numbers of conserved residues most severely impaired *in vitro* telomerase activity (Figs 2.1 and 2.2). Despite the low degree of C terminus sequence homology among members of the entire TERT family, our data and those derived from previous studies of the C termini of human, *Tetrahymena* and yeast TERTs indicate that the TERT C-terminus is essential for telomerase catalytic activity (Bachand and Autexier, 2001; Beattie *et al.*, 2001; Lai *et al.*, 2001; Peng *et al.*, 2001; Banik *et al.*, 2002).

2.6.1 *Functional complementation requires a hTERT molecule containing both catalytically essential C-terminal residues and intact RT motifs*

Inactive hTERT fragments can functionally complement each other to reconstitute telomerase activity, providing additional evidence that human telomerase exists as a dimer or multimer (Armbruster *et al.*, 2001; Beattie *et al.*, 2001; Wenz *et al.*, 2001; Arai *et al.*, 2002; Moriarty *et al.*, 2002b). The requirements for functional complementation include the association of hTR with only one active subunit of hTERT, intact hTR interaction sites in the N terminus of a hTERT molecule containing functional reverse transcriptase motifs, and catalytically important residues located C-terminal to amino acid 884 (Beattie *et al.*, 2001; Moriarty *et al.*, 2002b). This latter region includes

the residues 914-928 that are implicated in hTERT oligomerization (Arai *et al.*, 2002). A previous study reported that a truncated inactive version of hTERT encoding amino acids 1-884 cannot complement a motif A catalytic point mutant, indicating that an intact C terminus and RT motifs on the same hTERT molecule are required for functional multimerization (Armbruster *et al.*, 2001; Beattie *et al.*, 2001; Arai *et al.*, 2002; Moriarty *et al.*, 2002b). Our study, using hTERT variants containing small internal deletions at the C terminus supports this latter conclusion. Our results also indicate that the catalytic and functional complementation defects of the C-terminal variants were not due to the inability of the mutant hTERTs to associate with hTR (data not shown). Thus, functional complementation requires a hTERT molecule containing catalytically essential C-terminal residues and intact RT motifs.

We also report that all of the hTERT C-terminal mutants can physically associate with wild-type GST-hTERT protein, suggesting that the catalytic and functional complementation defects of the C-terminal variants were not due to inhibition of dimerization or oligomerization. The mechanisms mediating the enhanced interactions of all C-terminal variants with GST-hTERT are unknown; perhaps regions implicated in negatively regulating hTERT association have been disrupted, or the conformation of the mutant proteins have been slightly altered. However, the association of the hTERT variants with hTR suggests that the proteins are not grossly misfolded (data not shown) (Bachand and Autexier, 2001; Beattie *et al.*, 2001). Deletion of hTERT residues 936-945 resulted in a statistically significant ($p<0.01$) increase in hTERT interaction (approximately 3 fold). Arai and coworkers recently determined that amino acids 914-928 are essential for oligomerization (Arai *et al.*, 2002). The proximity of residues 936-

945 and 914-928 suggests that residues 936-945 could be part of a C-terminal region implicated in oligomerization. Thus C-terminal hTERT amino acids required for catalytic activity and functional complementation are not essential for the physical interaction of hTERT with another hTERT molecule or the telomerase RNA component. Recently, Arai and coworkers reported that the C terminus of hTERT can bind the N terminus of hTERT (Arai *et al.*, 2002). As our experiments were performed using wild-type hTERT and C-terminal hTERT mutants, the C terminus of wild-type hTERT may bind the N terminus of the hTERT mutants, possibly masking defects in multimerization normally mediated by C-terminal hTERT residue interactions.

2.6.2 *Role of the hTERT C terminus in DNA synthesis: TRAP versus the non-PCR-based elongation assay*

The results we obtained using the standard telomerase assay varied from those obtained using the TRAP technique in two important aspects. First, the non-PCR-based assay is more sensitive than the TRAP technique for detecting differences in levels of reconstituted telomerase activity or DNA synthesis. Secondly, short telomerase elongation products, representing the addition of the first few telomeric repeats onto the DNA substrate, are detectable by the conventional telomerase assay but not by TRAP.

Using the TRAP technique, the Δ1047-1056, Δ1108-1117, Δ1123-1132, L974A, L978A, L980A and S1041A hTERT mutants reconstituted similar, or slightly reduced levels of activity compared with the levels reconstituted by wild-type hTERT, suggesting that these residues are not essential for catalysis. Our results obtained using the TRAP technique also demonstrate an *in vitro* catalytic role for many vertebrate-conserved C-terminal residues. hTERT C-terminal amino acid residues important for

telomerase activity were recently identified at similar positions using telomerases reconstituted *in vivo* (Banik *et al.*, 2002). Deletion (this study) or substitution (Banik *et al.*, 2002) of hTERT residues at overlapping positions affected levels of telomerase activity similarly, suggesting that deletion of the hTERT residues does not grossly distort the overall structure of the protein.

Using the non-PCR-based telomerase elongation assay, we found that most of the vertebrate-conserved residues mutated in this study were required to reconstitute wild-type levels of *in vitro* catalytic activity (Fig. 2.3). Only the substitution of residues at positions 1030 and 1037, previously determined to be involved in regulatory interactions with 14-3-3 *in vivo*, did not affect levels of reconstituted telomerase activity assayed by the non-PCR-based technique. Interestingly, mutation of residues in the CRM1-interacting site of hTERT caused catalytic defects that were detected by the conventional assay, indicating that conserved residues in this motif may regulate the catalytic function of human telomerase in addition to modulating hTERT localization. Banik and colleages recently reported that substitution of the last six hTERT residues 1127 to 1132, which constitute a biologically essential DAT domain, reduces but does not abolish telomerase activity, as detected by TRAP (Banik *et al.*, 2002). In the present study, deletion of residues 1123-1132, which overlaps with the DAT domain, nearly abolished telomerase activity detected by the conventional assay but did not affect telomerase activity detected by TRAP. These results suggest that catalytic defects could in part mediate the telomere maintenance defects of the DAT mutant (Banik *et al.*, 2002). In fact, a correlation was recently found between telomerase activity and processivity

mediated by the C terminus of *S. cerevisiae* TERT, and telomere length *in vivo* (Peng *et al.*, 2001; Hossain *et al.*, 2002).

In the present study, we also report that mutants that are inactive when assayed by TRAP synthesize low levels of short elongation products detectable using the standard assay. It is likely that these short elongation products cannot be amplified by PCR due to the design of the TS and ACX primers as the shortest products detectable by TRAP are 50 nucleotides in length (Kim and Wu, 1997; Szatmari and Aradi, 2001; Gavory *et al.*, 2002). Thus the standard assay allows the analysis of all elongation products generated by telomerase. Detailed mechanistic studies of *S. cerevisiae* and recombinant *Tetrahymena* telomerases have been possible through the use of direct telomerase assays (Collins and Gandhi, 1998; Hardy *et al.*, 2001; Peng *et al.*, 2001; Hossain *et al.*, 2002). The non-PCR-based telomerase elongation assay that we report will now permit a detailed characterization of the human telomerase mechanism of action using recombinant telomerase reconstituted in RRL.

2.6.3 *The hTERT C terminus is a determinant of human telomerase repeat addition processivity*

Numerous studies have characterized the regulation of catalytic activity and processivity of endogenous telomerases (Greider, 1991; Cohn and Blackburn, 1995; Fulton and Blackburn, 1998; Greene and Shippen, 1998). The processivity of endogenous human telomerase *in vitro* can be modulated by temperature, substrate (primer and dNTP) concentrations, primer sequence and G-quadruplex interacting agents such as potassium ions (Morin, 1989; Maine *et al.*, 1999; Sun *et al.*, 1999). However, few studies have identified determinants within the TERT, telomerase RNA or associated

protein components that regulate telomerase enzyme processivity. Detailed characterization of the telomerase mechanism of action has previously been most commonly reported using a non-PCR-based elongation assay, and this assay is the method of choice for mechanistic analyses of telomerase. Recently a modified TRAP assay has also been reported for the analysis of processivity (specifically the generation of 9-10 repeats) and minimal template RNA length requirements of the human telomerase enzyme (Szatmari and Aradi, 2001; Gavory *et al.*, 2002).

Studies of *Euplotes crassus* telomerase indicate that the developmentally regulated association of proteins with telomerase can influence repeat addition processivity (Bednenko *et al.*, 1997; Greene and Shippen, 1998). *Tetrahymena* telomerase enzymes reconstituted *in vitro or in vivo* with mutant telomerase RNA subunits have type II processivity defects, implicating the RNA component in the regulation of enzyme processivity (Autexier and Greider, 1994; Autexier and Greider, 1995; Ware *et al.*, 2000). Hardy *et al.* (2001) also reported the decreased repeat addition processivity of recombinant *Tetrahymena* telomerases reconstituted in RRL with mutant telomerase RNA subunits (Hardy *et al.*, 2001). Recently, nucleotide determinants adjacent to the template region of human and mouse telomerase RNAs were identified as regulators of enzyme processivity (Chen and Greider, 2003).

However, to date there has been only one report characterizing the role of TERT in type II enzyme processivity. Bryan *et al.* (2000) identified a leucine at position 813 in motif C of *Tetrahymena* telomerase that affected the intrinsic processivity of the enzyme (Bryan *et al.*, 2000). Interestingly, in all retroviral RTs the residue at this position is a tyrosine that is critical for catalytic activity. Substitution of the *Tetrahymena*

telomerase leucine at position 813 to tyrosine resulted in increased repeat addition processivity (type II), creating an enzyme that resembles the highly processive classical RTs (Bryan *et al.*, 2000). Several of the C-terminal hTERT mutants that we characterized in the current study exhibited type II processivity defects, suggesting that a determinant of human telomerase repeat addition processivity resides in the hTERT C terminus, perhaps between residues 963 and 1117. The mechanism mediating defects in processivity remains to be determined. Defects in processivity could result from alignment of substrate at alternate template positions, defects in primer or dNTP binding or defects in primer extension or translocation. Human telomerase repeat addition processivity, like that of *Tetrahymena* telomerase, may also be mediated by other regions of hTERT, including the conserved RT motifs that are implicated in retroviral RT processivity.

2.6.4 *Type I processivity: Functional conservation between the C-terminal domains of human and yeast telomerase reverse transcriptase subunits*

Processivity defects that map to the *S. cerevisiae* TERT C terminus have previously been characterized (Peng *et al.*, 2001; Hossain *et al.*, 2002). C-terminal Est2p deletions affect type I processivity, and this region may be functionally analogous to the C-terminal extension of the HIV-1 RT (Peng *et al.*, 2001). Interestingly, the deletion of the Est2p C terminus does not completely abolish telomerase activity as measured in a direct telomerase assay, though levels of DNA synthesis are reduced (Peng *et al.*, 2001). Similar to the Est2p C-terminal mutants, many C-terminal hTERT mutants that we characterized in the current study exhibited significant type I processivity and overall DNA synthesis defects. Thus, despite weak conservation of C-terminal TERT sequences

from vertebrates and yeast, the identification of C-terminal hTERT residues involved in modulating type I enzyme processivity suggests a functional conservation between the C-terminal domains of human and yeast telomerases. The mechanistic basis of this functional similarity will require careful characterization. Our results indicate that the hTERT C terminus plays essential roles in several human telomerase functions: catalytic activity, functional complementation with other hTERT molecules and enzyme processivity.

2.7 *Acknowledgements*

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CHAPTER 3

Human Telomerase Enzyme catalyzes Nucleolytic Primer Cleavage

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3.1 *Preface*

In the previous chapter, extensive effort has been accomplished to understand the function of the C-terminal region of hTERT. We clearly demonstrated that the C-terminal region is essential for enzyme catalysis, nucleotide addition and processive DNA repeat addition. In addition to a processive DNA synthesis performed by the telomerase enzyme, previous studies have identified a telomerase-dependent nucleolytic cleavage activity in ciliates and yeasts. The *in vivo* functions of this telomerase-mediated DNA substrate cleavage are still unknown, but similar mechanisms may regulate both processivity and cleavage. We investigated the possibility that the vertebrate human telomerase enzyme catalyzes a nucleolytic primer cleavage. This study was accomplished by monitoring DNA synthesis and DNA cleavage with a direct primer extension assay performed with RRL-reconstituted human telomerase and partially purified telomerase from human cells.

3.2 *Abstract*

Telomerase is a reverse transcriptase that uses an integral RNA molecule to add *de novo* G-rich repeats onto telomeric DNA, or onto nontelomeric DNA generated during chromosome fragmentation and breakage events. A telomerase-mediated DNA substrate cleavage activity has been reported in ciliates and yeasts. Nucleolytic cleavage may serve a proofreading function, enhance processivity or ensure that nontemplate telomerase RNA sequences are not copied into DNA. We identified and characterized a human telomerase-mediated nucleolytic cleavage activity using enzyme reconstituted in a rabbit reticulocyte lysate *in vitro* transcription/translation system and native enzyme extracted from cells. We found that telomerase catalyzed the removal of nucleotides from DNA substrates including those that can form a mismatch with the RNA template or that contain nontelomeric sequences located 3' to a telomeric sequence. Unlike *Tetrahymena* telomerase, human telomerase catalyzed the removal of more than one nucleotide (up to 13) from telomeric primers. DNA substrates predicted to align at the 3'-end of the RNA template were not cleaved, consistent with cleavage being dictated by the template 5'-end. We also found some differences in the nuclease activity between RRL-reconstituted human telomerase and native enzyme.

3.3 *Introduction*

Telomeres form a nucleoprotein complex at the ends of linear eukaryotic chromosomes that serves as a protective cap conferring chromosome stability (reviewed in Blackburn, 2001). Telomeric DNA is composed of G-rich repeated sequences (TTAGGG in vertebrates) and is a substrate for the ribonucleoprotein (RNP) enzyme telomerase. Telomerase mediates *de novo* addition of telomeric TTAGGG DNA repeats onto the 3'-ends of chromosomes allowing the maintenance of telomere length and the long-term proliferation of eukaryotic cells (reviewed in Harrington and Robinson, 2002; reviewed in Harrington, 2003). In addition to elongating pre-existing telomere tracts, telomerase can also catalyze the synthesis of telomeric DNA onto nontelomeric DNA sequences generated by chromosome fragmentation and breakage events (Harrington and Greider, 1991; Melek *et al.*, 1996; Bednenko *et al.*, 1997; Wang and Blackburn, 1997).

The human telomerase enzyme is minimally composed of a protein catalytic subunit, the telomerase reverse transcriptase (hTERT), and the telomerase RNA (hTR) that is used as a template for telomeric DNA synthesis (Feng *et al.*, 1995). The expression of hTERT and hTR in a rabbit reticulocyte lysate (RRL) *in vitro* transcription/translation system is sufficient to reconstitute enzyme activity (Weinrich *et al.*, 1997; Beattie *et al.*, 1998). hTR contains a short template sequence of 11 nucleotides complementary to the telomeric repeats (Feng *et al.*, 1995; Chen *et al.*, 2000). Telomerase elongation and substrate specificity have been characterized *in vitro* for a wide range of organisms including protozoa, yeast and human (Morin, 1989; Harrington and Greider, 1991; Lue and Peng, 1998; Sun *et al.*, 1999; Baran *et al.*, 2002; Wallweber *et al.*, 2003). Short single-stranded oligonucleotides containing bases complementary to

the template region can serve as telomerase substrates in a DNA primer extension (conventional) assay (Sun *et al.*, 1999; Huard *et al.*, 2003). During telomere repeat synthesis, the 3'-end of the DNA substrate anneals to the telomerase RNA template. Nucleotides are added to the DNA until the 5' terminus of the template sequence is reached; subsequently, the newly synthesized 3'-end of the substrate is repositioned at the beginning (3' terminus) of the template (reviewed in Greider, 1996). Repeat addition processivity, defined as the successive rounds of nucleotide addition and primer translocation, is a key feature of most telomerase enzymes. The 5'-end of the substrate is also thought to bind an anchor site contributing to telomerase processivity (Morin, 1989; Greider, 1991; Harrington and Greider, 1991; Collins and Greider, 1993; Hammond *et al.*, 1997; Lue and Peng, 1998; Wallweber *et al.*, 2003).

Like a number of DNA and RNA polymerases, telomerase exhibits a DNA substrate cleavage activity in addition to its polymerase function (reviewed in Shevelev and Hübscher, 2002; reviewed in Arndt and Kane, 2003). In *Euplotes crassus*, *Tetrahymena thermophila*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, telomerase cleaves DNA substrates bound via the template sequence of the telomerase RNA (Collins and Greider, 1993; Cohn and Blackburn, 1995; Melek *et al.*, 1996; Lue and Peng, 1997; Niu *et al.*, 2000) and the cleavage can occur by an endonucleolytic mechanism (Melek *et al.*, 1996; Niu *et al.*, 2000). There is some evidence that this cleavage activity is inherent to telomerase. Nuclease activity remains associated with the telomerase complex after extensive purification (Greene *et al.*, 1998). Cleavage patterns are also altered in specific ways when the telomerase enzyme contains a telomerase RNA mutated in the template sequence (Ware *et al.*, 2000). RNA sequences outside the

template can also influence telomerase cleavage activity (Bhattacharyya and Blackburn, 1997). Moreover, expression of *Tetrahymena* TERT and TR in RRL is sufficient to reconstitute cleavage activity (Collins and Gandhi, 1998). Several primers have been tested and reported to be substrates for cleavage. Primers containing bases that can form mismatches with the RNA template sequence are cleaved; cleavage occurs preferentially at the junction of match-mismatch between the primer and the template (Collins and Greider, 1993; Melek *et al.*, 1996; Greene *et al.*, 1998; Niu *et al.*, 2000). In *E. crassus*, telomerase can also remove 3'-terminal nontelomeric DNA from a primer to expose telomeric sequences for elongation (Melek *et al.*, 1996; Greene *et al.*, 1998).

The nucleolytic cleavage activity of vertebrate telomerase has not been characterized. In this study, we report the characterization of a human telomerase-mediated nucleolytic cleavage activity using enzyme reconstituted in RRL and native enzyme extracted from cells. We demonstrated that telomerase catalyzes the removal of 1 to 13 nucleotides from telomeric substrates that can align at, or beyond, the 5'-end of the RNA template sequence, that can form a mismatch with the RNA template, or that contain nontelomeric sequences located 3' to a telomeric sequence. DNA substrates predicted to align at the 3'-end of the RNA template are not cleaved, consistent with cleavage being dictated by the template 5'-end. We also found some differences in the nuclease activity between RRL-reconstituted human telomerase and native enzyme partially purified from 293 cells.

3.4 *Materials and methods*

3.4.1 *Oligonucleotides*

Gel-purified 5'-biotinylated oligonucleotides were obtained from Operon (Alameda, CA) and resuspended in water. To generate the radiolabeled size markers carrying a 3'-terminal dideoxynucleotide, 5'-biotinylated oligonucleotides were 3'-end labeled using terminal deoxynucleotidyltransferase (Invitrogen) and [α-^{32}P]dATP (cordycepin: NEN Life Science Products) for 30 min at 37°C, followed by DNA precipitation.

3.4.2 *Plasmid constructions*

The construction of the pET28b-hTERT and phTR+1 expression plasmids has been described previously (Autexier *et al.*, 1996; Bachand and Autexier, 1999).

3.4.3 *In vitro transcription/translation*

The rabbit reticulocyte lysate (RRL) *in vitro* T7-coupled transcription/translation system (Promega) was used as described previously (Moriarty *et al.*, 2002; Huard *et al.*, 2003). Full length hTERT was synthesized in RRL in the presence of purified hTR. hTR was synthesized and purified on RNeasy spin columns (QIAGEN) from *Fsp*I-linearized phTR+1 plasmid (Moriarty *et al.*, 2002).

3.4.4 *DNA primer extension assay*

A non-PCR-based telomerase elongation assay was performed (Sun *et al.*, 1999; Huard *et al.*, 2003). hTERT protein expressed in 20 µl RRL in the presence of hTR was assayed for telomerase activity in a 40 µl final volume reaction using a gel-purified 5'-biotinylated oligonucleotide (Operon). Standard reaction conditions have

been described previously (Huard *et al.*, 2003). The proteinase K solution was 10 mM Tris-HCl pH 7.5, 0.5% SDS, 0.3 mg/ml proteinase K. The elongation products immobilized on magnetic beads were washed twice with buffer A (10 mM Tris-HCl pH 7.5, 1 M NaCl, 0.5 mM EDTA), once with buffer B (10 mM Tris-HCl pH 7.5) and analyzed by 8% or 10% polyacrylamide-urea gel electrophoresis. The relative amount of cleavage-derived products, expressed by the ratio of cleavage products/elongation products, was determined by densitometric analysis of the autoradiographs (ImageQuant software, Molecular Dynamics). The total counts for each signal were normalized to the amount of transcripts by dividing each product signal by the number of dGTPs it contains. The ratio was calculated using the normalized signals of the major elongation products above the input primer and the major cleavage products below the input primer.

3.4.5 *Human telomerase extracts*

The preparation of partially purified 293 cell extracts has been described previously (Autexier *et al.*, 1996). DNA primer extension assays were performed with 20 μg of this cellular extract as described above.

3.5 *Results*

3.5.1 *3'-end cleavage of telomeric DNA substrates*

Nucleolytic cleavage of DNA primer substrates can be reconstituted by expressing *Tetrahymena* TERT in the presence of TR in a RRL *in vitro* transcription/translation system, and detected using a DNA primer extension (conventional) assay (Collins and Gandhi, 1998). This result suggests that the DNA cleavage may be dependent on the minimal components required for telomerase activity, TERT and TR. To identify and characterize a human telomerase-mediated nucleolytic cleavage activity, we first reconstituted human telomerase in RRL by expressing hTERT in the presence of hTR, and performed a DNA primer extension assay using different 5'-biotinylated telomeric oligonucleotides (Fig. 3.1C). As previously reported, RRL-reconstituted human telomerase assayed by the primer extension method generates a ladder of elongation products with a six nucleotide pausing pattern (Fig. 3.1C, lanes 1, 2) (Chen and Greider, 2003; Huard *et al.*, 2003). Processive elongation of DNA primers by the telomerase enzyme is observed as the addition of multiple telomeric repeats (Fig. 3.1C, lanes 1, 2).

The *Tetrahymena* and *Euplotes* telomerase enzymes generate products that are shorter than the original oligonucleotide substrates, detectable as radiolabeled products that migrate below the input primers (Collins and Greider, 1993; Melek *et al.*, 1996). We first assayed telomeric oligonucleotide substrates [$(T_2AG_3)_3$ and $(T_2AG_3)_4$], and two others primers [$(G_2T_2AG)_4$ and $(G_2T_2AG)_3G_2T_2A_2G$] that mimic oligonucleotides cleaved by *Tetrahymena* telomerase (Fig. 3.1D). $(G_2T_2AG)_3G_2T_2A_2G$ is predicted to form a mismatch with the RNA template (Fig. 3.1B). Under standard

Figure 3.1

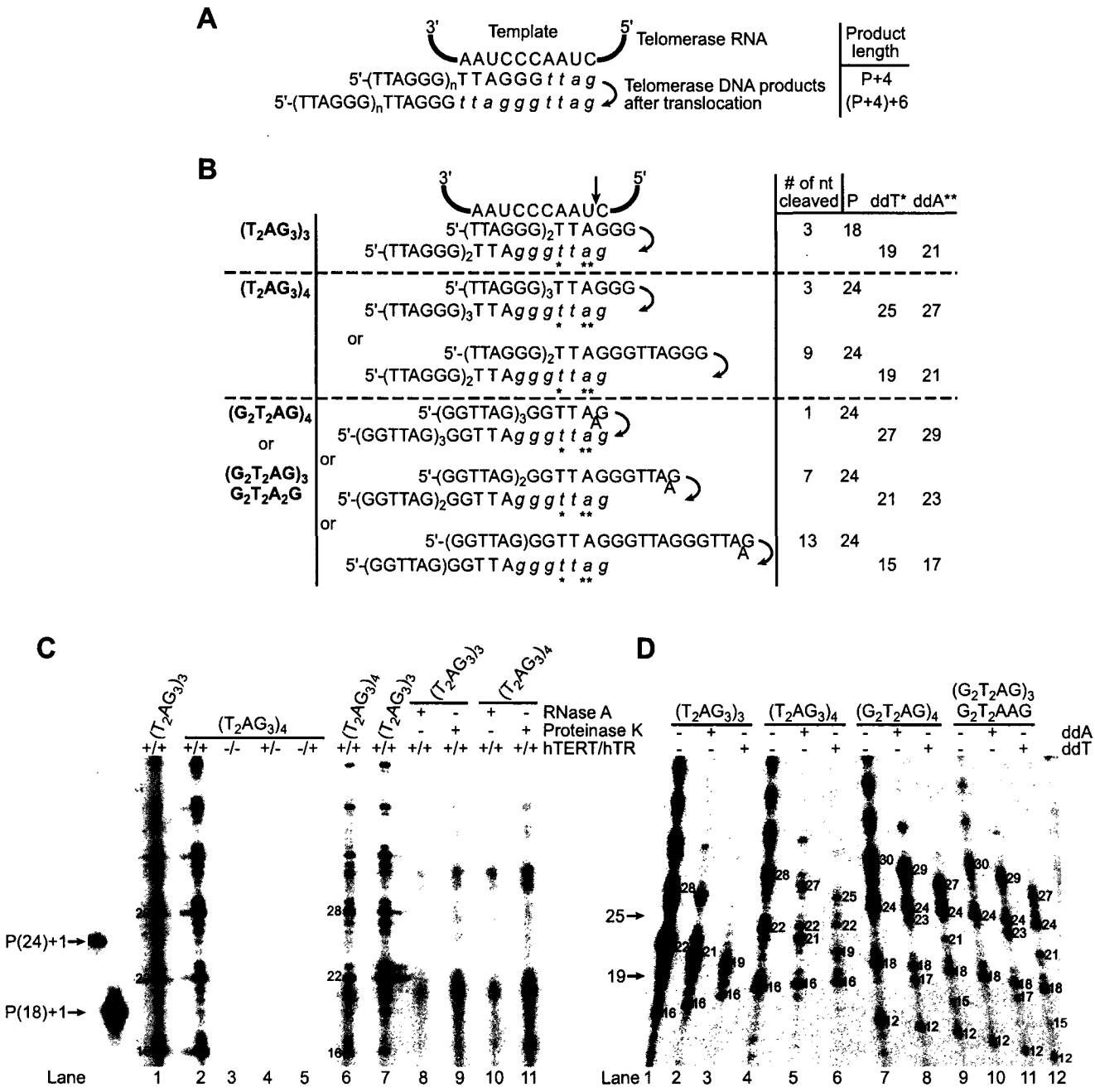
A
3'
Template
5'
Telomerase RNA
AAUCCCAAUC
5'-(TTAGGG)nTTAGGGttag
5'-(TTAGGG)nTTAGGGttagggttag
Telomerase DNA products after translocation
Product length
P+4
(P+4)+6
B
# of nt cleaved
P
ddT*
ddA**
(T2AG3)3
5'-(TTAGGG)2TTAGGG
5'-(TTAGGG)2TTAgggttag
3
18
19
21
(T2AG3)4
5'-(TTAGGG)3TTAGGG
5'-(TTAGGG)3TTAgggttag
3
24
25
27
or
5'-(TTAGGG)2TTAGGGTTAGGG
5'-(TTAGGG)2TTAgggttag
9
24
19
21
(G2T2AG)4
or
(G2T2AG)3G2T2A2G
5'-(GGTTAG)3GGTTAG
5'-(GGTTAG)3GGTTAgggttag
1
24
27
29
or
5'-(GGTTAG)2GGTTAGGGTTAG
5'-(GGTTAG)2GGTTAgggttag
7
24
21
23
or
5'-(GGTTAG)GGTTAGGGTTAGGGTTAG
5'-(GGTTAG)GGTTAgggttag
13
24
15
17
C
(T2AG3)3
(T2AG3)4
(T2AG3)4
(T2AG3)3
(T2AG3)3
(T2AG3)4
RNase A
Proteinase K
hTERT/hTR
+/+
+/+ -/- +/- -/+
+/+ +/+ +/+ +/+ +/+ +/+
P(24)+1
P(18)+1
Lane 1 2 3 4 5 6 7 8 9 10 11
D
(T2AG3)3
(T2AG3)4
(G2T2AG)4
(G2T2AG)3 G2T2AAG
ddA
ddT
25
19
Lane 1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.1 Cleavage of telomeric 3'-ends. (**A** and **B**) Potential alignments of various telomeric oligonucleotides (tested in **C** and **D**) with the hTR template, before and after translocation. Compared to $(G_2T_2AG)_4$, $(G_2T_2AG)_3G_2T_2A_2G$ has an extra A predicted to form a mismatch with the RNA template. Italicized letters represent nucleotides added by the telomerase enzyme onto the 3'-end of the primer during the elongation step. The diagram implies that cleavage and nucleotide addition to the RNA template 5'-end precede translocation. P: primer length. ddA and ddT: dideoxyATP and dideoxyTTP. Numbers in A-D represent the lengths of the primers, elongation or chain termination products. The proposed cleavage site is shown by the vertical arrow. Asterisks indicate the positions of the first ddA or ddT incorporated during the DNA primer extension assay. (**C** and **D**) DNA primer extension assays were performed with the indicated oligonucleotides using RRL-reconstituted telomerase enzyme. The elongation products were resolved on 8% polyacrylamide-urea gels and subjected to autoradiography. The 3'-end radiolabeled 5'-biotinylated primers, $(T_2AG_3)_3$ and $(T_2AG_3)_4$, migrate respectively as 19 and 25 mers [P(18)+1 and P(24)+1 respectively].

reaction conditions, we observed radiolabeled primer-sized products or products below the input primers (Fig. 3.1C, lanes 1, 2, 6, 7 and Fig. 3.1D, lanes 1, 4, 7, 10). This product profile results from the removal of one or several terminal primer nucleotides followed by incorporation of [α-^{32}P]dGTP during elongation by the telomerase enzyme. These short products were not visualized when 5'-end radiolabeled $(T_2AG_3)_3$ or 3'-end radiolabeled 5'-biotinylated $(T_2AG_3)_3$ primers were added to RRL reactions in the absence of human telomerase components, even in the presence of dNTPs (data not shown) (Huard *et al.*, 2003). These radiolabeled primers were neither cleaved nor elongated. These observations suggest that short products are not generated by random nucleases that might be present in the lysate, and are consistent with cleavage being a telomerase-mediated process. Both telomerase elongation activity and the generation of products shorter than the input primer were hTERT and hTR dependent (Fig. 3.1C, lanes 3-5), and RNase A and proteinase K sensitive (Fig. 3.1C, lanes 8-11).

The first major product generated by telomerase-mediated elongation of $(T_2AG_3)_4$ is expected to be 28 nucleotides (nt) in length [primer(P)+4] (Fig. 3.1A). This product results from enzyme pausing or dissociation upon reaching the 5'-end of the RNA template (Fig. 3.1A and Fig. 3.1D, lane 4). Interestingly, telomerase reactions with this primer also led to the formation of cleavage-derived products 16 and 22 nt in length due to the removal of 9 and 3 nt respectively (Fig. 3.1B and Fig. 3.1D, lane 4). The length of shorter products suggested that cleavage occurred 3' to the A residue within the primer (Fig. 3.1B). To characterize the short products and identify the position of the cleavage within $(T_2AG_3)_4$, DNA primer extension reactions were performed in the presence of the chain terminators ddATP or ddTTP. Substitutions of dATP with ddATP or TTP with

ddTTP in the conventional assays generated specific chain termination products of different sizes (Fig. 3.1B and Fig. 3.1D, lanes 5, 6). In the absence of cleavage, chain termination products would be undetectable since the incorporation of ddATP or ddTTP prevents the incorporation of [α-^{32}P]dGTP (Fig. 3.1A). Therefore the chain termination products we detected were generated by cleavage followed by subsequent addition of radiolabeled dGTP and ddATP or ddTTP onto the primer (Fig. 3.1B). These reactions also generated cleavage-derived products 16 and 22 nt in length resulting only from the incorporation of [α-^{32}P]dGTP (Fig. 3.1D, lanes 5, 6). The results of this experiment indicated that 3 or 9 nt could be cleaved from the 3'-end of $(T_2AG_3)_4$ depending on the potential alignments of this oligonucleotide with the RNA template, including alignment of the primer beyond the RNA template 5'-end (Fig. 3.1B).

Primer extension reactions were also performed with $(T_2AG_3)_3$, $(G_2T_2AG)_4$ or $(G_2T_2AG)_3G_2T_2A_2G$ (Fig. 3.1D, lanes 1-3, 7-12). Reactions conducted with $(T_2AG_3)_3$ generated cleavage-derived products 16 nt in length consistent with the removal of 3 nt from the 3'-end of the primer (Fig. 3.1B and Fig. 3.1D, lane 1). Substitutions of dATP with ddATP or TTP with ddTTP generated chain termination products of 21 or 19 nt, respectively, consistent with the incorporation of [α-^{32}P]dGTP subsequent to cleavage and prior to chain termination (Fig. 3.1B and Fig. 3.1D, lanes 2, 3). These reactions also generated cleavage-derived products 16 in length resulting only from the incorporation of radiolabeled dGTP (Fig. 3.1D, lanes 2, 3). Reactions with $(G_2T_2AG)_4$ or $(G_2T_2AG)_3G_2T_2A_2G$ (which forms a mismatch with the RNA template) generated cleavage-derived products 24 (primer-size), 18 and 12 nt in length (Fig. 3.1D, lanes 7, 10), consistent with the cleavage of 1, 7 or 13 nt, depending on the primer alignments

with the RNA template (Fig. 3.1B). Substitutions of dATP with ddATP or TTP with ddTTP in the telomerase assays using $(G_2T_2AG)_4$ or $(G_2T_2AG)_3G_2T_2A_2G$ as substrates generated chain termination products of 29, 23 and 17 nt (with ddATP: Fig. 3.1D, lanes 8, 11) or 27, 21 and 15 nt (with ddTTP: Fig. 3.1D, lanes 9, 12), as well as cleavage-derived products 24, 18 and 12 nt in length resulting only from the incorporation of [α-^{32}P]dGTP (Fig. 3.1D, lanes 8, 9, 11, 12). The length of cleavage-derived and chain termination products suggests that cleavage occurred 3' to the TTA residues within the telomeric sequence primers (Fig. 3.1B).

3.5.2 *Effects of different reaction conditions on telomerase-mediated primer cleavage*

Previous studies in *Tetrahymena*, *Euplotes*, yeast and human have shown that an increase in either dGTP or primer concentration in the telomerase assay affects the nucleolytic primer cleavage activity as well as the processivity of the telomerase enzyme (Hammond *et al.*, 1997; Collins and Gandhi, 1998; Sun *et al.*, 1999; Niu *et al.*, 2000; Hardy *et al.*, 2001). To determine whether the extent of cleavage is affected by these reaction conditions, we varied either the concentration of dGTP or primer in the telomerase reactions using RRL-reconstituted human telomerase. Unlabeled dGTP was used to increase the total concentration of dGTP from 1.25 μM to 13.75 μM, which did not affect the relative amount of cleavage-derived products, but promoted the generation of longer products, consistent with an increase in enzyme processivity (Fig. 3.2A). A similar increase in processivity also occurred by varying the $(T_2AG_3)_4$ concentration from 0.25 μM to 5 μM, but the relative amount of cleavage-derived products was also not affected (data not shown).

Figure 3.2

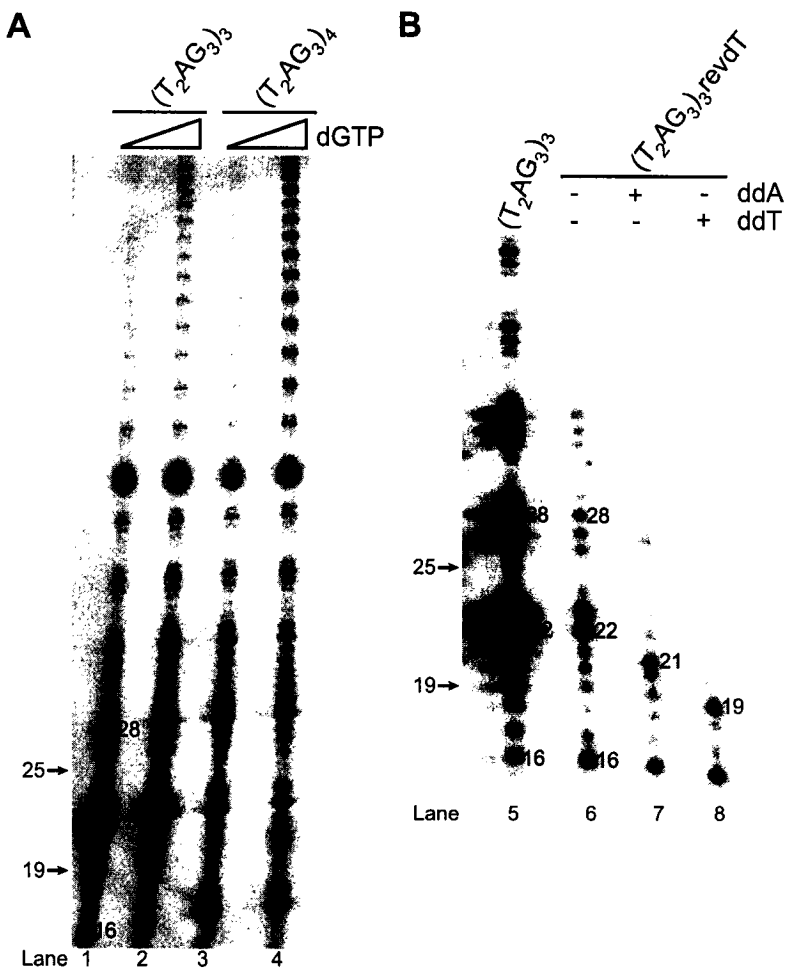
A
$(T_2AG_3)_3$
$(T_2AG_3)_4$
dGTP
25
19
16
Lane 1 2 3 4
B
$(T_2AG_3)_3$
$(T_2AG_3)_3$revdT
ddA
ddT
25
19
28
22
21
19
16
Lane 5 6 7 8

Figure 3.2 Effects of different parameters on telomerase-mediated primer cleavage. (**A**) Effects of dGTP concentration on telomerase-mediated cleavage activity. DNA primer extension assays were conducted using different concentrations of dGTP. In addition to 1.25 μM [α-^{32}P]dGTP, unlabeled dGTP was added. Lanes 1 and 3 contain no unlabeled dGTP. Lanes 2 and 4 contain 12.5 μM of unlabeled dGTP. (**B**) Cleavage of a telomeric primer containing a chain terminator (revdT) at the 3'-end. Direct DNA primer extension assays were performed with the indicated oligonucleotides. The 3'-end radiolabeled 5'-biotinylated primers, $(T_2AG_3)_3$ and $(T_2AG_3)_4$, migrate respectively as 19 and 25 mers at the positions indicated by the arrows. ddA and ddT: dideoxyATP and dideoxyTTP. dGTP: deoxyGTP. Numbers in (**A**) and (**B**) represent the lengths of the elongation or chain termination products.

We speculated that cleavage could be due to pyrophosphorolysis. This reaction can occur when the base at the 3' terminus of a DNA chain reacts with pyrophosphate ions in solution to generate a free dNTP molecule and a DNA chain that is one base shorter. As we observed the removal of 3 or 9 nt from $(T_2AG_3)_4$, the cleavage activity we have described is not likely to be due to pyrophosphorolysis. Moreover, the addition of 5 units of pyrophosphatase, an enzyme used to inhibit pyrophosphorolysis, did not abolish the cleavage of $(T_2AG_3)_3$ (data not shown).

To further characterize the cleavage reaction, we assayed a telomeric oligonucleotide substrate containing a modification that prevents chain extension [$(T_2AG_3)_3$revdT]. This oligonucleotide can only be elongated by the telomerase enzyme if the chain terminating modification is removed. Primer extension assays performed with $(T_2AG_3)_3$revdT in the presence of either dNTPs or ddNTPs generated cleavage-derived products 16 nt in length, consistent with the removal of 3 nt prior to elongation (Fig. 3.2B, lanes 6-8).

3.5.3 *Telomerase-mediated primer cleavage is specified by primer alignments with the telomerase RNA template sequence*

In *Tetrahymena* and *Euplotes*, primer-RNA template interactions regulate telomerase-mediated nucleolytic cleavage activity (Collins and Greider, 1993; Melek *et al.*, 1996; Greene *et al.*, 1998). In general, mismatches between the primer and the RNA template sequence promote primer cleavage at the junction of match-mismatch. As previously described in figure 3.1, cleavage of $(G_2T_2AG)_3G_2T_2A_2G$, a primer which can form a mismatch with the RNA template, produced products 24 nt in length. Such products would be generated by the removal of the last two 3' nucleotides prior to the

incorporation of [α-^{32}P]dGTP during DNA synthesis (Fig. 3.1D, lane 10). Moreover, certain primers containing nontelomeric sequences that are predicted to form mismatches with the RNA template are also good cleavage and subsequent elongation substrates for *Euplotes* telomerase (Melek *et al.*, 1996; Greene *et al.*, 1998). These primers consist of an internal telomeric cassette flanked by nontelomeric DNA sequences. *Euplotes* telomerase can remove the 3' nontelomeric sequences to expose the telomeric sequences for elongation. RRL-reconstituted human telomerase was assayed using primers [CACTATCGAC-**G_3T_2A**-CAT] and [CACTATC-**G_3T_2A**-GATCAT] abbreviated as (10-**G_3T_2A**-3) and (7-**G_3T_2A**-6) respectively (the telomeric cassettes are indicated in bold). These primers are predicted to align with the RNA template as indicated in figure 3.3A. Telomerase reactions performed with (10-**G_3T_2A**-3) or (7-**G_3T_2A**-6) led to the formation of products that were shorter than the input primer at 17 and 14 nt respectively (Fig. 3.3B, lanes 2, 3). Product size was consistent with primer cleavage occurring 3' to the telomeric cassette to remove the 3' nontelomeric sequences (3 or 6 nt). Substitutions of dATP with ddATP or TTP with ddTTP in telomerase assays confirmed that 3 or 6 nt were removed, eliminating the nontelomeric DNA before the initiation of DNA synthesis (Fig. 3.3A and data not shown).

To determine whether nucleolytic cleavage activity is dictated by the alignment of the primer substrate at the 5'-end of the RNA template, we tested an oligonucleotide [$(TG)_8$TAG] that mimics a primer predicted to align at the 3'-end of the *Tetrahymena* telomerase RNA template (Fig. 3.3A) (Collins and Greider, 1993). Primer extension reactions with RRL-reconstituted human telomerase did not generate primer-sized products (19 nt) or products below the input primer, but favoured the direct

Figure 3.3

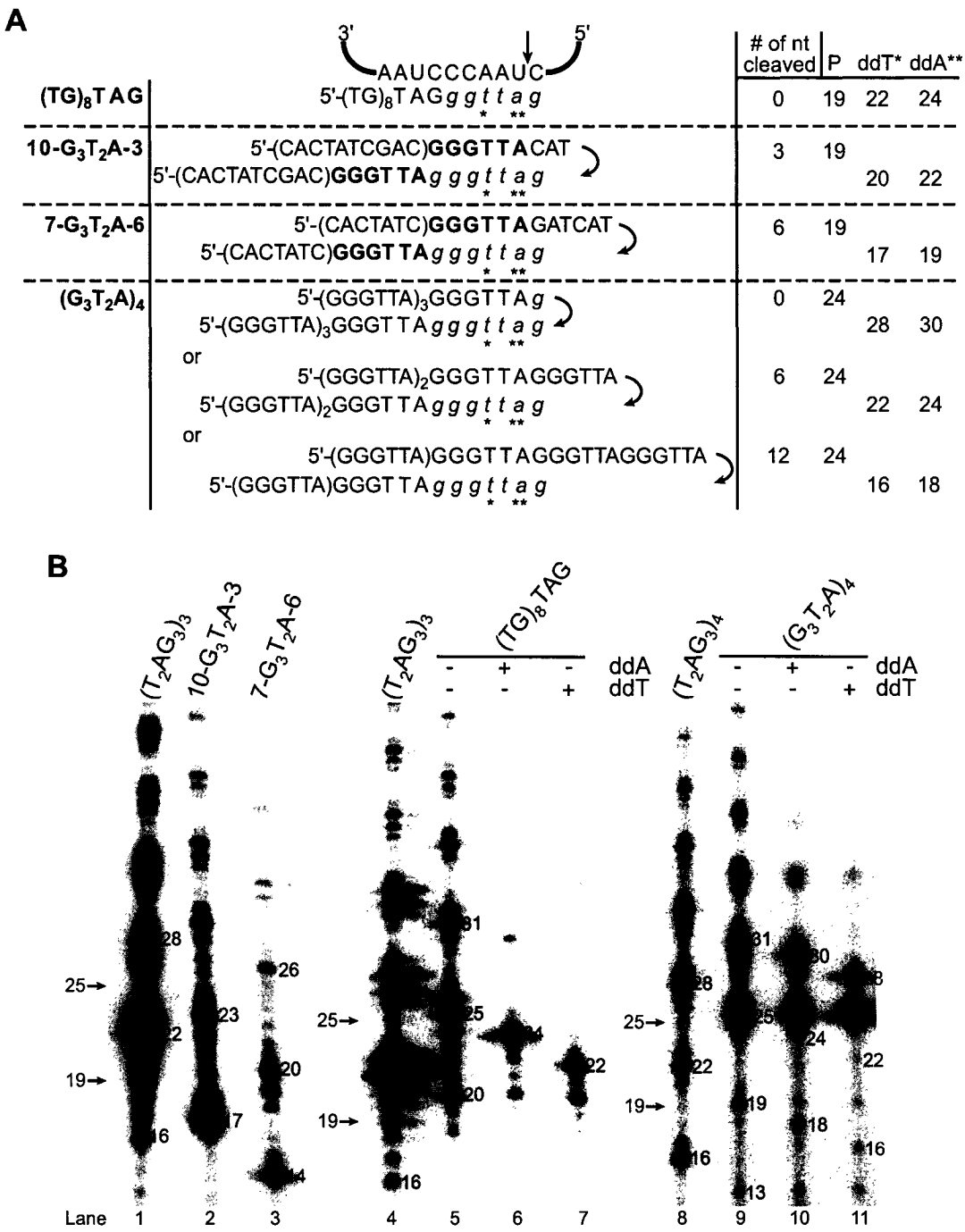
A
3'
5'
AAUCCCAAUC
# of nt cleaved
P
ddT*
ddA**
(TG)8TAG
5'-(TG)8TAGggttag
0 19 22 24
10-G3T2A-3
5'-(CACTATCGAC)GGGTTACAT
5'-(CACTATCGAC)GGGTTAggttag
3 19 20 22
7-G3T2A-6
5'-(CACTATC)GGGTTAGATCAT
5'-(CACTATC)GGGTTAggttag
6 19 17 19
(G3T2A)4
5'-(GGGTTA)3GGGTTAg
5'-(GGGTTA)3GGGTTAggttag
0 24 28 30
or
5'-(GGGTTA)2GGGTTAGGGTTA
5'-(GGGTTA)2GGGTTAggttag
6 24 22 24
or
5'-(GGGTTA)GGGTTAGGGTTAGGGTTA
5'-(GGGTTA)GGGTTAggttag
12 24 16 18
B
(T2AG3)3
10-G3T2A-3
7-G3T2A-6
(T2AG3)3
(TG)8TAG
ddA
ddT
(T2AG3)4
(G3T2A)4
ddA
ddT
Lane 1 2 3 4 5 6 7 8 9 10 11

Figure 3.3 Cleavage is specified by the primer alignment relative to the RNA template sequence. (**A**) Potential alignments of various oligonucleotides (tested in **B**) with the hTR template, before and after translocation. Italicized letters represent nucleotides added by the telomerase enzyme onto the 3'-end of the primer during the elongation step. P: primer length. ddA and ddT: dideoxyATP and dideoxyTTP. The proposed cleavage site is shown by the vertical arrow. Asterisks indicate the positions of the first ddA or ddT incorporated during the DNA primer extension assay. (**B**) Direct DNA primer extension assays were performed with the indicated oligonucleotides. 10-**G_3T_2A**-3: CACTATCGAC-**GGGTTA**-CAT; 7-**G_3T_2A**-6: CACTATC-**GGGTTA**-GATCAT, where the bold nucleotides represent a telomeric cassette. The 3'-end radiolabeled 5'-biotinylated primers, $(T_2AG_3)_3$ and $(T_2AG_3)_4$, migrate respectively as 19 and 25 mers at the positions indicated by the arrows. Numbers in (**A**) and (**B**) represent the lengths of the primers, elongation or chain termination products.

extension pathway leading to the formation of products ≥ 20 nt in length (Fig. 3.3B, lanes 5-7). This primer generated a six nucleotide pausing pattern starting at 25 nt as expected if it is aligned with the template RNA 3'-end 3'-(A)AUC-5' (Fig. 3.3A). These results are comparable to those obtained with *Tetrahymena* telomerase and a similar primer [$(TG)_8TTG$], supporting the predicted alignment of the $(TG)_8TAG$ with the 3'-end of the RNA template (Collins and Greider, 1993).

The cleavage activity does not appear to fulfill a classical proofreading function since entirely telomeric substrates are cleaved (Fig. 3.1) (Collins and Greider, 1993; Melek *et al.*, 1996). In *Euplotes*, *de novo* telomere formation is extremely precise, where all new telomeres start with the sequence 5'-GGGGTTTT-3' (Wang and Blackburn, 1997). We speculated that the preferred substrate for elongation might be one ending with GGGTTA, and predicted that such a substrate might not be cleaved. We assayed $(G_3T_2A)_4$ and found that RRL-reconstituted human telomerase favoured the direct extension pathway leading predominantly to the formation of products ≥ 25 nt in length (Fig. 3.3B, lane 9). The generation of shorter products 13 and 19 nt in length derived from the cleavage of 12 and 6 nt from $(G_3T_2A)_4$ was reduced compared to the generation of shorter products derived from the cleavage of $(T_2AG_3)_4$ (Fig. 3.3B, compare lane 8 with lane 9). The sizes of the cleavage-derived products and the chain termination products generated when substituting dATP with ddATP or TTP with ddTTP were consistent with the potential alignments of the $(G_3T_2A)_4$ with the RNA template (Fig. 3.3A), and a nucleolytic cleavage activity that occurs 3' to the A residue. We also found that the cleavage of the telomeric primer $[(AG_3T_2)_3]$ was reduced compared to the cleavage of $(T_2AG_3)_4$ (data not shown). The reduced cleavage of certain telomeric

primers may suggest that these primers are preferred elongation substrates. Alternatively, these primers may preferentially align such that their 3' sequences do not extend beyond the 5'-end of the template (Fig. 3.3A).

3.5.4 *Primer length requirement of the telomerase-mediated cleavage reaction*

Previous studies have shown that the efficient extension of different length oligonucleotides by human and *Tetrahymena* telomerases is dependent on telomerase assay reaction conditions (Morin, 1989; Collins and Greider, 1993; Collins and Gandhi, 1998; Baran *et al.*, 2002). We determined the primer length requirement of RRL-reconstituted human telomerase-mediated nucleolytic cleavage activity under our standard reaction conditions. We tested primers containing different numbers of the same telomeric repeat permutation: [$(G_2T_2AG)_4$, $(G_2T_2AG)_3$, $(G_2T_2AG)_2$, (G_2T_2AG)] and primers shorter than $(G_2T_2AG)_2$ designed by removing one to six nucleotides from the 5'-end (Fig. 3.4). We found that primer-sized cleavage-derived products could be visualized in telomerase reactions with primers 9 nt in length or longer (Fig. 3.4, lanes 2-7). Primers 6, 7 and 8 nt in length were weakly elongated under standard reaction conditions (Fig. 3.4, lanes 9-11). Upon a 3-fold increase in the primer concentration, it was possible to detect primer-sized products with the 8 nt primer (Fig. 3.4, lane 13). However, under the same reaction conditions, we could not confirm cleavage of the 6 and 7 nt primers due to their less efficient elongation by telomerase and to the altered mobility of the lowest products derived from these primers (Fig. 3.4, compare lanes 10, 11 with lanes 14, 15).

Figure 3.4

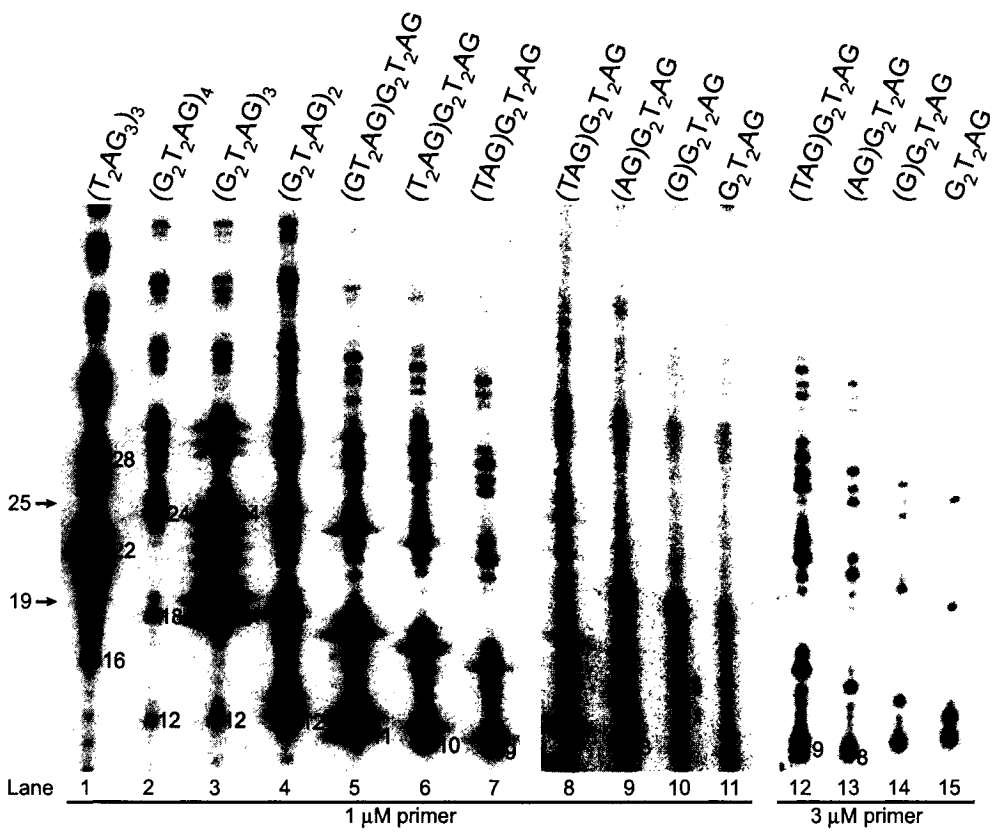
$(T_2AG_3)_3$
$(G_2T_2AG)_4$
$(G_2T_2AG)_3$
$(G_2T_2AG)_2$
$(GT_2AG)G_2T_2AG$
$(T_2AG)G_2T_2AG$
$(TAG)G_2T_2AG$
$(TAG)G_2T_2AG$
$(AG)G_2T_2AG$
$(G)G_2T_2AG$
G_2T_2AG
$(TAG)G_2T_2AG$
$(AG)G_2T_2AG$
$(G)G_2T_2AG$
G_2T_2AG
28
25
24
22
19
18
16
12
12
10
9
9
8
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 μM primer
3 μM primer

Figure 3.4 Primer length requirement of the telomerase-mediated cleavage activity at different primer concentrations. DNA primer extension assays were performed with the indicated oligonucleotides. The elongation products were resolved on 10% polyacrylamide-urea gels. The 3'-end radiolabeled 5'-biotinylated primers, $(T_2AG_3)_3$ and $(T_2AG_3)_4$, migrate respectively as 19 and 25 mers at the positions indicated by the arrows. Numbers represent the lengths of the elongation products.

3.5.5 *Mechanism of telomeric substrate cleavage*

Characterization of *E. crassus* and *S. cerevisiae* telomerases indicates that the primer cleavage proceeds by an endonucleolytic mechanism. Since human telomerase-mediated primer cleavage activity eliminates nucleotides that are complementary to the template residues, it did not seem to use a classical proofreading mechanism. Therefore we were also interested to determine whether the cleavage activity proceeds by an endonucleolytic mechanism. We designed oligonucleotides containing a phosphorothioate group (represented by *), a nuclease-resistant phosphodiester substitution that replaces an oxygen of the phosphodiester bond with a sulfur group. Primer extension telomerase assays performed with $(T_2AG_3)_4$ resulted in the removal of 3 and 9 nt, generating cleavage-derived products 22 and 16 nt in length as described previously (Fig. 3.5, lane 1). $(T_2AG_3)_4$ was modified to contain a phosphorothioate substitution three nucleotides $[(T_2AG_3)_3T_2A*G_3]$ or one nucleotide $[(T_2AG_3)_3T_2AG_2*G]$ from the 3' terminus respectively. An endonucleolytic cleavage reaction eliminating 3 nt would be affected by the nuclease-resistant substitution located three nucleotides from the 3'-end of $(T_2AG_3)_3T_2A*G_3$. However, the endonucleolytic cleavage removing 3 nt should still proceed if the same modification is located one nucleotide from the 3'-end of $(T_2AG_3)_3T_2AG_2*G$. Primer cleavage was similarly abolished by the modifications at either position and led to the formation of products ≥ 28 nt in length (Fig. 3.5, lanes 2, 3). Cleavage-derived products 16 nt in length generated by the removal of 9 nt from $(T_2AG_3)_4$ were also inhibited by the modifications at the same positions, suggesting that the cleavage proceeds by an exonucleolytic mechanism. Substitutions of dATP with ddATP or TTP with ddTTP in telomerase assays generated

Figure 3.5

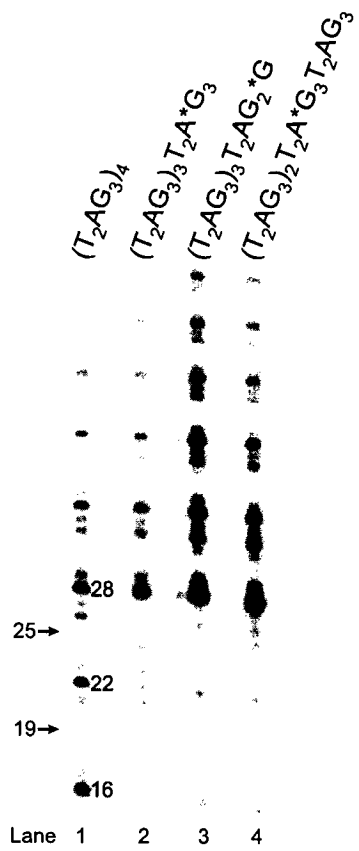
$(T_2AG_3)_4$
$(T_2AG_3)_3T_2A^*G_3$
$(T_2AG_3)_3T_2AG_2^*G$
$(T_2AG_3)_2T_2A^*G_3T_2AG_3$
28
25
22
19
16
Lane 1 2 3 4

Figure 3.5 Cleavage of telomeric substrates by human telomerase may proceed by an exonucleolytic mechanism. DNA primer extension assays were performed with the indicated oligonucleotides. The 3'-end radiolabeled 5'-biotinylated primers, $(T_2AG_3)_3$ and $(T_2AG_3)_4$, migrate respectively as 19 and 25 mers at the positions indicated by the arrows. Numbers represent the lengths of the elongation products. The asterisks indicate the positions of the phosphorothioate modifications within the primers.

no detectable chain termination products due to the incorporation of ddATP or ddTTP prior to the incorporation of [α-^{32}P]dGTP (data not shown). These results were consistent with an inhibition of primer cleavage prior to the elongation.

To determine whether the telomerase-mediated cleavage activity proceeds by an exonucleolytic mechanism, we designed an oligonucleotide carrying a phosphorothioate substitution located nine nucleotides from the 3'-end of $(T_2AG_3)_2T_2A*G_3T_2AG_3$. We predicted that the removal of 9 nt but not 3 nt from the 3'-end of $(T_2AG_3)_2T_2A*G_3T_2AG_3$ would be affected by the nuclease-resistant substitution if the cleavage was exonucleolytic. However, the internal modification in $(T_2AG_3)_2T_2A*G_3T_2AG_3$ abolished the cleavage and inhibited the removal of both 9 and 3 nt and the generation of both shorter products 16 and 22 nt in length (Fig. 3.5, lane 4). If the cleavage activity is exonucleolytic, we might also expect to see products derived from the removal of fewer than 3 nt for both $(T_2AG_3)_3T_2A*G_3$ and $(T_2AG_3)_2T_2A*G_3T_2AG_3$ and products derived from the removal of fewer than 9 nt for the latter primer. However, such products were not detected. These results do not confirm that the cleavage mechanism is exonucleolytic. However, they do not support an endonucleolytic mechanism. Our results raise the possibility that cleavages at multiples sites, whether exonucleolytic or endonucleolytic, might not be independent.

3.5.6 *Cleavage activity of endogenous human telomerase*

RRL-reconstituted human telomerase functions similarly to endogenous telomerase with respect to the basic *in vitro* elongation. However, a comparison of RRL-reconstituted *Tetrahymena* telomerase to endogenous enzyme has revealed some differences, including differences in repeat addition processivity (Collins and Gandhi,

1998; Bryan *et al.*, 2000; Hardy *et al.*, 2001). To identify and characterize endogenous human telomerase-mediated nucleolytic cleavage activity, we performed primer extension assays using native telomerase enzyme partially purified from 293 cell extracts. As shown in figure 3.6, endogenous telomerase catalyzed nucleolytic primer cleavage under standard reaction conditions. We tested a series of oligonucleotides that we had characterized for cleavage using RRL-reconstituted human telomerase. $(T_2AG_3)_3$ and $(T_2AG_3)_4$ were cleaved and native telomerase generated cleavage-derived products of the same length (16 and 22 nt) as those generated by RRL-reconstituted telomerase (Fig. 3.6, compare lane 1 with lanes 2, 3). Telomerase reactions were also performed with $(T_2AG_3)_4$ using different concentrations of dGTP or primer as those we used with RRL-reconstituted human telomerase. The relative amount of cleavage-derived products was not affected (data not shown).

Similarly to our previous results using RRL-reconstituted human telomerase, $(TG)_8TAG$ and $(G_3T_2A)_4$ were not cleaved and the native enzyme favoured the direct extension pathway leading to the formation of products ≥ 20 or 25 nt in length respectively (compare Fig. 3.3B, lanes 5, 9 with Fig. 3.6, lanes 7, 8). However, $(G_2T_2AG)_3$ and $(G_2T_2AG)_4$ were also cleaved, but generated only primer-sized products (compare Fig. 3.4, lanes 2, 3 with Fig. 3.6, lanes 4, 6). These results differ from those obtained using RRL-reconstituted telomerase enzyme where more than one nucleotide were cleaved from these primers. To determine if primers containing nontelomeric sequences are also good cleavage substrates for endogenous human telomerase, we performed telomerase reactions with (10-$\mathbf{G_3T_2A}$-3) or (7-$\mathbf{G_3T_2A}$-6). Such reactions also generated products at 17 and 14 nt respectively, corresponding to the cleavage of the

Figure 3.6

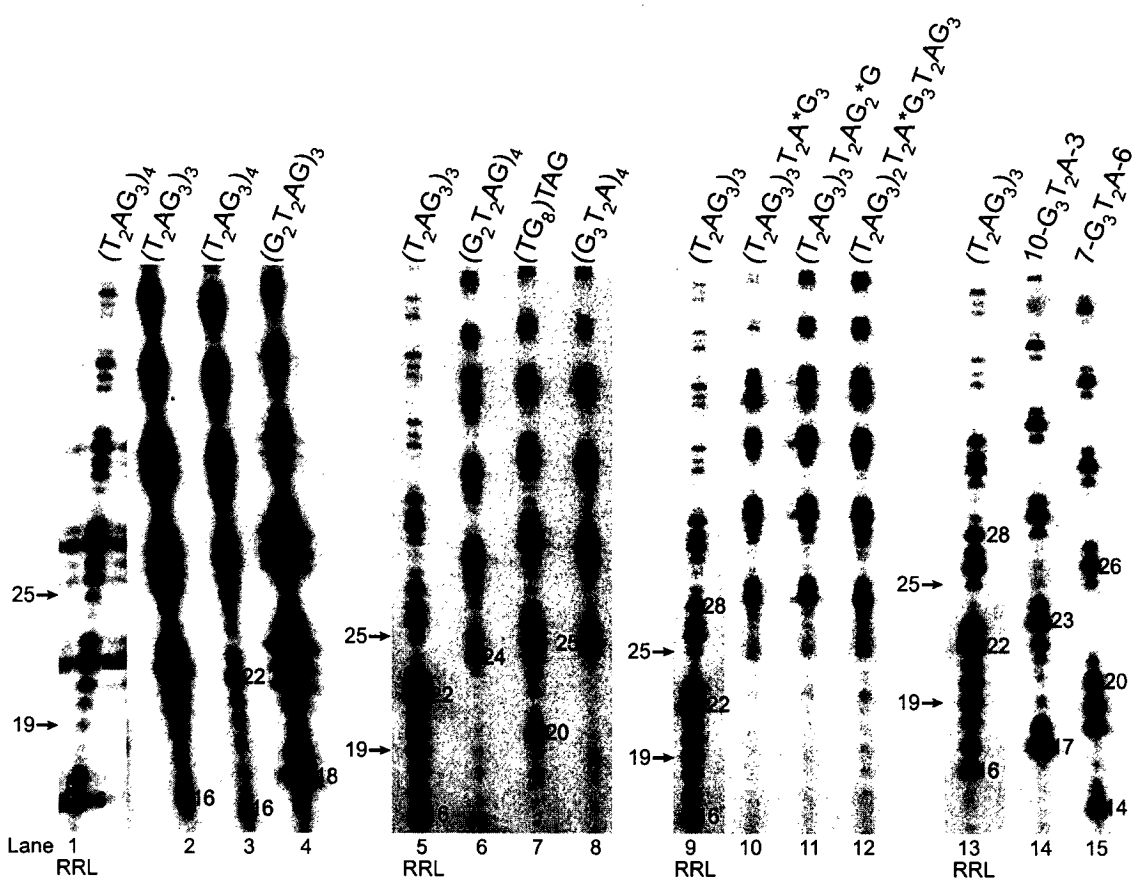
(T2AG3)4
(T2AG3)3
(T2AG3)4
(G2T2AG)3
(T2AG3)3
(G2T2AG)4
(TG8)TAG
(G3T2A)4
(T2AG3)3
(T2AG3)3T2A*G3
(T2AG3)3T2AG2*G
(T2AG3)2T2A*G3T2AG3
(T2AG3)3
10-G3T2A-3
7-G3T2A-6
25
19
28
26
23
22
20
17
16
14
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
RRL RRL RRL RRL

Figure 3.6 Telomerase-mediated cleavage activity of endogenous human telomerase. DNA primer extension assays were performed with the indicated oligonucleotides using RRL-reconstituted telomerase enzyme (lanes 1,5,9,13) or enzyme partially purified from 293 cell extracts (lanes 2-4, 6-8, 10-12, 14, 15). The 3'-end radiolabeled 5'-biotinylated primers, $(T_2AG_3)_3$ and $(T_2AG_3)_4$, migrate respectively as 19 and 25 mers at the positions indicated by the arrows. Numbers represent the lengths of the elongation products.

nontelomeric sequences (3 or 6 nt) by the enzyme (compare Fig. 3.3B, lanes 2, 3 with Fig. 3.6, lanes 14, 15). Lastly, to determine whether native human telomerase mediates telomeric primer cleavage via an endonucleolytic mechanism like other endogenous telomerase enzymes, we performed telomerase reactions with telomeric primers containing phosphorothioate substitutions. Native telomerase generated similar products as the RRL-reconstituted telomerase enzyme with the modified primers, suggesting that human telomerase may mediate cleavage by an exonucleolytic mechanism (compare Fig. 3.5, lanes 2-4 with Fig. 3.6, lanes 10-12).

3.6 *Discussion*

Properties of the nuclease activity described in our studies suggest that the cleavage reaction is dependent on human telomerase and not a nonspecific nuclease. First, our data indicates that the cleavage reactions occurring with RRL-reconstituted human telomerase and native enzyme share some common features. Similarly to RRL-reconstituted *Tetrahymena* telomerase (Collins and Gandhi, 1998), the human TERT and TR expressed in RRL appear sufficient to cleave a primer DNA substrate. Second, end-radiolabeled primers that are incubated with RRL in the absence of telomerase components are neither cleaved nor elongated. Third, the sites of cleavage within the primer DNA substrates are nonrandom, occurring 3' to the A residue in the human telomeric sequence repeat, GGTTAG, and appear to be dependent on the primer alignment with the 5'-end of the template. Lastly, though we did not extensively purify human telomerase from 293 cells, primer cleavage and DNA synthesis appear to be functionally coupled following a partial purification, suggesting that it either is an inherent property of the enzyme or is catalyzed by an associated factor.

3.6.1 *The nucleolytic cleavage reaction*

We found that the RRL-reconstituted human telomerase-mediated cleavage reactions were primer length-dependent, as was reported for *Tetrahymena* telomerase (Collins and Greider, 1993). The minimal length required for primers to be cleaved using 1 μM or 3 μM primer concentration was 9 and 8 nt respectively. Primers less than 10 nt, such as (G_4T_2G), are not cleaved by highly purified preparations of endogenous *Tetrahymena* telomerase, though at high primer concentrations (51 μM), this primer is nonprocessively elongated (Collins and Greider, 1993). Inefficient elongation

of short primers may be due to a requirement for primers to interact with an anchor site in addition to binding the telomerase RNA template (Harrington and Greider, 1991; Morin, 1991; Collins and Greider, 1993; Baran *et al.*, 2002; Wallweber *et al.*, 2003).

Reaction parameters that favour processivity have been reported to reduce cleavage activity, including high concentrations of dGTP or primer (Niu *et al.*, 2000). Factors that stimulate cleavage have also been reported, including primers with mismatches or nontelomeric sequences (Melek *et al.*, 1996; Niu *et al.*, 2000). Although we increased the dGTP concentration to 13.75 μM or the primer concentration to 5 μM, we did not observe a change in the amount of cleaved products generated from $(T_2AG_3)_3$ or $(T_2AG_3)_4$, despite a noted effect on processivity. We speculate that due to the efficient elongation of telomeric primers, such substrates may not be as susceptible to regulation by dGTP or primer concentration. Though a detailed comparison of endogenous and RRL-reconstituted telomerase enzymes has not been performed in most organisms, there are differences with respect to the requirement of dGTP for repeat addition processivity (Collins and Gandhi, 1998; Hardy *et al.*, 2001). Specifically RRL-reconstituted *Tetrahymena* telomerase requires high dGTP concentrations for repeat addition processivity. As factors that inhibit processivity stimulate cleavage, the concentrations of dGTP or primer that regulate elongation and primer cleavage may differ between endogenous and RRL-reconstituted telomerase enzymes. However, our experiments do not indicate a difference between endogenous or RRL-reconstituted human telomerase in the regulation of telomeric primer cleavage by dGTP or moderate primer concentrations.

3.6.2 *Regulation of human telomerase-mediated cleavage by primer alignments with the RNA template*

Our studies indicate that primer-RNA interactions affect cleavage consistent with results of studies with yeast and ciliate telomerase-mediated nuclease activity (Collins and Greider, 1993; Autexier and Greider, 1994; Melek *et al.*, 1996; Prescott and Blackburn, 1997; Collins and Gandhi, 1998; Greene *et al.*, 1998; Lue and Peng, 1998). The potential alignments of each primer with the RNA template allowed us to propose a potential cleavage site relative to the RNA template, between the U and C in the sequence 3'-AAUCCCAAUC-5'. The pausing pattern generated by the elongation of the cleavage-derived products is consistent with the addition of radiolabeled dGTP at the 5'-end of the template after a cleavage event. A primer [$(TG)_8$TAG] predicted to align at the 3'-end of the RNA template is not cleaved by human telomerase, leading us to propose that primer cleavage is dependent on primer alignment with the RNA template 5'-end. Similar locations in the *Tetrahymena* and *E. crassus* telomerase RNA template were reported to dictate cleavage (Collins and Greider, 1993; Melek *et al.*, 1996), though alternate internal sites have also been observed to mediate cleavage *in E. crassus* (Greene *et al.*, 1998). Primers would be cleaved when they are aligned at the 5'-end or beyond the 5'-end of the template to ensure that only residues within the RNA template are copied during telomere synthesis.

Consistent with the potential alignments of primers with the 5'-end of the template, the proposed cleavage site and a possible proofreading function, RRL-reconstituted and endogenous human telomerase catalyzed the removal of nucleotides that form a mismatch with the template similarly to Euplotes and Tetrahymena telomerase (Collins and Greider, 1993; Melek *et al.*, 1996). In our study, such primers

included substrates with one nucleotide mismatch [$(G_2T_2AG)_3G_2T_2A\underline{AG}$] and primers bearing a telomeric sequence repeat surrounded by nontelomeric sequences [10-G_3T_2A-3 and 7-G_3T_2A-6]. Consistent with studies of *Euplotes* and yeast telomerase, our studies indicate that human telomerase mediated cleavage at the junction of the telomeric and nontelomeric sequences (Melek *et al.*, 1996; Niu *et al.*, 2000). Thus the removal of mismatched sequences from telomerase substrates is consistent with a proofreading function for telomerase-mediated cleavage and the enzyme's high fidelity to retain only residues that are complementary to the template.

3.6.3 *Role of telomerase-mediated cleavage activity in the generation of preferred substrates*

Cleavage of telomeric primers by *Tetrahymena* telomerase has been reported, but appears to be restricted to the removal of one nucleotide (Collins and Greider, 1993). Unlike *Tetrahymena* telomerase, RRL-reconstituted human telomerase catalyzed the removal of more than one nucleotide from primers containing permutations of 5'-GGTTAG-3'. $(T_2AG_3)_4$ was cleaved at either of two positions to generate products 22 and 16 nt in length. $(G_2T_2AG)_4$ and $(G_2T_2AG)_3$ also generated more than one product (24, 18 and 12 nt in length or 18 and 12 nt, respectively). We proposed above that several alignments of the $(T_2AG_3)_4$ and $(G_2T_2AG)_4$ primers could occur relative to the RNA template to generate two or three different sizes of cleavage-derived products respectively. Some of the shorter products would thus be generated by cleavage of sequences that extend past the 5'-end of the RNA template, as has been documented for *Tetrahymena* telomerase (Collins and Greider, 1993). However, the function for the cleavage of telomeric primers and their cleavage at multiple sites is unclear. Cleavage of

telomeric primers by *S. cerevisiae* telomerase to generate labelled products shorter than the input primer by up to eight nucleotides has also been reported though a function for the cleavage of telomeric substrates has not been determined (Cohn and Blackburn, 1995). We propose that a requirement for the cleavage of telomeric primers *in vivo* might be to generate a preferred substrate for human telomerase. For example, *de novo* telomere formation in *E. crassus* is extremely precise, with new telomeres initiating with the sequence 5'-GGGGTTTT-3' (Klobutcher *et al.*, 1981). *S. cerevisiae* also initiate telomere formation with $TG_{1\text{-}3}$ repeats (Kramer and Haber, 1993). As cleavage of the primer ending in 5'-GGGTTA-3' is reduced, we suggest that new telomeres in human chromosomes might initiate with the sequence 5'-GGGTTA-3'. However, cleavage of the telomeric primer $(AG_3T_2)_3$ was also reduced. Human telomerase can also add different permutations of the human telomeric sequence to primers derived from the chromosome 16 breakpoint sequence (Morin, 1991). Thus the reduced cleavage of certain telomeric primers may not indicate that they are preferred substrates for elongation by telomerase. Alternatively, such primers may preferentially align such that their 3' sequences do not extend beyond the 5'-end of the template.

Characterization of the human telomerase-dependent nuclease of telomeric substrates revealed that cleavage may occur by an exonucleolytic mechanism. Our findings were somehow unexpected as primer cleavage by endogenous *E. crassus* and *S. cerevisiae* telomerases occurs by an endonucleolytic mechanism (Melek *et al.*, 1996; Niu *et al.*, 2000). We obtained similar results whether we used RRL-reconstituted or partially purified native human telomerase enzyme. Though our results suggest that cleavage occurs by an exonucleolytic mechanism, our findings using the primer

$(T_2AG_3)_2T_2A^*G_3T_2AG_3$ were also surprising, as an internal nuclease-resistant modification should not have affected the cleavage that can occur at the 3'-end of the primer. The modification does not appear to alter the primer alignment with the RNA template, since all the modified primers were efficiently elongated by RRL-reconstituted and endogenous telomerases to generate the expected six nucleotide product pausing pattern. However, one possibility may be that $(T_2AG_3)_3T_2A^*G_3T_2AG$ aligns only with the modification opposite to the 5'-end of the template that dictates cleavage. Another possibility may be that cleavages at the multiple sites are not independent, and abolishing cleavage at the internal site may affect cleavage at the more 3'-terminal site. Alternatively the modified primers may preferentially align at the 3'-end of the template and are not substrates for a cleavage-mediated reaction that is dependent on primer alignment at the RNA template 5'-end. If either of these scenarios are indeed occurring, we must also consider the possibility that such events may be consistent with an endonucleolytic mode of action.

3.6.4 *Difference between RRL-reconstituted telomerase enzyme and native enzyme*

Processivity is a property common to many enzymes that perform critical functions of DNA synthesis. Processivity of RRL-reconstituted and native *Tetrahymena* telomerase enzymes differs (Collins and Gandhi, 1998; Bryan *et al.*, 2000; Hardy *et al.*, 2001). In our study, we found that processivity and cleavage differed between RRL-reconstituted human telomerase and native enzyme. The reconstituted enzyme complex could be lacking required associated regulatory factors and/or be assembled in a different conformation. As for *Tetrahymena* telomerase, RRL-reconstituted human enzyme was

less processive than endogenous telomerase (Collins and Gandhi, 1998; Bryan *et al.*, 2000; Hardy *et al.*, 2001). Using the primers $(G_2T_2AG)_3$ or $(G_2T_2AG)_4$, the native enzyme generated mainly primer-sized products, consistent with cleavage of similar substrates ending in G_3T_2G by endogenous *Tetrahymena* telomerase (Collins and Greider, 1993). These results suggest a preferential binding of these primers at the 5'-end of the hTR in the native enzyme, and do not support the alignment of such primers beyond the 5'-end of the template. This is in contrast to the results we obtained using RRL-reconstituted enzyme where cleaved products were consistent with the primers aligning beyond the template 5'-end. Moreover, the relative amount of cleaved versus elongated products derived from $(T_2AG_3)_3$ and $(T_2AG_3)_4$ is less for the native enzyme than the RRL-reconstituted enzyme. The cleavage, particularly marked for the RRL-reconstituted complex, may result from a high frequency of stalling by the human telomerase at several positions along the RNA template. We speculate that the nuclease activity may be an error-correcting mechanism or a way of rescuing pause/arrested complexes, which facilitates the elongation of DNA substrates (Sastry and Ross, 1997). The native enzyme or telomerase-associated factors may more stringently regulate the alignment and binding of DNA substrates with the RNA template. Further studies will be required to better characterize the human telomerase-mediated cleavage activity and the dependence of this activity on the minimal telomerase components (sequence or structure of hTERT or hTR) and/or additional protein components.

3.7 *Acknowledgements*

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CHAPTER 4

Expression of Recombinant Human Telomerase Enzyme in Insect Cells

4.1 *Preface*

The previous chapters describe works characterizing the biochemical properties of the human telomerase, focusing on the C-terminal region of hTERT and the intrinsic nucleolytic cleavage activity performed by the enzyme. The lack of an abundant source of active telomerase is currently limiting the structural and functional studies of this enzyme, including mechanistic studies of processivity and cleavage. Several heterologous protein expression systems have been used to express telomerase, but the levels of expression and solubility of the recombinant telomerase enzyme are low. In this study, we established the experimental conditions to produce active recombinant human telomerase in insect cells.

4.2 *Introduction*

Human telomerase is a ribonucleoprotein (RNP) complex that synthesizes telomeric DNA repeats at the ends of eukaryotic chromosomes (reviewed in Nugent and Lundblad, 1998). The telomerase enzyme is minimally composed of a protein catalytic subunit, the telomerase reverse transcriptase (hTERT), and the telomerase RNA (hTR) which provides the template for telomere synthesis (reviewed in Greider, 1996). Actually, detailed biochemical analyses of human telomerase are difficult due to the low amounts of endogenous enzyme detected in cells, and are currently limited by the lack of an abundant source of catalytically active recombinant enzyme. Several investigations have reported the reconstitution of human telomerase *in vitro* and demonstrated the essential role of hTERT and hTR subunits during DNA repeat synthesis.

Catalytically active human telomerase was initially reconstituted by incubating micrococcal nuclease (MNase)-treated human cell extracts with *in vitro*-transcribed hTR (Autexier *et al.*, 1996). Active enzyme was also generated by the synthesis of hTERT in rabbit reticulocyte lysate (RRL) in the presence of *in vitro*-transcribed hTR (Weinrich *et al.*, 1997; Beattie *et al.*, 1998), and by the ectopic expression of the human telomerase subunits *in vivo* in both telomerase-positive and telomerase-negative cells (Marušic *et al.*, 1997; Bodnar *et al.*, 1998; Wen *et al.*, 1998; Tesmer *et al.*, 1999; Beattie *et al.*, 2000; Guiducci *et al.*, 2001; Banik *et al.*, 2002; Ly *et al.*, 2003). Studies with RRL-reconstituted human telomerase suggested that hTERT and hTR are the minimal components necessary to generate an active enzyme (Weinrich *et al.*, 1997; Beattie *et al.*, 1998). However, the poor expression and reconstitution of telomerase in RRL limit its utility.

Several other methods of protein expression have been described to produce recombinant telomerase necessary to study the molecular mechanisms of the enzyme and to further characterize the telomerase RNP complex. The attempts to express and purify soluble full length hTERT from *Escherichia coli* (*E. coli*) were unsuccessful (Autexier, C., *laboratory observations*). However, a modified hTERT gene containing codons optimized for expression in bacteria was expressed in *E. coli* rapid translation system (RTS) (Roche Applied Science) in the presence of chaperone proteins (Steigerwald *et al.*, 2002). Typically 10-20 μg/ml of codon modified hTERT are produced in RTS in contrast to 2 μg/ml of wild-type hTERT in RRL (Collins and Gandhi, 1998; Steigerwald *et al.*, 2002). hTERT was also synthesized in insect cells and partially purified by conventional chromatography before reconstituting the telomerase complex by the addition of *in vitro*- transcribed hTR (Masutomi *et al.*, 2000). Insect cell extracts containing hTERT were similarly incubated with *in vitro*-transcribed hTR, and hTERT was subsequently purified by affinity against its RNA subunit (Wenz *et al.*, 2001). The coexpression of the minimal components of human telomerase, hTERT and hTR, also generated catalytically active telomerase in yeast (Bachand and Autexier, 1999) and in insect cells (Mikuni *et al.*, 2002). Although several protein expression systems have been used to successfully produce recombinant hTERT, steps still requiring optimization during hTERT production and purification include the increase of hTERT expression levels and solubility and the enhancement of affinity purification.

The production of a recombinant source of an RNP such as telomerase can be complicated by the fact that the RNA component may need to be in close proximity to the protein subunit during translation to help their folding into an active RNP (reviewed

in Weeks, 1997). Our laboratory has developed a hTERT-hTR *cis* construct, in which the sequence encoding hTR is fused at the 3'-end of the hTERT coding sequence (Bachand *et al.*, 2000). The proximity of hTERT to hTR provided by the bicistronic expression of hTERT-hTR *cis* construct may promote the efficient folding and assembly of telomerase during translation of hTERT from hTERT-hTR *cis* transcript. This strategy was shown to produce an active telomerase in RRL and in yeast (Bachand *et al.*, 2000). However, the amounts of hTERT from those sources are limited. In the present work, we reported the experimental conditions for the expression of hTERT-hTR *cis* in *sf21* insect cells upon infection with a baculovirus carrying the hTERT-hTR *cis* gene, a commonly used system for recombinant protein expression.

4.3 *Materials and methods*

4.3.1 *Plasmid constructions*

To generate the hTERT-hTR *cis* construct, a *SalI/Not*I hTERT-hTR fragment from the BSSK/hTERT-hTR plasmid (Bachand *et al.*, 2000) was cloned into the pFastBac-HTb donor vector (Life Technologies) that was previously digested with *Sal*I and *Not*I (Bachand, F. and Autexier, C., *unpublished*). To generate the hTERT construct, hTERT cDNA was amplified by PCR from the pGRN121 plasmid (Nakamura *et al.*, 1997) using the 5' primer 5'-CCGGAATTCTATGCCGCGCGCTCCCC-3' and the 3' primer 5'-GAATGCGGCCGCTCAGTCCAGGATGGTCTTG-3' containing *Eco*RI and *Not*I restriction sites, respectively (Autexier, C., *unpublished*). The *Eco*RI/*Not*I-digested PCR products were cloned into the pT7/T3D-Pac vector. A 2.9 kb *Mlu*I-*Bsm*I hTERT fragment within pT7/T3D-Pac was replaced by an equivalent 2.9 kb *Mlu*I-*Bsm*I hTERT fragment from pGRN121 plasmid to reduce potential PCR-generated mutations. Finally, a *Eco*RI/*Not*I hTERT fragment from the pT7/T3D-Pac plasmid was cloned into the pFastBac-HTc donor vector (Life technologies) that was previously digested with *Eco*RI and *Not*I.

4.3.2 *Cells and viruses*

Spodoptera frugiperda (*Sf21*) insect cell line was maintained in serum-free SF900 II medium supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin. *Sf21* were grown at 27°C as monolayer cultures or in suspension cultures. The recombinant viruses were generated by using Bac-to-Bac® baculovirus expression system (Life Technologies) according to the supplier's instructions (hTERT-hTR *cis*: Bachand, F. and Autexier, C., *unpublished*; hTERT: Autexier, C., *unpublished*).

Additional cell infection stages were performed on monolayer cultures to amplify recombinant viruses and generate high-titer viral stocks (10^7-10^9 pfu/ml) required for recombinant protein production. The baculovirus suspension carrying either hTERT-hTR *cis* or hTERT transgene was titered by end-point dilution assay.

4.3.3 *Expression of recombinant human telomerase*

Exponentially growing *sf21* insect cells in suspension cultures were infected with recombinant viruses at different multiplicities of infection (MOI) and for different time intervals. The baculovirus-infected insect cells were harvested and washed once with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4). The pellet was directly lysed for protein analysis or was frozen at -80°C.

4.3.4 *Extraction of recombinant human telomerase*

All the steps were carried out at 4°C. Baculovirus-infected insect cells were harvested at high-speed centrifugation, washed once with PBS, resuspended in four volumes of cold lysis buffer (20 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol, 10% glycerol, 0.1 mM PMSF and protease inhibitor cocktail) and incubated on ice for 5 min. The cells were subjected to a gentle homogenization (25 strokes) in a Dounce (working capacity: 7 ml) with a loose pestle (clearance: 0.05 mm) and were left on ice for 15 min. The cell suspension was centrifuged and the supernatant was collected. The pellet was subjected to a second round of a gentle homogenization (20 strokes) in two volumes of cold lysis buffer. All the supernatants were pooled together and the soluble recombinant telomerase enzyme was immediately purified. Approximately 40-50% of the recombinant telomerase enzyme was soluble. The protein concentration was determined

by a Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as a standard. The reconstitution of catalytically active telomerase was performed as previously described by the addition of *in vitro*-transcribed hTR to the supernatant containing recombinant hTERT (Wenz *et al.*, 2001).

293 cells were collected, harvested by centrifugation and washed once with PBS. The cells were washed again in with cold washing buffer (10 mM HEPES-KOH pH 7.5, 1.5 $MgCl_2$, 10 mM KCl, 1mM DTT). The washed cells were resuspended in cold lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 1 mM DTT, 0.5% CHAPS, 10% glycerol and protease inhibitor cocktail) and incubated on ice for 30 min. The cell suspension was harvested at high-speed centrifugation, and the supernatant was collected and stored at -80°C. The protein concentration was determined by a Bradford assay (Bradford, 1976) with BSA as a standard.

4.3.5 *Affinity purification of recombinant human telomerase*

Affinity purification of the human telomerase complex against its RNA subunit from insect and 293 cell extracts was carried out as previously described (Schnapp *et al.*, 1998), with some modifications. The buffer used during the whole purification procedure was the lysis buffer (with 0.5 mM DTT and without β-mercaptoethanol) employed during the extraction of recombinant human telomerase. To purify the telomerase complex by affinity, the oligonucleotide 5'-biotin-CTAGACCTGTCATCAGUUAGGGUUAG (residues underlined = 2'-*O*-methylribonucleotides) was used (Operon). Telomerase complexes bound to the

Ultralink-immobilized Neutravidin Plus beads (Pierce) were eluted with the displacement oligonucleotide 5'-CTAACCCTAACTGATGACAGGTCTAG-3'.

4.3.6 *SDS-PAGE and immunoblotting*

Expression and purification of recombinant human telomerase were monitored by SDS-PAGE (Laemmli, 1970). For immunoblotting analysis, equal amounts of proteins were subjected to electrophoresis by SDS-PAGE and transferred on a nitrocellulose membrane. hTERT was detected by an affinity-purified polyclonal hTERT antibody (Moriarty *et al.*, 2002). Equal amounts of proteins were also visualized by Coomassie blue staining.

4.3.7 *Telomerase activity assays*

A PCR-based telomerase repeat amplification protocol (TRAP) assay was performed on equal amounts of proteins as described previously (Kim and Wu, 1997; Moriarty *et al.*, 2002).

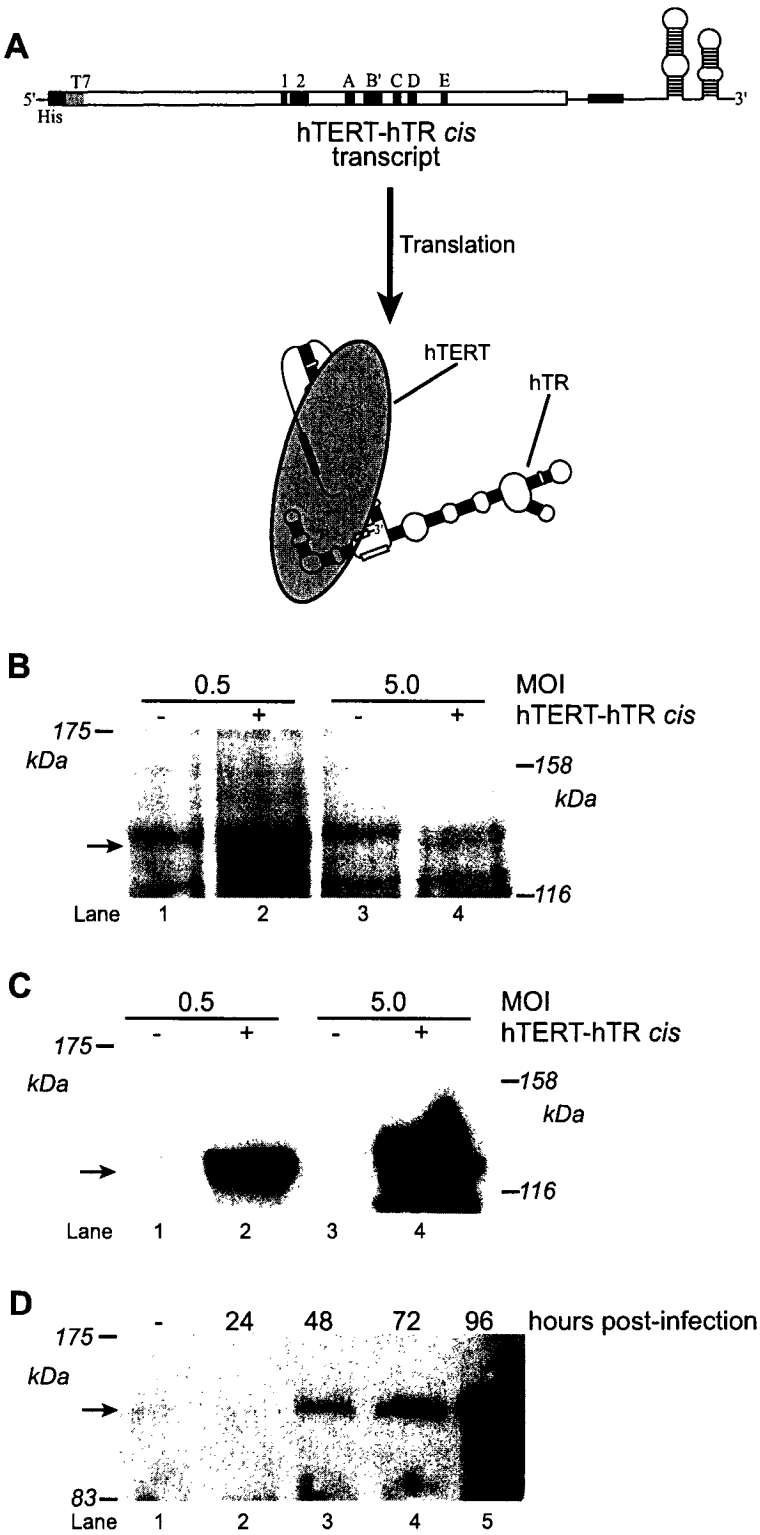
4.4 *Results*

4.4.1 *Expression of recombinant human telomerase in sf21 insect cells*

Insect cells have already been used to express recombinant hTERT, but its levels of expression and solubility need to be improved (Masutomi *et al.*, 2000; Wenz *et al.*, 2001; Arai *et al.*, 2002; Mikuni *et al.*, 2002). We have independently developed a strategy to express hTERT in insect cells upon infection with a baculovirus carrying the hTERT cDNA. Our laboratory designed a hTERT-hTR *cis* construct (Fig. 4.1A), in which the 3'-end of the hTERT coding sequence is extended by the sequence encoding hTR (Bachand *et al.*, 2000). The bicistronic expression of hTERT-hTR *cis* construct has previously been shown to produce active human telomerase in RRL and in yeast (Bachand *et al.*, 2000), allowing to reconstitute the enzyme without subsequent addition of *in vitro*-transcribed hTR to insect cell extracts. This construct may favor the folding or assembly of the telomerase complex (Fig. 4.1A), as previously described for the hepatitis B virus (HBV) polymerase (Lanford *et al.*, 1995).

We first optimized the infection parameters (MOI and time course infection) with the recombinant baculovirus carrying the hTERT-hTR *cis* transgene to produce high levels of recombinant telomerase. We infected insect cells at an MOI of 0,5 and 5 pfu/cell. Baculovirus-infected cells were harvested at 96 hours post-infection, lysed, and protein extracts were subjected to Coomassie blue staining (Fig. 4.1B) and immunoblotting (Fig. 4.1C) analysis. Proteins of the expected molecular weight (approximately 127 kDa) were detected upon viral infection with the recombinant hTERT-hTR *cis* baculovirus (Fig. 4.1B and 4.1C, compare lanes 1 and 2, 3 and 4). We did not find major differences in the levels of hTERT expression when the cells were

Figure 4.1


A
T7
His
5'
1 2
A B' C D E
3'
hTERT-hTR *cis* transcript
Translation
hTERT
hTR
B
0.5
5.0
MOI
hTERT-hTR *cis*
175—
kDa
—158
kDa
—116
Lane 1 2 3 4
C
0.5
5.0
MOI
hTERT-hTR *cis*
175—
kDa
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kDa
—116
Lane 1 2 3 4
D
- 24 48 72 96 hours post-infection
175—
kDa
83—
Lane 1 2 3 4 5

Figure 4.1 Expression of hTERT under different infection parameters (MOI and time course infection). (**A**) Structure of hTERT-hTR *cis* construct. Sequence encoding hTR is fused 18 nt downstream from the 3'-end of the hTERT coding sequence. hTERT is represented as a box containing the reverse transcriptase-like motifs and His and T7 tags fused to the N-terminus. The template sequence of hTR is shown as a black rectangle in its secondary structure. (**B**) Protein extracts made from insect cells infected with the recombinant hTERT-hTR *cis* baculovirus were subjected to electrophoresis on SDS-PAGE 6% and stained with Coomassie blue. *Lanes 1 and 3*, protein extracts from noninfected insect cells. *Lanes 2 and 4*, protein extracts from insect cells infected at different MOIs and harvested at 96 hours post-infection. (**C**) SDS-PAGE 6% as described in (**B**) was subjected to immunoblotting. *Lanes 1 and 3*, protein extracts from noninfected insect cells. *Lanes 2 and 4*, protein extracts from insect cells infected at different MOIs and harvested at 96 hours post-infection. (**D**) SDS-PAGE 7,5% as described in (**B**) was subjected to immunoblotting. *Lane 1*, protein extract from noninfected cells. *Lanes 2-5*, protein extracts from insect cells infected at an MOI of 0,5 pfu/cell and harvested after different time of infection. The arrows indicate the recombinant hTERT protein. Equal amounts of proteins were loaded on each SDS-PAGE.

infected at different MOIs (Fig. 4.1B and 4.1C, compare lanes 2 and 4). At an MOI of 0,5 pfu/cell, we determined that the levels of hTERT expression were higher at 96 hours post-infection compared to 24, 48 or 72 hours (Fig. 4.1D). The cells were completely growth arrested and enlarged, a good parameter indicating viral infection. Finally, we performed a TRAP assay to determine whether hTERT and hTR can generate an active telomerase complex upon viral infection. Catalytically active telomerase is detected in baculovirus-infected cell extracts (Fig. 4.3A, compare lanes 2 and 3), suggesting that the single hTERT-hTR transcript is well translated and that sufficient amounts of telomerase RNP are assembled to generate activity, consistent with studies in RRL and in yeast (Bachand *et al.*, 2000).

During the course of our experimentations, independent investigators reported the reconstitution of active telomerase by incubating insect cell extracts containing hTERT with *in vitro*-transcribed hTR (Masutomi *et al.*, 2000; Wenz *et al.*, 2001; Arai *et al.*, 2002; Mikuni *et al.*, 2002), a similar method to the RRL system. We determined whether the expression of hTERT from a recombinant baculovirus carrying the hTERT transgene may produce an active enzyme by the same reconstitution procedure. Upon a viral infection for 96 hours with the recombinant hTERT baculovirus, we found that the expression of hTERT was higher at an MOI of 1,0 pfu/cell compared to an MOI of 10 pfu/ml (Fig. 4.4A, compare lanes 2 and 3). Based on the procedure provided by Wenz *et al.*, (2001), we reconstituted catalytically active telomerase that is dependent on the addition of *in vitro*-transcribed hTR to insect cell extracts (Fig. 4.4B, compare lanes 1 and 2).

Figure 4.2

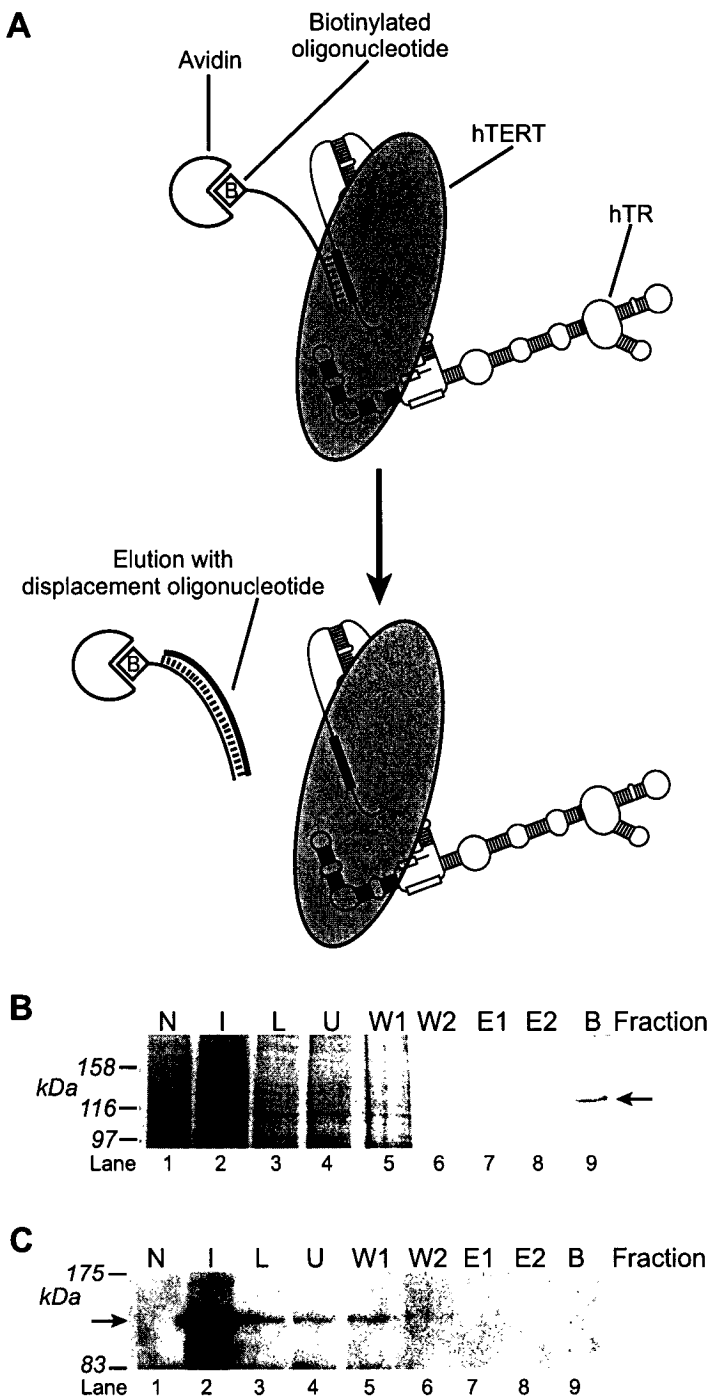
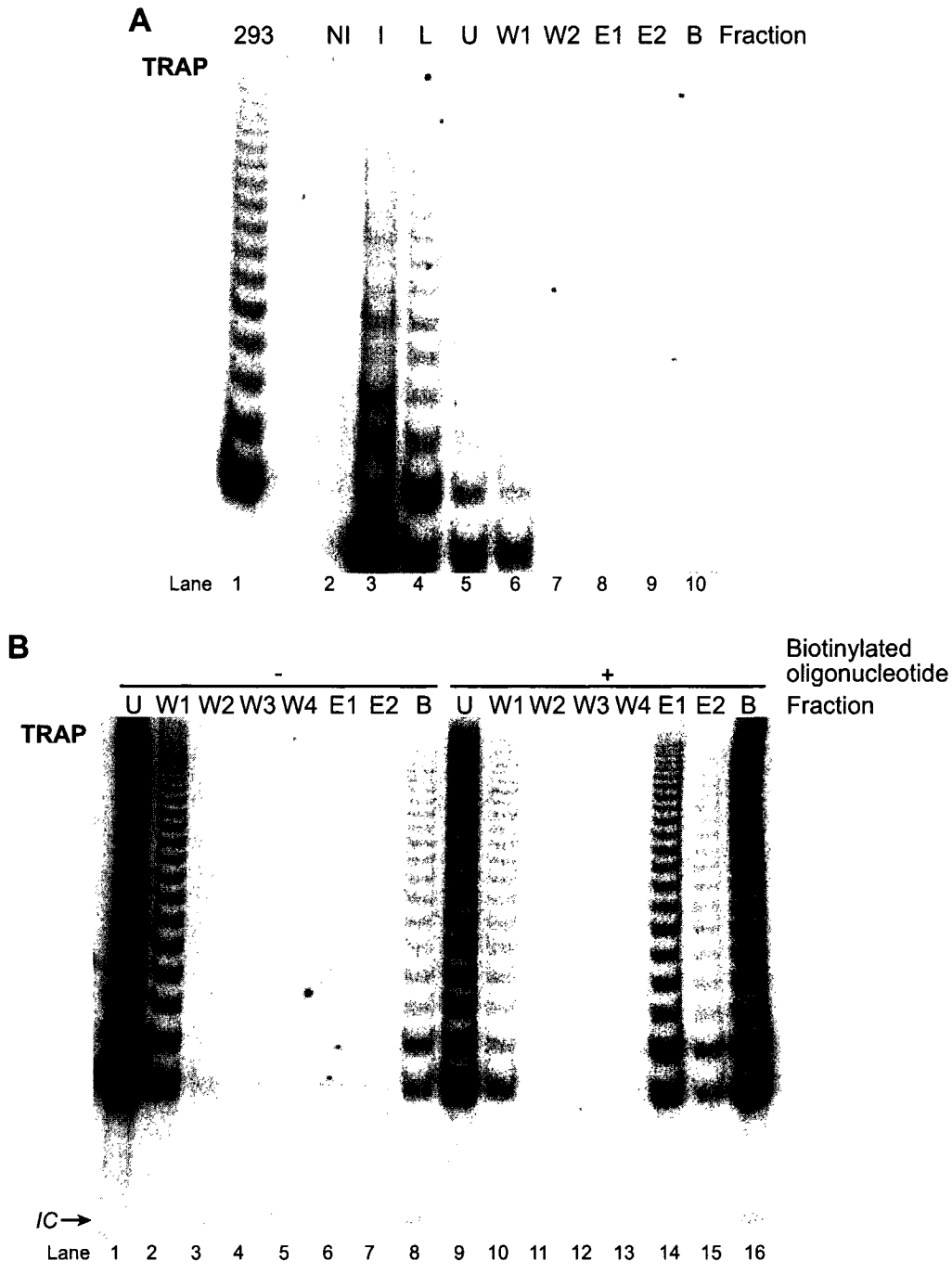

A
Avidin
Biotinylated oligonucleotide
hTERT
hTR
Elution with displacement oligonucleotide
B
N I L U W1 W2 E1 E2 B Fraction
158
kDa
116
97
Lane 1 2 3 4 5 6 7 8 9
C
N I L U W1 W2 E1 E2 B Fraction
175
kDa
83
Lane 1 2 3 4 5 6 7 8 9

Figure 4.2 Distribution of recombinant hTERT proteins during the affinity purification against hTR template. (**A**) Human telomerase complex is bound to avidin beads via a biotinylated oligonucleotide complementary to the hTR template and eluted with a displacement oligonucleotide. (**B**) Recombinant human telomerase contained in insect cell extracts was subjected to affinity purification against hTR template followed by electrophoresis on SDS-PAGE 7,5%. Proteins were visualized by Coomassie blue staining. The insect cells were infected with the recombinant hTERT-hTR *cis* baculovirus at an MOI of 0,5 pfu/cell and harvested at 96 hours post-infection. (**C**) SDS-PAGE 7,5% as described in (**B**) was subjected to immunoblotting. *Lane 1*, protein extract from noninfected (NI) insect cells. *Lane 2*, protein extract of infected (I) insect cells. *Lanes 3-9*, total proteins corresponding to each fraction of the affinity purification. L: load; U: unbound; W: wash; E: elution; B: bound. The arrows indicate the recombinant hTERT protein. Equal amounts of proteins were loaded on each SDS-PAGE.

Figure 4.3


A
293
NI
I
L
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W1
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E1
E2
B
Fraction
TRAP
Lane 1 2 3 4 5 6 7 8 9 10
B
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Biotinylated oligonucleotide
U W1 W2 W3 W4 E1 E2 B U W1 W2 W3 W4 E1 E2 B
Fraction
TRAP
IC→
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 4.3 Telomerase activity of each fraction of the affinity purification. **(A)** Recombinant human telomerase contained in insect cell extracts was subjected to an affinity purification against hTR template followed by TRAP assay. The insect cells were infected with the recombinant hTERT-hTR *cis* baculovirus at an MOI of 0,5 pfu/cell and harvested at 96 hours post-infection. **(B)** Endogenous human telomerase from 293 cell extracts was subjected to an affinity purification against the hTR template followed by TRAP assay. NI: noninfected insect cells; I: infected insect cells; L: load; U: unbound; W: wash; E: elution; B: bound. Equal amounts of proteins were subjected to TRAP assay. IC: PCR internal control.

Figure 4.4

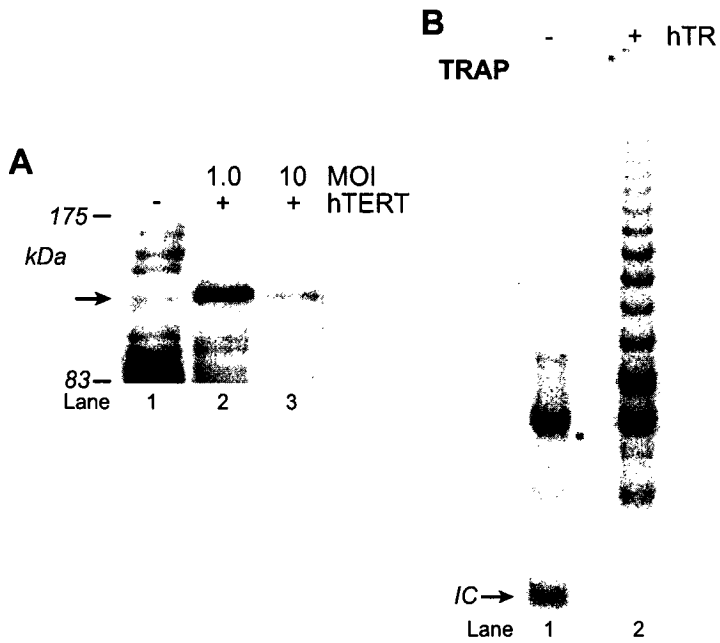

Figure 4.4 Reconstitution of active telomerase. **(A)** Protein extracts made from insect cells infected with the recombinant hTERT baculovirus were subjected to electrophoresis on SDS-PAGE 7,5% followed by immunoblotting analysis. *Lane 1*, protein extract from noninfected insect cells. *Lanes 2 and 3*, protein extracts from insect cells infected at different MOIs and harvested at 96 hours post-infection. Equal amounts of proteins were loaded on the SDS-PAGE. **(B)** Reconstitution of recombinant telomerase by incubating protein extracts containing hTERT with *in vitro*-transcribed hTR. Telomerase activity was monitored by TRAP assay. Equal amounts of proteins were subjected to TRAP assay. IC: PCR internal control.

4.4.2 *Affinity purification of recombinant human telomerase*

We successfully generated active human telomerase in insect cells infected with the recombinant hTERT-hTR *cis* baculovirus. Several methods of purification, including different epitope tags attached to the catalytic protein subunit or successive chromatography, have been used to purify *in vitro*-reconstituted telomerase or endogenous telomerase for biochemical and functional studies (Autexier *et al.*, 1996; Bednenko *et al.*, 1997; Bryan *et al.*, 2000; Masutomi *et al.*, 2000; Peng *et al.*, 2001; Lai *et al.*, 2002; Livengoog *et al.*, 2002; Chen and Greider, 2003; Wallweber *et al.*, 2003). Affinity purification against the telomerase RNA component is also an effective method to purify recombinant telomerase RNP complexes (Lingner and Cech, 1996; Schnapp *et al.*, 1998; Wenz *et al.*, 2001). We decided to purify recombinant human telomerase from insect cells by this method and compare it to a purification of endogenous telomerase from 293 human cells.

Briefly, biotinylated oligonucleotide that is complementary to the template sequence of telomerase RNA is used to interact with the telomerase complex (Fig. 4.2A). Avidin beads are subsequently added to immobilize biotinylated oligonucleotide/telomerase binary complexes (Fig. 4.2A). Following binding and extensive washing steps, the telomerase RNP complex is eluted from the biotinylated matrix by competition with an excess of primer complementary to the biotinylated oligonucleotide (Fig. 4.2A). We first performed the affinity purification of telomerase against hTR with 293 cell extracts and monitored telomerase activity after each critical step of the procedure by TRAP assay. Following the addition of biotinylated oligonucleotides complementary to the hTR template sequence, catalytically active

telomerase was detected in the elution fraction (Fig. 4.3B, compare lanes 6-7 and 14-15) and lower amounts of telomerase complex were present in the unbound fraction (Fig. 4.3B, compare lanes 1 and 9), demonstrating that the affinity purification against hTR could be successfully performed with native enzyme. Nonspecific binding was also detected between avidin beads and telomerase (Fig. 4.3B, lane 8), but it was eliminated after the first washing step (Fig. 4.3B, compare lanes 2 and 10), confirming that most of the telomerase complex were bound to the beads by the biotinylated oligonucleotide.

We next executed the same affinity purification with insect cells previously infected with the recombinant hTERT-hTR *cis* baculovirus. Protein extracts obtained after each step of the purification procedure were subjected to Coomassie blue staining (Fig. 4.2B), immunoblotting (Fig. 4.2C) and TRAP assay (Fig. 4.3A) analysis in order to evaluate the efficiency of the method. We did not succeed to elute significant amounts of active recombinant hTERT (Fig 4.2B and 4.2C, lanes 7, 8; Fig. 4.3C, lanes 7, 8). However, proteins of the expected molecular weight were detected in the bound fraction (Fig 4.2B, lane 9), but we were unable to confirm the identity of these proteins as hTERT (compare Fig. 4.2B, lane 9 with Fig 4.2C, lanes 2-5, 9). Moreover, most of telomerase did not bind to the biotinylated oligonucleotide, because bands corresponding to hTERT have the same intensity (Fig. 4.2C, compare lanes 3-5) and telomerase activity is detected in the load and unbound fractions (Fig. 4.3A, compare lanes 4 and 5). Catalytically active telomerase was also detected in the first low stringent wash fraction (Fig. 4.2C, lane 5 and Fig. 4.3A, lane 6), but it was absent after further step of purification (Fig. 4.2C, lanes 7-9 and Fig. 4.3A, lanes 8-10), suggesting that the recombinant telomerase complex is loosely attached to the biotinylated matrix. The

telomerase complex may be not properly folded which prevent the efficient binding of hTR template to the biotinylated oligonucleotide.

4.5 *Discussion*

Structural studies are hindered by the difficulty to obtain large amounts of active telomerase enzyme. Several investigations reported the production of the hTERT subunit in protein expression systems followed by the *in vitro* reconstitution of telomerase in the presence of *in vitro*-transcribed hTR (Weinrich *et al.*, 1997; Beattie *et al.*, 1998; Masutomi *et al.*, 2000; Steigerwald *et al.*, 2002). The goal of our study was to express hTERT and hTR as a single transcript in order to purify telomerase already reconstituted in insect cells without further step of reconstitution as described above. The bicistronic expression of hTERT and hTR components from the same RNA molecule may be a convenient method to facilitate the production of recombinant human telomerase. This approach has previously been established in yeast and in RRL (Bachand *et al.*, 2000). In our experiments, the hTERT-hTR *cis* construct expressed in insect cells produces an active telomerase enzyme, but low amounts of recombinant telomerase are detected. Theoretically, in our expression system, hTERT mRNA is expressed as a fusion with the hTR component. It is not clear yet if the single RNA molecule generated by the transcription of the hTERT-hTR *cis* transgene remains as a 4.0 kb RNA transcript, which could affect the levels of telomerase reconstitution and the affinity purification (Bachand *et al.*, 2000). As an alternative, the coexpression of both subunits in insect cells as two different RNA transcripts may improve the levels of expression and reconstitution of telomerase (Mikuni *et al.*, 2002), but it needs to be compared with our strategy.

Several investigations reported the partial purification of telomerase by conventional chromatography (Autexier *et al.*, 1996; Bednenko *et al.*, 1997; Masutomi *et al.*, 2000; Peng *et al.*, 2001; Wallweber *et al.*, 2003). Affinity purification against the

telomerase RNA component appears to be an efficient method to purify recombinant telomerase RNP complexes and native enzyme (Lingner and Cech, 1996; Schnapp *et al.*, 1998; Wenz *et al.*, 2001). This method was initially developed to purify telomerase from *Euplotes* (Lingner and Cech, 1996) and then adapted to the human cells (Schnapp *et al.*, 1998; Wenz *et al.*, 2001). Based on the affinity purification protocol, a 70-fold enrichment of the recombinant telomerase activity from insect cell extracts can be obtained (Wenz *et al.*, 2001). Although recombinant telomerase was not abundantly expressed in insect cells, the one-step affinity method may allow a rapid and efficient purification of the enzyme without the inconvenience of traditional purification techniques. The purification of the telomerase complex is necessary to provide an experimental system in which we can understand more clearly the multimerization state of telomerase, identify potential telomerase-associated proteins and characterize domains in both telomerase subunits that are essential in enzyme catalysis. In our experiments, we did not succeed to purify detectable amounts of recombinant human telomerase from insect cells in contrast to the native enzyme from human cells subjected to the same affinity purification procedure. Although we generated an active telomerase, most of the enzyme may not fold properly and/or may not bind to the biotinylated oligonucleotide. The native enzyme from 293 cells may be more stringently regulated. The optimization of the experimental conditions promoting the binding of the enzyme to the biotinylated oligonucleotide or the folding of telomerase by the coexpression of chaperone proteins may be useful. In the study, we also reported a convenient method of expressing hTERT in insect cells followed by the *in vitro* reconstitution of the telomerase enzyme in the

presence of *in vitro*-transcribed hTR. Eventually, the availability of large amounts of recombinant telomerase will constitute a valuable source of enzyme for structural studies.

4.6 *References*

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CHAPTER 5

Discussion

The telomerase enzyme is a specialized reverse transcriptase (RT) that maintains telomere length by synthesizing DNA repeats at the chromosomes ends. In contrast to the viral RTs that can reverse transcribe any sequences of potential RNA substrates transiently associated with the enzyme into DNA, hTERT and hTR form a highly stable complex that reverse transcribes repeatedly a well defined template region of its RNA subunit (reviewed in Nugent and Lundblad, 1998). The combination of these unique features results in an enzyme specialized for processive addition of telomeric DNA repeats at the chromosome ends.

5.1 *Reminder of the work hypothesis*

The work presented in this thesis focuses mainly on the biochemical and functional aspects of the human telomerase ribonucleoprotein. We were interested to understand the biochemical properties of human telomerase at the chromosome ends. We characterized particular features of the mechanism of action of the human telomerase enzyme. We analysed the role of the C-terminal domain of hTERT *in vitro* in a number of telomerase-specific functions (chapter 2). We also examined the initiation of extension of different DNA substrates *in vitro*, and we reported a telomerase-mediated nucleolytic cleavage activity (chapter 3). In our analyses, we identified human telomerase determinants of processivity and fidelity. The implications of these studies on how telomerase recognizes its DNA substrates and uses its template are discussed below.

5.2 *Functional role of the C-terminal region of hTERT*

We designed an *in vitro* experimental approach where hTERT containing amino acid deletions or substitutions located in its C-terminal domain were tested for

enzymatic activity. We provided evidence that the C-terminal region of hTERT is involved directly in the polymerase function (Huard *et al.*, 2003). In this study, the loss of catalytic activity of telomerase is attributed to the deleted or substituted residues, whereas the binding of hTR to these hTERT C-terminal mutants is not compromised. These functional results are consistent with other published observations (Beattie *et al.*, 2000; Bachand *et al.*, 2001; Lai *et al.*, 2001; Banik *et al.*, 2002), and we concluded that the C-terminal domain is dispensable for telomerase RNA binding, but it is clearly required for telomerase catalysis.

However, the role played by the C-terminal domain of hTERT *in vivo* has recently begun to be explored, but it remains largely uncharacterized. We found that one hTERT C-terminal mutant that we characterized contains an amino acid deletion which overlaps with a previously reported mutation in the C-terminal DAT region (Banik *et al.*, 2002). Although the exact function of the DAT region of hTERT remains to be resolved, the DAT mutant is catalytically active *in vitro*, but its ability to maintain telomere length *in vivo* is abolished (Banik *et al.*, 2002). The DAT region seems to be involved in the recruitment or targeting of hTERT to telomeres (Armbruster *et al.*, 2003; Armbruster *et al.*, 2004), since the nuclear localization of the DAT mutant and its ability to interact with hTR are not altered (Banik *et al.*, 2002). The telomere maintenance defect of the C-terminal DAT mutant may reflect an altered interaction between this mutant and telomeres, compromising telomere elongation once catalytically functional enzyme is assembled in the nucleus (Banik *et al.*, 2002; Lee *et al.*, 2003). We also found that telomerase activity requires catalytically essential C-terminal residues and intact RT-like

motifs on the same hTERT molecule (Huard *et al.*, 2003), consistent with previously published analyses of hTERT C-terminal truncations (Beattie *et al.*, 2001).

Other studies suggested that the C-terminal domain of hTERT may stabilize the telomere structure and maintain its integrity (Huang *et al.*, 2002; Huang *et al.*, 2003). Overexpression of the C-terminal fragment of hTERT induces telomere dysfunction and sensitizes HeLa cells to oxidative stress (Huang *et al.*, 2002; Huang *et al.*, 2003). In these studies, the C-terminal fragment of hTERT seems to act as a dominant negative mutant that could interfere with various components of telomeres and modulate cellular signalling pathways to stress responses (Huang *et al.*, 2002; Huang *et al.*, 2003).

We and others found that the C-terminal domain of TERT from several organisms has low sequence homology, suggesting species-specific biological functions of this region (Peng *et al.*, 2001; Banik *et al.*, 2002; Huard *et al.*, 2003). Perhaps the most interesting observation is that the first measurement of the levels of activity at nucleotide resolution revealed that some hTERT C-terminal mutants have defects in processivity (Huard *et al.*, 2003). Processivity is a common property of enzymes that carry out critical functions with nucleic acid metabolism (Subramanian *et al.*, 2003). The processivity of the telomerase complex can be defined as the number of nucleotides incorporated into a substrate during DNA synthesis before its dissociation from the DNA products. The molecular mechanisms that enable telomerase to remain stably bound to its substrate during catalysis are poorly understood, but they may involve the contribution of an anchor site which maintains the primer bound to the enzyme (Greider, 1991; Harrington and Greider, 1991; Morin, 1991; Collins and Greider, 1993; Hammond *et al.*,

1997; Lue and Peng, 1998). Since the processivity is governed by the relative probability of dissociation of the enzyme from its DNA substrate (Greider, 1991), the efficiency of DNA synthesis initiation, the nucleotide incorporation or the realignment of the extended primer on the template may affect the processivity of hTERT C-terminal mutants. As yeast TERT C-terminal truncation analyses also revealed defects in telomerase processivity (Peng *et al.*, 2001; Hossain *et al.*, 2002), the mechanistic similarity between human and yeast TERT may be greater than the apparent sequence identity anticipates (Peng *et al.*, 2001; Huard *et al.*, 2003).

Based on the results of our study, we propose that the annealing of the DNA substrate is unable to satisfy the molecular restriction imposed by telomerase containing hTERT C-terminal mutations. The DNA substrate may not be optimally annealed leading to the formation of inert complexes favouring the pausing or the dissociation of the primer. This pausing would temporarily inhibit nucleotide addition, resulting in an inefficiency of DNA polymerization and translocation events. The transcript release has also been proposed when transcriptional arrests of *Escherichia coli* RNA polymerase is induced by an RNA transcript that is inappropriately positioned during RNA synthesis (Artsimovitch and Landick, 2000; Neuman *et al.*, 2003). In our system, the influence of specific features which change the dynamic of the enzyme/substrate complex affects the processivity. The biological significance of these hypotheses needs to be further addressed.

Furthermore, *in vivo* experiments expressing hTERT C-terminal mutants would allow us to determine whether the *in vitro* processivity of telomerase that we observed correlates with the *in vivo* processivity of the enzyme at telomeres. Mouse

telomerase is a nonprocessive enzyme *in vitro*, but it maintains long telomeres *in vivo* (Prowse *et al.*, 1993; Prowse and Greider, 1995). On the other hand, *Tetrahymena thermophila* telomerase is very processive *in vitro*, but it is not processive *in vivo* (Yu *et al.*, 1990; Greider, 1991). This may imply the role of associated proteins that regulate telomerase functions. Biochemical fractionation of cellular protein extracts would be useful to identify potential factors mediating the processivity in addition to telomerase.

5.3 *Telomerase-mediated nucleolytic cleavage activity*

During DNA synthesis, the telomerase enzyme must be accurate. Serious functional consequences leading ultimately to cell death may occur by altering the sequence of telomeric DNA repeats at the chromosome ends (Yu *et al.*, 1990; McEachern and Blackburn, 1995; Gilley and Blackburn, 1996; Marušic *et al.*, 1997; Ware *et al.*, 2000; Guiducci *et al.*, 2001). The binding of telomere-associated proteins may be altered, compromising the telomere capping function. Thus the fidelity of DNA replication is a key determinant of telomere stability.

One aspect of the DNA synthesis that has received much interest is how polymerases prevent misincorporation of nucleotides. Occasionally, an error is made in DNA replication and an incorrect nucleotide is incorporated into the DNA chain being synthesized. The mismatched base has a very high probability to be excised before the next base in the chain is added. Error rates for single-base substitutions due to proofreading-deficient DNA polymerases vary from 10^{-3} to 10^{-6} per nucleotide, depending on the identity of the DNA polymerase, the type of mismatch and the local sequence environment (reviewed in Kunkel and Bebenek, 2000).

An *in vitro* study measured the error rates for single-base substitutions of human telomerase at all positions within the telomeric DNA repeat TTAGGG. The error rate of human telomerase was estimated at $2x10^{-3}$ per nucleotide (Kreiter *et al.*, 1995), being similar to DNA polymerase. This error rate was more significant at the A position, suggesting that the misincorporation of nucleotides at different positions within the repeat seems to be differently tolerated by the enzyme (Kreiter *et al.*, 1995). Another study demonstrated that *Paramecium tetraurelia* telomerase is inherently imprecise (McCormick-Graham and Romero, 1996). Frequent dTTP misincorporations are responsible for the occasional detection of variable repeats at telomeres (McCormick-Graham *et al.*, 1997; Ye *et al.*, 1999). The hypothesis of the presence of several telomerase RNAs with different template sequences in the cell has been ruled out (McCormick-Graham and Romero, 1996). Moreover, *Tetrahymena thermophila* telomerase bearing a specific mutation in its template exhibits an infrequent misincorporation of dATP (Gilley *et al.*, 1995), whereas a specific template mutation of *Paramecium tetraurelia* telomerase inhibits the tendency of dTTP misincorporations (McCormick-Graham *et al.*, 1997; Ye *et al.*, 1999). The molecular mechanisms underlying these phenotypes are unknown. Different biochemical properties of *Saccharomyces cerevisiae* telomerase also contribute to synthesize variable sequences of telomeric DNA repeats *in vivo* (Förstemann and Lingner, 2001). This repeat diversity results from several alignment possibilities of the DNA substrate with the telomerase RNA template and frequent incomplete reverse transcription of the template by telomerase rather than a presumed tendency of dNTP misincorporations (Förstemann and

Lingner, 2001). All together, these studies suggest that the fidelity of telomerase is highly regulated.

The high fidelity of DNA synthesis is also achieved through molecular mechanisms which correct the mistakes after incorporation. In addition to their DNA polymerization activity, several polymerases exhibit an exonucleolytic activity (reviewed in Shevelev and Hübscher, 2002). Polymerases can proofread and enhance the accuracy of DNA synthesis by excising incorrectly polymerized nucleotides. This proofreading ability results in an extremely low error frequency in inserting the wrong base during DNA replication. In our study, we presented an experimental approach to monitor a potential cleavage activity performed by telomerase (Huard and Autexier, 2004). We assayed different DNA substrates in order to characterize the specificity of the nucleolytic cleavage (Huard and Autexier, 2004). Unfortunately, we have been unable to develop a nucleolytic cleavage assay that is independent of elongation activity. However, the nuclease activity remains associated with the partially purified telomerase enzyme, and this argues in favour of a mechanistically linked cleavage/elongation process (Huard and Autexier, 2004; Oulton and Harrington, 2004).

We found that human telomerase can cleave nucleotides from telomeric DNA substrates that are fully complementary with the template, suggesting that the enzyme does not follow the classical rules of proofreading (Huard and Autexier, 2004). We and others also found that mismatches which have been introduced by alignment of DNA substrates with the RNA template are excised prior to substrate elongation (Huard and Autexier, 2004; Oulton and Harrington, 2004). The nucleolytic cleavage performed by the enzyme seems to be directed at a specific position within the template (Huard and

Autexier, 2004; Oulton and Harrington, 2004). Taken together, these data reveal several unique features of telomeric DNA synthesis that distinguish this enzyme from the other members of the reverse transcriptase family.

The nucleolytic cleavage activity that is reported for several telomerase enzymes from different organisms is analogous to the intrinsic endonuclease activity of RNA polymerase (reviewed in Fish and Kane, 2002). RNA polymerase cleavage is enhanced at the position of the incorrectly polymerized nucleotides and is often accompanied by a backward movement of the enzyme on the substrate prior to the cleavage (Lee *et al.*, 1994; Wang and Landick, 1997). We speculate, as have others, that the telomerase-mediated nucleolytic cleavage activity may serve a proofreading function (Collins and Greider, 1993; Melek *et al.*, 1996; Greene *et al.*, 1998; Niu *et al.*, 2000; Huard and Autexier, 2004; Oulton and Harrington, 2004). However, the cleavage activity may be involved in other functions, as described below.

As we demonstrated, the cleavage, particularly marked in the RRL-reconstituted telomerase compared to the native enzyme partially purified from human cells, may result from a high frequency of stalling of human telomerase at several positions along the RNA template. An high frequency of stalling and substrate dissociation of yeast telomerase has been reported to generate irregular telomeric DNA repeats (Cohn and Blackburn, 1995; Förstemann and Lingner, 2001). We propose that the nuclease activity may be a way to prevent or rescue stalled complexes, which would facilitate the substrate elongation (Collins and Greider, 1993; Melek *et al.*, 1996; Greene *et al.*, 1998; Niu *et al.*, 2000; Huard and Autexier, 2004; Oulton and Harrington, 2004). It is possible that the more processive nature of the native telomerase or telomerase-

associated factors regulate more stringently the alignment and binding of DNA substrates with the RNA template, preventing possible arrests of DNA synthesis. It would be consistent with the role of TFIIS, GreA and GreB as cleavage stimulatory factors that enhance the cleavage of the nascent RNA transcript to recover the RNA polymerase from an arrested state (reviewed in Fish and Kane, 2002). The new RNA transcript 3'-end is repositioned within the polymerase catalytic center in order to continue the transcription process (reviewed in Arndt and Kane, 2003).

A better conformation of the catalytic core of the native telomerase is also possible and may regulate the cleavage activity more efficiently than the RRL-reconstituted enzyme. The identification of telomerase-associated factors that mediate the cleavage activity would be useful to address the biological significance of these hypotheses. Ultimately, the development of an assay monitoring the DNA cleavage that is independent of the elongation activity would be important for future investigations. Oligonucleotides that would be detectable after a cleavage event without being elongated need to be developed and tested.

5.4 *Concluding remarks*

In vitro studies of RRL-reconstituted telomerase demonstrate that the minimal catalytic core, TERT and TR, is sufficient to generate telomerase activity, but other experiments indicate that additional factors are required for *in vivo* maturation, assembly, recruitment and/or activation of the RNP. Genetic studies in yeast have identified several factors that are essential for *in vivo* activity of telomerase at telomeres, suggesting that the *in vivo* regulation of telomerase is much more complex than *in vitro*. Our emphasis on structural and biochemical studies of human telomerase provides new

insights about the importance of the efficiency and fidelity of telomeric DNA synthesis. In our study, the processivity and cleavage activity appear specifically to modulate the interactions between the telomerase complex and the DNA substrate during the DNA polymerization cycle. Finally, detailed structural and functional analyses of the human telomerase enzyme are currently limited by the lack of an abundant recombinant source of active enzyme. As we and others set up experimental conditions to express recombinant telomerase in insect cells, the ultimate goal would be the possibility to generate a crystal structure of the enzyme to perform detailed studies of the mechanism of action of telomerase and ultimately create potent and specific inhibitors.

CHAPTER 6

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