FUNCTIONAL RECONSTITUTION AND RNA-PROTEIN INTERACTIONS OF HUMAN TELOMERASE

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À Audrey, ma mère et mon père

ABSTRACT

Telomerase is a ribonucleoprotein (RNP) enzyme responsible for the replenishment of repetitive DNA sequences present at the ends of most eukaryotic chromosomes. Telomerase is minimally composed of a protein catalytic subunit, the telomerase reverse transcriptase (TERT), and an RNA subunit. Using a small single-stranded segment (7-11 nucleotides) of the telomerase RNA as a template, the active site of the TERT catalytic subunit adds complementary nucleotides onto telomeric DNA. The primary goal of the work presented in this thesis was to biochemically and functionally characterize the human telomerase reverse transcriptase (hTERT) and the human telomerase RNA (hTR), as well as to identify novel telomerase-associated proteins. First, we demonstrated that the budding yeast *Saccharomyces cerevisiae* possesses the cellular machinery to fully assemble a catalytically active human telomerase RNP *in vivo*. We also analyzed telomerase activity and binding of hTR to hTERT in rabbit reticulocyte lysates by expressing different hTERT and hTR variants. Our results identified two distinct regions of hTR that can independently bind hTERT *in vitro*. Furthermore, sequences or structures that include the conserved CR4-CR5 domain of hTR were found to be important for hTERT-hTR interactions and telomerase activity reconstitution. Human TERT carboxy- and amino-terminal amino acid deletions indicated that the polymerase and RNA binding functions of hTERT are separable. We also found that the product of the *survival of motor neuron* (*SMN*) gene, a protein involved in the biogenesis of certain RNPs, is a telomerase-associated protein. Our results demonstrate that the human TR and the human TERT are not associated with Sm proteins, in contrast to *Saccharomyces cerevisiae* telomerase. Taken together, the work presented in this thesis

indicate that the reconstitution of human telomerase activity *in vitro* requires regions of hTERT that (i) are distinct from the conserved reverse transcriptase motifs; and (ii) bind nucleotides distal to the hTR template sequence. Furthermore, our work establishes SMN as a novel telomerase-associated protein that is likely to function in human telomerase biogenesis.

SOMMAIRE

La télomérase est l'enzyme responsable du maintien des séquences d'ADN répétitives présentes aux bouts des chromosomes chez la plupart des organismes eukaryotes. La télomérase consiste essentiellement en une sous-unité catalytique (TERT: telomerase reverse transcriptase) et une molécule d'ARN (TR). Une portion de cet ARN contient la séquence complémentaire de la répétition d'ADN télomérique et agit ainsi comme molécule matrice lors de l'addition de nouveaux nucléotides. L'objectif majeur de cet ouvrage était de caractériser biochimiquement les propriétés et les fonctions des composantes protéiques (hTERT) et ARN (hTR) de la télomérase humaine, ainsi que la biogénèse de ce complexe ribonucléoprotéique. Dans un premier temps, nous avons démontré que la levure *Saccharomyces cerevisiae* possède toutes les composantes cellulaire *in vivo* afin d'assembler une télomérase humaine catalytiquement active. L'activité de la télomérase ainsi que la liaison de la composante ARN avec la sous-unité protéique ont été étudiées en exprimant différentes molécules de hTERT et hTR mutées dans un système d'expression de réticulocytes de lapin. Nos résultats ont identifiés deux régions de hTR capable de se lier avec hTERT de façon indépendante *in vitro*. De plus, des séquences ou des structures secondaires qui contiennent le domaine conservé CR4-CR5 de hTR on été démontrées comme étant importante pour l'association entre hTERT et hTR ainsi que pour la reconstitution de l'activité de la télomérase. Des études de délétion aux extrémités N- et C-terminale de hTERT ont démontré que les fonctions de polymérisation et de liaison à l'ARN de hTERT sont indépendantes. Nous avons aussi trouvé que le produit du gène *survival of motor neuron* (*SMN*) est associé à la télomérase humaine. Nos résultats démontrent que, contrairement aux composantes de la télomérase

de la levure *Saccharomyces cerevisiae*, hTR et hTERT ne se fixe pas aux différentes protéines Sm. L'ensemble des observations presentées dans cette thèse démontre que la reconstitution d'une télomérase active *in vitro* nécessite des régions de hTERT (i) distinctes des motifs conservés de transcriptase inverse; et (ii) se fixant à des séquences de hTR autres que la région matrice. De plus, le travail présenté dans cet ouvrage établit SMN comme étant une protéine associée à la télomérase qui est probablement impliquée dans l'assemblage de ce complexe ribonucléoprotéique.

PREFACE

This Ph.D. thesis was written in accordance with the Guidelines for Thesis preparation from the Faculty of Graduate Studies and Research of McGill University. I have exercised the option of writing the thesis as a manuscript-based thesis. For this, the guidelines state: "...Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. .....The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory. ....The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary. ....In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. .....In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. ....."

As chapters of this thesis, I have included the texts and figures of three original manuscripts that have been published or accepted for publication. Each of these chapters (Chapters 2, 3, and 4) contains its own summary, introduction, materials and methods, results, discussion, and references sections. In addition, a preface is included at the beginning of each chapter in order to introduce and bridge the papers with connecting texts. A general introduction and literature review is presented in Chapter 1, whereas a final discussion is included in Chapter 5. The references for chapters 1 and 5 are included at the end of the thesis.

Papers included in this thesis:

Chapter 2 **Bachand, F.** and C. Autexier. (1999) Functional reconstitution of human telomerase expressed in *Saccharomyces cerevisiae*. J. Biol. Chem. **274**: 38027-38031.

Chapter 3 **Bachand, F.** and C. Autexier. (2001) Functional regions of human telomerase reverse transcriptase and human telomerase RNA required for telomerase activity and RNA-protein interactions. Mol. Cell. Biol. **21**: 1888-1897.

Chapter 4 **Bachand, F.**, Boisvert, FM., Coté, J., Richard, S., and C. Autexier. (2002) The product of the *survival of motor neuron* (*SMN*) gene is a human telomerase-associated protein. Mol. Biol. Cell. **13**: 3192-3202.

Contribution of Authors:

The candidate performed most of the research presented in this thesis and wrote all of the included manuscripts with support from Dr. Autexier. The contribution of other authors to this work is described below:

In Chapter 5, François-Michel Boisvert constructed the YFP-SmB plasmid used in Fig. 4-4, whereas Dr Jocelyn Côté constructed plasmids expressing myc-tagged SmB and SmD3 proteins used in Fig. 4-3. Dr Stéphane Richard provided the anti-myc (9E10) antibody, DNA oligos specific for the U1 and U6 snRNAs (Fig. 4-3), and the myc-SmB, myc-SmD3, YFP-SmB, and YFP-SmD1 expression constructs.

The different studies were all conducted under the supervision of Dr Chantal Autexier.

In addition to the papers included in this thesis, the candidate contributed to the following studies, which have been published or submitted:

Bachand, F., Kukolj, G., and C. Autexier. (1999) Expression of hTERT and hTR in cis reconstitutes an active human telomerase ribonucleoprotein. RNA. **6**: 778-784.

Bachand, F*., Triki, I*., and C. Autexier. (2001) Human telomerase RNA-protein interactions. Nucleic Acids Res. **29**: 3385-3393. (*these authors contributed equally to the work)

Boisvert, FM., Côté J., Boulager, MC., **Bachand F.**, Autexier, C., and S. Richard. (2002) Arginine methylation is required for SMN localization in Cajal bodies and nuclear pre-mRNA splicing. Submitted.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF ABBREVIATIONS

ATM	mutated in ataxia-telangiectasia
ALT	alternative lengthening of telomeres
C-	carboxy
CB	Cajal bodies or coiled bodies
CDC	cell division cycle
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CR	conserved region
DAPI	4, 6-diamidino-2-phenylindole
DBD	DNA binding domain
DKC	dyskeratosis congenita
DN	dominant-negative
DNA	deoxyribonucleic acid
DSB	double-stranded break
EST	ever shorter telomeres
GFP	green fluorescent protein
GST	glutathione-S-transferase
HIV	human immunodeficiency virus
hnRNP	heterogeneous ribonucleoprotein
HPV	human papilloma virus
HSP	heat shock protein
hTERT	human telomerase reverse transcriptase
hTR	human telomerase RNA
IC	internal control
kb	kilobase
kDa	kiladalton
LT Ag	large T antigen
mRNA	messenger RNA
N-	amino

NHEJ	non-homologous end joining
PAGE	polyacrylamide gel
PCR	polymerase chain reaction
PBS	phosphate buffered saline
Pol	polymerase
RAP	repressor and activator protein
Rb	retinoblastoma
RFC	replication factor C
RIF	rap1-interacting factor
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein
RRL	rabbit reticulocyte lysate
rRNA	ribosomal RNA
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SIR	silent information regulator
SMN	survival of motor neuron
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
SV40	simian virus 40
TAZ	telomere-associated in *Schizosaccharomyces pombe*
TEP	telomerase-associated protein
TLC	telomerase RNA component
TMG	trimethylguanosine
TPE	telomere position effect
TRAP	telomeric repeat amplification protocol
TRF	TTAGGG repeat factor
UAS	upstream activator sequence
YFP	yellow fluorescent protein

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The evolution of a single genetic failure into a metastatic tumor is a complex and multi-faceted process. Cancer is chaos within the highly regulated and ordered machine that is the mammalian cell. For many years now, cancer biologists have focused on how specific genetic alterations interfere with the ordered process of cell cycle control. Indeed, genes coding for important cell cycle checkpoint proteins are mutated in many different types of human cancers. However, the work of many different scientists on several aspects of chromosome structure and how chromosomal ends are replicated created a whole new field in oncology: the field of telomere biology.

Telomeres are essential nucleoprotein structures present at the ends of chromosomes, whereas telomerase is the specialized reverse transcriptase that replenishes telomeres with new DNA sequences (Collins and Mitchell, 2002). The critical importance of telomeres and telomerase in biology is demonstrated by their conservation throughout most of the eukaryotic kingdom (Wellinger and Sen, 1997; Collins, 2000). Notably, the finding that telomerase activity is present in over 85% of human cancer cells, whereas it is absent from most human somatic cells led to the recognition of the critical role played by telomerase in cellular immortalization and cancer biology (Kim et al., 1994). Conversely, data from the genetically engineered telomerase-negative mouse model and the discovery that the human degenerative disease dyskeratosis congenita results from defects in the telomerase machinery led to the notion that telomerase could

potentially be used as a therapeutic agent in degenerative diseases (Lee *et al.*, 1998; Mitchell *et al.*, 1999b). Telomerase is a ribonucleoprotein complex that minimally consists of an RNA component and a protein catalytic subunit (Nugent and Lundblad, 1998). Telomerase exhibits RNA-dependent DNA polymerase activity and uses a small subset of the telomerase RNA sequence as template for the synthesis of complementary sequences at the 3' ends of linear chromosomal DNA.

The work presented in this thesis focuses on the biochemical characterization of the human telomerase ribonucleoprotein. A better understanding of the biochemical properties of mammalian telomerase will help in the development of molecules that could either inhibit or activate telomerase. In this chapter, I will first review the biology and the major components of telomeres and telomerase in different model organisms. Then, I will describe the concept of replicative senescence and the role played by telomeres and telomerase in cellular immortalization and cancer.

1.2 Telomeres

Telomeres are specific DNA-protein complexes that cap the ends of eukaryotic chromosomes. In the absence of a distinctive telomere structure, the cellular proteins responsible for the recognition and repair of double-stranded DNA breaks (DSBs) would be incapable of distinguishing between a normal chromosomal end and a fortuitous DSB within genomic DNA. Unprotected telomeres are also subject to nuclease degradation, DNA recombination, and chromosome end-to-end fusions, all of which can lead to the loss of coding sequences and/or aberrant gene expression (Harrington and Robinson, 2002). Thus, telomeres are essential structures that provide stability to the genome. In

the following sections, I will review the sequence organization of telomeric DNA and their associated proteins in different model organisms. The organization of telomere-proximal DNA sequences is surprisingly well conserved in eukaryotes and can be divided in three distinct regions as shown in Figure 1-1: (1) subtelomeric or telomere-associated sequences; (2) the double-stranded (ds) telomeric repeats; and (3) the single-stranded 3' overhang.

1.2.1. Subtelomeres

Subtelomeric sequences are particular regions of the eukaryotic genome that establish the transition between chromosome-specific sequences and the telomere tandem repeats (discussed below). Though different in primary nucleotide sequences, the overall organization of the subtelomeric regions of human, yeast, and trypanosomes is remarkably conserved (Wellinger and Sen, 1997; Mefford and Traks, 2002). First, long stretches of sequences (that can reach a few hundred kilobases in humans) are shared by certain chromosome ends in their centromere-proximal portion. Second, shorter blocks of sequences that generally consists of different stretches of tandem repeats can be found in the most distal part of the subtelomeric region. Third, these proximal and distal subtelomeric domains are usually separated by degenerate blocks of telomeric sequence repeats. The DNA sequences within the more distal subtelomeric region are usually more homogeneous between chromosomal ends than the larger centromere-proximal region (Mefford and Traks, 2002). This subtelomere organization is often referred as the two-domain structure. This is based mostly on sequence comparison of several human and yeast telomeres (Flint *et al.*, 1997), and thus, might not be universal. The growing

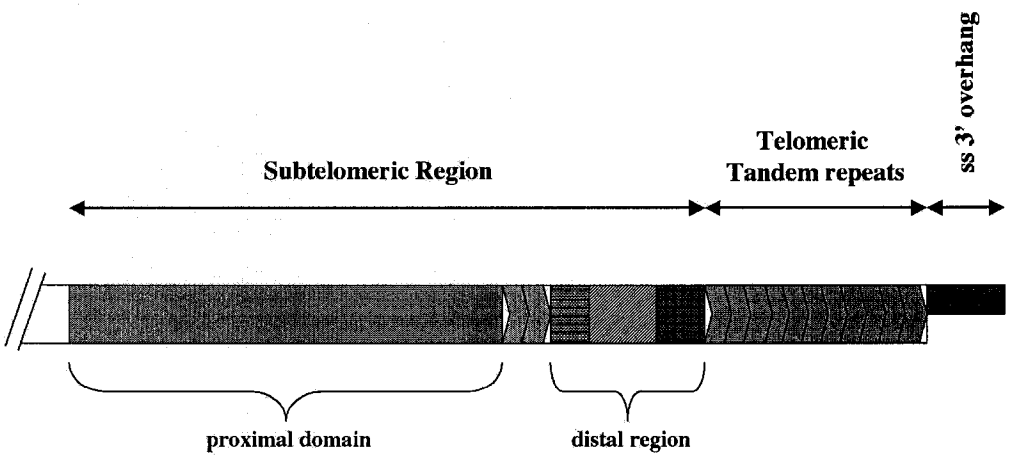

Figure 1-1. The Organization of Telomere-Proximal Sequences in Eukaryotes

number of complete genome sequences from a variety of organisms will enable a better comparison between the subtelomeres of different eukaryotes.

The functional role played by subtelomeric sequences is not clearly understood, yet using genetically tractable organisms such as *S. cerevisiae* and *Drosophila*, it was initially established that reporter genes inserted nearby telomeres are in most cases transcriptionally repressed (Levis *et al.*, 1985; Gottschling *et al.*, 1990). This phenomenon is known as telomere position effect (TPE). However, it is not yet clear whether subtelomeric sequences have a direct role in TPE (Mefford and Traks, 2002; Tham and Zakian, 2002). The potential of subtelomeres for recombination might also be involved in the maintenance of telomeres in the absence of telomerase. By combining the sequences available from the working draft of the human genome and previously isolated telomere-terminal fragments, a recent study reveals that many human subtelomeric regions appear to be rich in gene-coding sequences, including some known expressed genes (Riethman *et al.*, 2001). It will be interesting to determine whether these human genes are transcriptionally silenced due to TPE.

1.2.2 Double-Stranded Telomere Repeats

The identification of the first tandem telomere repeats was from unicellular protozoa such as *Tetrahymena*, *Oxytricha*, and *Trypanosoma* species (Blackburn and Gall, 1978; Klobutcher *et al.*, 1981; Blackburn and Challoner, 1984). During a specific stage of their lifecycle, the micronuclear genome of these organisms is converted to transcriptionally active macronuclei by the specific fragmentation of chromosomes into thousands of telomere-capped DNA molecules (Asai and Forney, 2000). The abundance of

chromosomal ends within the macronuclei of these protozoa was instrumental to the identification of the first telomeric sequences.

With the avenue of the polymerase chain reaction and cloning techniques, telomere sequence repeats from a variety of organisms were identified. As can be seen in Table 1-1, the sequence of the repeat segment of telomeres are clearly related, in contrast to the lack of conservation between subtelomeric sequences of different organisms. In most eukaryotes, telomeric DNA consists of repeated arrays of 6-8 bp sequences. The polarity of the telomere repeats is also conserved throughout most eukaryotes; with one strand rich in guanosine and thymidine and the other strand rich in cytosine and adenine (see Table 1-1). The vast majority of telomerase-generated telomeric sequences are relatively simple and highly repetitive, yet there are some exceptions such as for the yeast *Saccharomyces* and *Schizosaccharomyces* where the repeat pattern is more complex (Table 1-1). This might reflect some inherent differences in the biochemical properties of the telomerase reverse transcriptases (Forstemann and Lingner, 2001). The actual number of telomere repeats, and thus the length of telomeres, varies considerably between organisms (Table 1-1). Furthermore, the number of repeats can also vary between individual chromosome ends in the same organism and different cell types (Forstemann *et al.*, 2000; Suda *et al.*, 2002). This heterogeneity in telomere length is reflected by the smeary pattern of telomeric sequences when restriction fragments of genomic DNA are analyzed by Southern blotting using a telomere-specific probe.

Although most eukaryotes maintain the length of their telomeres using the telomerase-based addition of simple sequence repeats, some organisms do not have telomeric sequences consisting of arrays of small tandem repeats. The best-studied

Table 1-1. ***Telomeric simple-sequence repeats***

Organisms	Repeat sequence[a]	Telomere length
Protozoa		
Tetrahymena	T_2G_4	~300 bp
Oxytricha	T_4G_4	~20 bp[b]
Euplotes	T_4G_4	~20 bp[b]
Trypanosoma	T_2AG_3	10-20 kb
Fungi		
Saccharomyces	$T(G)_{2-3}(TG)_{1-6}$	200-350 bp
Kluyveromyces	$G_2TATGTG_2TGT$	250-500 bp
Schizosaccharomyces	$T_{1-}2ACA_{0-1}C_{0-1}G_{1-6}$	250-500 bp
Plants		
Arabidopsis	T_3AG_3	2-4 kb
Vertebrates		
Homo sapiens	T_2AG_3	5-20 kb
Mus musculus	T_2AG_3	10-100 kb

[a] 5' to 3' toward the physical end of the chromosomes
[b] macronuclear chromosomes

7

example is the fruit fly *Drosophila melanogaster* where telomeres consist of a mixture of large sequence elements generated by the transposition at telomeres of two major reverse transcribed non-LTR polyadenylated RNAs (Pardue and DeBaryshe, 1999). Other insect species such as the midge *Chironomus* and the mosquito *Anopheles gambiae* also lack short tandem arrays of telomeric repeats and maintain their telomeres via a recombinational pathway rather than using retrotransposition (Roth *et al.*, 1997; Lopez *et al.*, 1999).

1.2.3 Telomeric Single-Stranded 3' Overhang

The initial analysis of the structural configuration of the telomeric extremities from ciliates suggested the presence of a 12-16 nt overhang (Klobutcher *et al.*, 1981; Henderson and Blackburn, 1989). The demonstration that the telomeric G-rich strand generates a 3' overhang has been reported for several species including protozoa, yeast, plants, and vertebrates (Henderson and Blackburn, 1989; Dionne and Wellinger, 1996; Makarov *et al.*, 1997; Riha *et al.*, 2000). Using variations of a primer extension-nick translation protocol, 3' overhangs in the range of 12-16 nt in ciliates to 150-200 nt in humans have been detected. Interestingly, the 3' overhang of *S. cerevisiae* is only detected in late S phase and disappears thereafter (Dionne and Wellinger, 1996), whereas ciliate and human 3' overhangs are detectable throughout the cell cycle (McElligott and Wellinger, 1997; Wright *et al.*, 1997; Jacob *et al.*, 2001). As will be discussed in more detail in the next sections, different organisms have made use of specific telomere-binding proteins to protect their telomeric 3' overhangs from potential nuclease digestion. Furthermore, as telomerase has only been shown to extend single-stranded

oligonucleotides *in vitro*, the G-rich 3' overhang is likely to be the *in vivo* substrate for telomerase. Although the specific role of the 3' overhang is still ill defined, it is clear that it is critical for telomere function.

1.3 Telomere-Binding Proteins

Analysis of telomere structure using conventional assays for histone-mediated nucleosomal patterns determination revealed that most of the telomeric DNA is non nucleosomal (Wright *et al.*, 1992). Later, non-histone proteins that bind the telomere simple sequence repeats were identified in several organisms. These proteins are likely to be responsible for many, if not all, of the different functions related to telomeres. In the following sections, I will introduce the major telomere-binding proteins of (1) humans, (2) yeast, and (3) protozoa.

1.3.1 Humans

The identification of a protein that binds oligonucleotide duplexes harboring multiple repeats of the TTAGGG sequence was the first report of a putative human telomere repeat-binding protein (Zhong *et al.*, 1992). The protein responsible for this specific DNA-binding activity was named TRF1 for TTAGGG repeat factor 1. Cloning of the TRF1 cDNA confirmed that the encoded protein was indeed a human telomere binding protein. First, immunofluorescence analysis using TRF1-specific antibodies demonstrated the colocalization of this protein with telomeric DNA (Chong *et al.*, 1995). Second, overexpression of TRF1 in immortalized and telomerase-positive cells resulted in the shortening of human telomeres (van Steensel and de Lange, 1997).

Homodimerization of TRF1 is an absolute requirement for its TTAGGG-binding activity (Bianchi *et al.*, 1997). Overexpression of a dominant-negative deletion mutant of TRF1 capable of homodimerization, but not of DNA-binding activity sequesters the endogenous TRF1 protein from the telomeres and results in telomere elongation (van Steensel and de Lange, 1997). Thus, based on these studies it is clear that TRF1 is a human telomere-binding protein involved in telomere length homeostasis, possibly through the negative regulation of telomerase-mediated elongation.

TRF2 is another key mammalian telomere-binding protein identified by two independent groups based on its sequence homology to TRF1 (Bilaud *et al.*, 1997; Broccoli *et al.*, 1997). The sequences of the human TRF1 and TRF2 proteins are surprisingly similar and sequence comparison reveals the conservation of two domains. First, their carboxy-terminal region harbors a helix-turn-helix DNA-binding domain related to the Myb transcriptional regulator. The second conserved region is a ~200 amino acids domain (TRF homology (TRFH)) that was previously characterized to be responsible for TRF1 dimerization (Bianchi *et al.*, 1997). The crystal structures of the TRFH domains of TRF1 and TRF2 was recently resolved and reveal different and specific dimerization interfaces that prevent possible heterodimerization between TRF1 and TRF2 (Fairall *et al.*, 2001). Like TRF1, TRF2 localizes to human telomeres (Broccoli *et al.*, 1997) and its overexpression affects telomere homeostasis (Smogorzewska *et al.*, 2000). However, whereas the overexpression of dominant-negative (DN) mutants of TRF1 results in telomere elongation (van Steensel and de Lange, 1997), the expression of a DN TRF2 induces a very different phenotype, including loss of the single-stranded 3' overhangs, chromosomal end-to-end fusions, and ATM/p53-

dependent apoptosis (van Steensel *et al.*, 1998; Karlseder *et al.*, 1999). Thus by 1999, the studies on TRF1 and TRF2 suggested that TRF1 is responsible for telomere length regulation and that TRF2 functions in (1) the protection of chromosomal ends by preventing the recognition of telomeres as DNA damage, and (2) in the activation of DNA damage response pathways in situation of inappropriate telomeric DNA exposure.

What type of telomere structure would explain the experimental data provided for TRF1 and TRF2? In May of 1999, it was reported that mammalian telomeres end as large terminal (T) loops, rather than ending as linear molecules as was previously thought (Griffith *et al.*, 1999). Incubation of recombinant TRF2, but not TRF1, with DNA molecules consisting of unique sequences followed by tandem arrays of ds(TTAGGG) including a 150-200 nt 3' overhang results in the formation of T-loops *in vitro* (Griffith *et al.*, 1999). By isolating telomeric DNA from different human cell lines, Griffith and colleages more importantly showed that T-loops exist at human telomeres *in vivo* (Griffith *et al.*, 1999). Based on this study and more recent findings (Stansel *et al.*, 2001), a model for the high-order structure of human telomeres was proposed and is shown in Figure 1-2. This model proposes that human telomeres are sequestered from DNA repair pathways by the formation of large duplex loops where the 3' overhang invades the duplex region into preceding telomeric tracts to form a lariat-like structure (Figure 1-2). Indeed, blunt-ended or 5' overhang-containing telomeric DNA templates are inefficient in loop formation *in vitro* (Griffith *et al.*, 1999; Stansel *et al.*, 2001). Because TRF2 is sufficient for efficient formation of T-loops *in vitro* and can be visualized directly at the loop junction (Stansel *et al.*, 2001), it is thought to stabilize the duplex loops. Consequently, this structure hides the telomeric 3' overhang from the machinery that

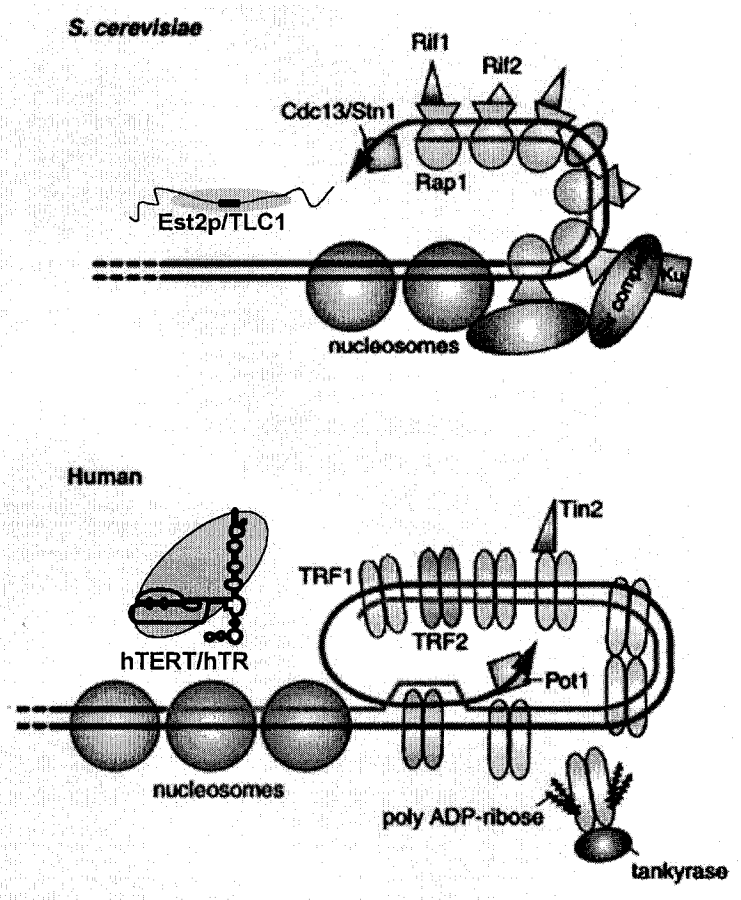

Figure 1-2. Higher-Order Structure of human and Yeast Telomeres with Associated Telomere-Binding Proteins **(Adapted from Blackburn (2001) Cell, *106*: 661)**

12

scans for broken DNA ends. Furthermore, the T-loop model explains the rapid and detrimental effects caused by the overexpression of mutant TRF2 that perturbs the integrity of the ss 3' overhangs without affecting the length of the double-stranded TTAGGG repeats (van Steensel *et al.*, 1998). Depending on the cell line, the effect of DN TRF2 is sufficient to (1) activate the ATM/p53-dependent DNA damage pathway and (2) induce end-to-end chromosome fusions (van Steensel *et al.*, 1998; Karlseder *et al.*, 1999). The association of the p53 DNA-binding protein with telomeric ss 3' overhangs and T-loop junctions *in vitro* was recently reported (Stansel *et al.*, 2002), providing further support to these results. T-loops were also isolated and visualized in *Trypanosoma* and *Euplotes*, two species of unicellular protozoa, suggesting that this telomere structure was conserved during evolution (Murti and Prescott, 1999; Munoz-Jordan *et al.*, 2001).

TRF1 and TRF2 lack ssDNA-binding activity and human telomeres harbor 100-250 nt 3' overhangs. Consequently, the question as to whether T-loops are sufficient to completely protect the ss 3' overhang or if a human ss telomeric-binding protein exists was open. Telomeric ssDNA-binding proteins were identified in *S. cerevisiae* (Nugent *et al.*, 1996), *Oxytricha* (Gottschling and Zakian, 1986), and *Euplotes* (Wang *et al.*, 1992). The recent cloning of a single-stranded telomeric DNA-binding protein from the fission yeast *S. pombe* (Pot1, for protection of telomeres) led to the identification of its human ortholog (Baumann and Cech, 2001). Though human Pot1 specifically binds ss TTAGGG oligonucleotides as analyzed by electrophoretic mobility shift assays (Baumann and Cech, 2001), functional evidence demonstrating that Pot1 is a true human telomere binding protein is still lacking.

Using TRF1 as bait in the yeast two-hybrid system, two new human telomere-binding proteins were identified: Tankyrase (Smith *et al.*, 1998) and the TRF1-interacting nuclear protein 2 (TIN2; (Kim *et al.*, 1999)). Tankyrase contains 24 ankyrin repeats and a catalytic domain for poly (ADP-ribose) polymerase. ADP-ribosylation inhibits the ability of TRF1 to bind telomeric DNA *in vitro* (Smith *et al.*, 1998). Consistently, overexpression of tankyrase in human telomerase-positive cells leads to reduced levels of TRF1 at telomeres and to a gradual and progressive elongation in telomere length (Smith and de Lange, 2000), suggesting that tankyrase-mediated ADP-ribosylation of TRF1 opens the telomeric complex to allow telomerase access.

1.3.2. Yeast

Yeast has been the model of choice to study the regulation of telomere length control due to the ease with which they can be genetically manipulated. The major and most characterized duplex telomere-binding protein in *S. cerevisiae* and *S. pombe* are Rap1p (Repressor and activator protein) and Taz1p (Telomere-associated in *Schizosaccharomyces pombe*), respectively. Similarly to human TRF1 and TRF2, the Rap1p and Taz1p proteins use Myb-like DNA-binding domains to directly associate with tandem telomeric DNA repeats (Buchman *et al.*, 1988; Konig *et al.*, 1996; Cooper *et al.*, 1997). Once telomere-bound, the presumably exposed carboxy-terminal domain of Rap1p tethers the Rif1p and Rif2p (Rap1-interacting factors) proteins to telomeres (Hardy *et al.*, 1992; Wotton and Shore, 1997). Thus, Rap1p is the key player that orchestrates the non-nucleosomal structure associated with budding yeast telomeres (see Figure 1-2). Tel2p, a protein previously characterized to modulate telomere length control *in vivo*, was

also reported to bind yeast telomeric DNA templates *in vitro* (Kota and Runge, 1998; Kota and Runge, 1999).

In the past few years, the *S. cerevisiae* model has generated a wealth of information regarding the recruitment of the telomerase holoenzyme to telomeres. Much of this information has come from studies on the product of the *CDC13* gene. Cdc13p is a single-stranded DNA-binding protein that demonstrates specific and high affinity binding to telomeric sequences *in vitro* (Lin, 1996; Nugent *et al.*, 1996). As mentioned in a previous section, a telomeric 3' overhang in yeast is generated in late S phase (Wellinger *et al.*, 1993). The generation of this overhang is independent of telomerase and is presumably induced by the action of a putative 5' to 3' nuclease that would act on the telomeric C-rich strand (Wellinger *et al.*, 1996; Dionne and Wellinger, 1998), thus providing a GT-rich single-stranded binding substrate to Cdc13p. Through the combination of elegant genetic and hybrid protein experiments, independent laboratories have defined two separate functions for Cdc13p in telomere length control: (1) in the recruitment of an end-protection complex that includes the Stn1 protein, and (2) in telomerase-dependent telomere elongation by recruiting the telomerase complex via the interaction with a subunit of the holoenzyme, Est1p (Grandin *et al.*, 1997; Evans and Lundblad, 1999; Grandin *et al.*, 2000; Qi and Zakian, 2000; Chandra *et al.*, 2001; Pennock *et al.*, 2001). Experiments using specific alleles of *CDC13* demonstrated that mutated Cdc13p showing a reduced association with the DNA polymerase α complex results in elongated telomeres. These results suggest that the recruitment of the conventional DNA replication machinery for coordinating CA-strand synthesis with GT-

strand synthesis by telomerase may be an additional mechanism of telomere length control (Qi and Zakian, 2000; Chandra *et al.*, 2001).

Although there is presently no direct evidence for T-loop structures at *S. cerevisiae* telomeres, recent experimental evidence suggest the existence of some type of loop-back structures at the end of yeast telomeres (de Bruin *et al.*, 2000). First, telomere-bound Rap1p associates with subtelomeric DNA located several kilobases upstream from the telomeric repeat tracts as determined by chromatin immunoprecipitations (ChIP) (Strahl-Bolsinger *et al.*, 1997). By artificially forcing transcription through the telomere repeat tracts, the ability of Rap1p to associate with subtelomeric DNA significantly decreases; this was interpreted as a disruption of Rap1p-induced telomere looping by transcription through telomeres (de Bruin *et al.*, 2000). Second, *GAL4*-dependent upstream activator sequences (UAS) located several kilobases away from a reporter gene are competent in transactivating this reporter gene when the UAS is placed in a telomere-proximal position, but not at an internal chromosomal locus (de Bruin *et al.*, 2001). As *S. cerevisiae* Rap1p can lead to structural bending of telomeric DNA *in vitro* (Gilson *et al.*, 1993; Muller *et al.*, 1994), it is possible that Rap1p-mediated looping is stabilized by Rif oligomerization, thus generating a closed telomere structure (see Figure 1-2).

Other important yeast telomere-associated proteins are the Sir (Silent information regulators) and the Ku complexes (Figure 1-2), both involved in TPE. The Sir complex is essentially composed of the product of the *SIR*2, *SIR*3, and *SIR*4 genes. Disruption of any of these genes significantly reduces transcriptional silencing at telomere-proximal loci (Aparicio *et al.*, 1991). The Sir complex is recruited to telomeres by association with the C-terminal domain of Rap1p (Moretti *et al.*, 1994; Strahl-Bolsinger *et al.*, 1997). The

finding that Sir2p possesses NAD-dependent deacetylase activity and deacetylates histone proteins *in vitro* (Imai *et al.*, 2000; Landry *et al.*, 2000) provides an explanation for the presence of hypoacetylated histones at telomere-proximal regions. Together, the recruitment of SIR complexes by telomere-bound Rap1 would lead to deacetylation of telomere-proximal histones by Sir2p, leading to the generation of a telomeric heterochromatin-like structure essential for TPE. The inability of a telomere-proximal UAS to transcriptionally activate a distantly positioned gene in a Δ*SIR3* yeast strain (de Bruin *et al.*, 2001); see above) suggest that SIR complexes are likely involved in the generation of the proposed closed telomeric loop-back structure.

In *S. cerevisiae*, the Ku complex consists of heterodimers of 70 and 80 kDa subunits that bind the ends of duplex DNA in a sequence independent fashion *in vitro* (Dynan and Yoo, 1998; Featherstone and Jackson, 1999). Although the Ku heterodimer is best characterized for its role in the non-homologous end joining (NHEJ) DNA repair pathway (Featherstone and Jackson, 1999), the Ku complex is also found at telomeres in budding and fission yeast, as well as in mammals (Gravel *et al.*, 1998; Hsu *et al.*, 1999; Baumann and Cech, 2000). While the precise role played by Ku in telomere function is not well defined, it is clear that yeast and human cells lacking functional Ku heterodimer have unstable telomeres (Gravel *et al.*, 1998; Hsu *et al.*, 1999; Samper *et al.*, 2000; d'Adda di Fagagna *et al.*, 2001).

1.3.3 Protozoa

As for the characterization of the first telomeric DNA sequences, the extremely high number of chromosomal ends (up to 1 X 10^8) in the macronucleus of some unicellular

protozoa was instrumental for the identification of the first telomere-binding proteins. In *Oxytricha nova*, the telomere-binding complex consists of two protein subunits (α and β), whereas a single telomere-binding protein is present in *Euplotes* (Gottschling and Zakian, 1986; Price and Cech, 1987; Fang *et al.*, 1993). The *Oxytricha* αβ heterodimer binds single-stranded oligonucleotides containing the telomeric sequence $(G_4T_4)_n$ (Gottschling and Zakian, 1986; Price and Cech, 1987). A ternary complex consisting of single-stranded $(G_4T_4)_n$ bound by recombinant αβ heterodimers is not a substrate for *Oxytricha* telomerase (Froelich-Ammon *et al.*, 1998). Furthermore, the αβ heterodimer displaces telomerase that is prebound to a $(G_4T_4)_n$ oligonucleotide, suggesting that the *Oxytricha* telomere-binding protein might regulate telomere elongation by competing with telomerase for the ssDNA substrate (Froelich-Ammon *et al.*, 1998). In 1998, the first three-dimensional structure of a telomere-binding protein was reported based on work that resolved the structure of crystals consisting of ssDNA-bound αβ heterodimers (Horvath *et al.*, 1998). The crystal structure suggests that the *Oxytricha* 3' telomeric overhang is completely buried within the complex, and thus protected from nuclease digestion. Interestingly, the *S. pombe* single-stranded telomeric DNA-binding protein Pot1p was identified based on its limited sequence similarity to the α subunit of the *Oxytricha* heterodimer (Baumann and Cech, 2001). The recent report of the solution structure of the *S. cerevisiae* single-stranded telomeric DNA-binding domain of Cdc13p suggests a type of ssDNA-recognition fold similar to the protozoan telomere-binding protein (Mitton-Fry *et al.*, 2002). It will be extremely interesting to compare the three-dimensional structures of the αβ heterodimer and Cdc13p with other ssDNA telomere-binding proteins.

1.4 End Replication Problem

The end replication problem was proposed about 30 years ago to describe how the ends of a linear DNA molecule cannot be fully replicated by the conventional replication machinery (Watson, 1972; Olovnikov, 1973). Recently, experiments using an *in vitro* reconstitution system for DNA replication clearly support the existence of an end replication problem (Ohki *et al.*, 2001). In this section, I will summarize the end replication problem, describing the two current views: (1) the lagging-strand problem and (2) the leading-strand problem.

The conventional DNA replication machinery follows three basic criteria. First, DNA polymerase replicates DNA only in the 5' to 3' direction. Second, polymerases need a 3' hydroxyl group in order to add a new nucleotide. Third, polymerases require the presence of a complementary strand to provide a template during replication. The current view of how duplex DNA is replicated can be summarized as follows (Waga and Stillman, 1998). Subsequent to the generation and to the partial extension of an RNA primer with DNA, leading-strand synthesis follows the movement of the replication fork via the processive action of DNA polymerases δ and ε. Lagging-strand synthesis is not processive, however; it requires the generation of repeated Okazaki fragments initiated by the DNA polymerase α-primase complex and that emerge in the opposite direction of the replication fork. The extended Okazaki fragments are eventually extended and ligated to generate a continuous lagging strand.

A schematic of the lagging-strand problem is shown in Figure 1-3. As illustrated, conventional DNA polymerases cannot fully replicate the sequences present at the

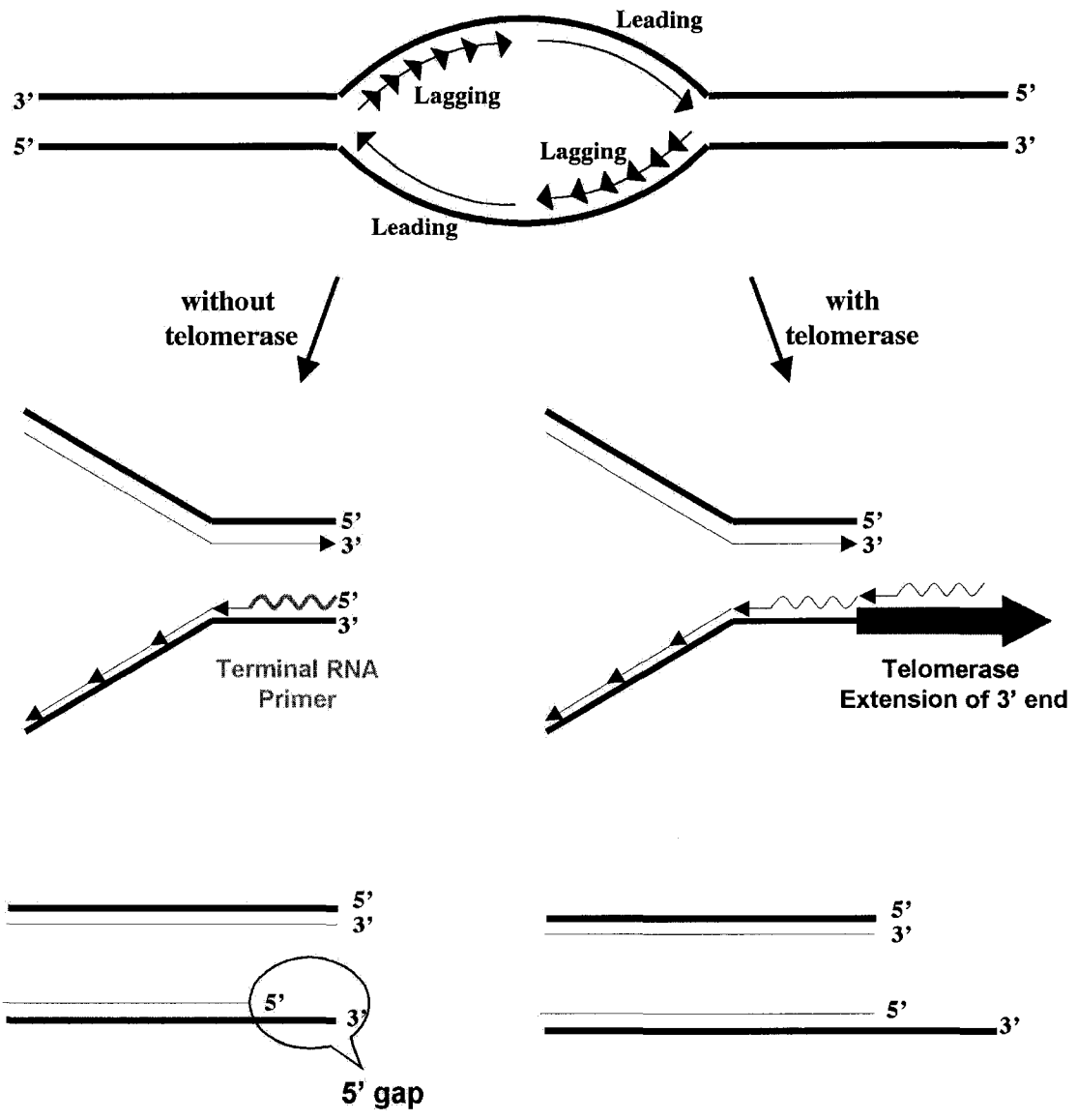

Figure 1-3. The Lagging-Strand Problem

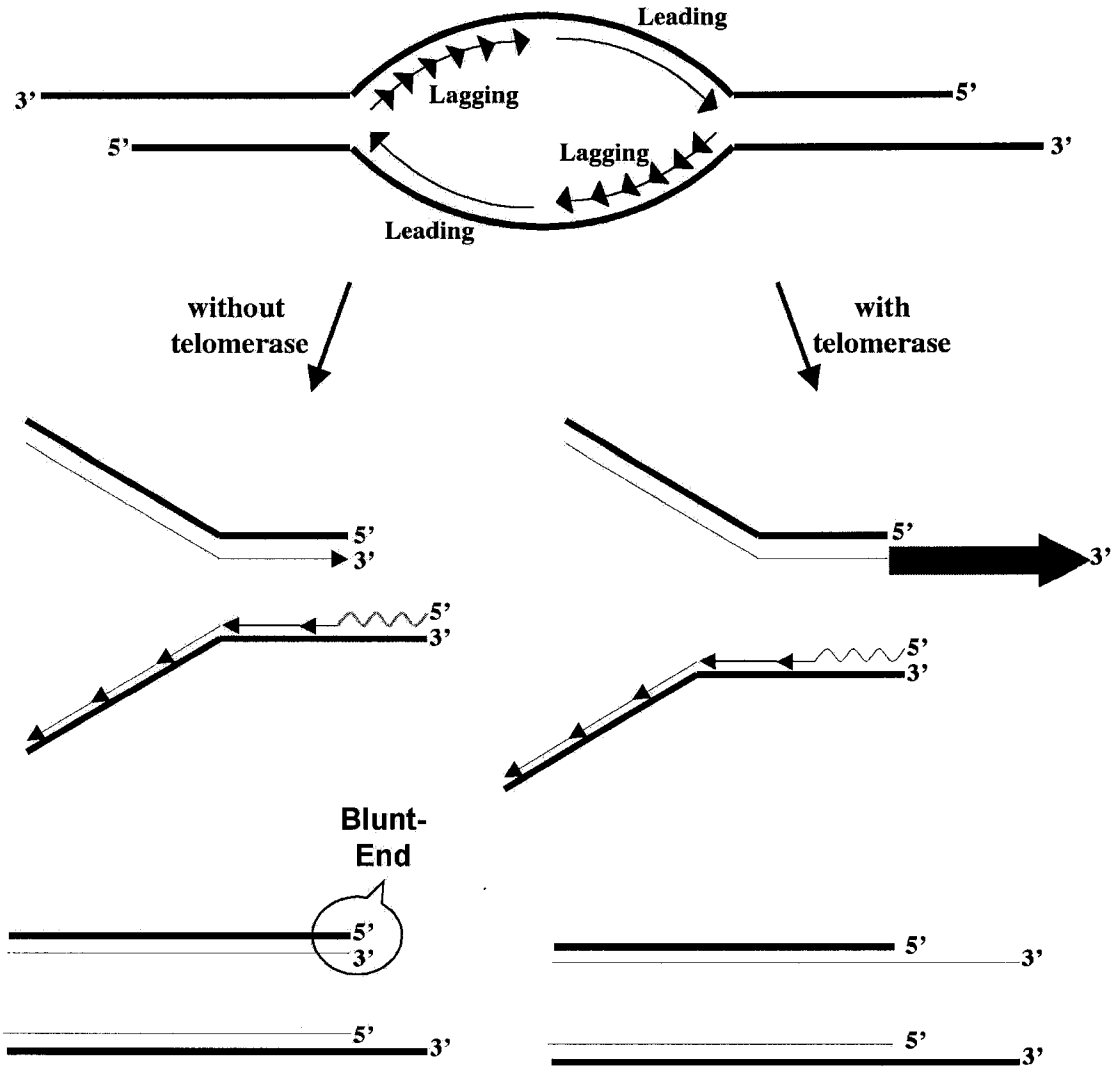

Figure 1-4. The Leading-Strand Problem

extreme 3' end of a blunt-ended linear molecule (see without telomerase model). Indeed, in the absence of complementary template DNA, the removal of the terminal RNA primer required for lagging-strand synthesis gives rise to a newly synthesized daughter molecule with a 5'-terminal gap. This end replication problem is caused by deficiencies in lagging-strand synthesis and was recently confirmed by elegant *in vitro* experiments (Ohki *et al.*, 2001). In this model, elongation of the 3' end by telomerase prior to the action of the conventional DNA replication machinery compensates for the loss of DNA generated by the lagging-strand problem (see Figure 1-3).

However, as was discussed in earlier sections, the terminal structure of telomeres in most organisms suggests the presence of TG-rich 3' overhangs. This led to the proposal of a different model for the end replication problem; the leading-strand problem (Lingner *et al.*, 1995). As illustrated in Figure 1-4, there is no loss of sequence information upon lagging-strand synthesis when the end of a linear DNA molecule harbors a 3' overhang. The 5'-terminal gap created upon removal of the extreme lagging-strand RNA primer in these conditions (see without telomerase model) does not lead to loss of original sequences. Yet, synthesis of the leading strand using this type of template molecule (containing 3' overhangs) leads to the loss of the 3' overhang, thus generating a blunt-ended terminal structure. This will lead to a lagging-strand problem (Figure 1-3) if a 3' overhang is not reestablished on this blunt-ended telomere. In this model, 3'-extension of the blunt-ended DNA molecules by telomerase following DNA replication prevents the future lagging-strand problem and circumvents the leading-strand problem (see with telomerase model in Figure 1-4). Whether it is a lagging or a leading strand problem, Figures 1-3 and 1-4 clearly illustrate that a linear DNA molecule loses sequence

information during every replication cycle in the absence of a specific mechanism to balance for this loss of DNA. Most eukaryotic genomes consist of linear DNA chromosomes and are faced with the end replication problem. Indeed, in most human primary cells where telomerase activity is not detected, telomere length decreases with increasing number of cell divisions *in vitro*, and with age *in vivo* (Harley *et al.*, 1990; Hastie *et al.*, 1990).

1.5 Telomerase

According to the concept discussed above, without a specialized enzymatic machinery responsible for the maintenance of chromosomal ends, the end replication problem will result in the loss of telomeric sequences at each cell division. The initial proposition for a telomere-specific polymerase originated from genetic experiments using the yeast *Saccharomyces cerevisiae*. In these experiments, the introduction of *Tetrahymena* and *Oxytricha* telomeres into yeast cells resulted in the recognition and the maintenance of these heterologous telomeres with yeast-specific telomeric sequences (Pluta *et al.*, 1984; Shampay *et al.*, 1984). In 1985, Carol Greider and Elizabeth Blackburn reported the identification of a polymerase activity from *Tetrahymena* cell extracts (which they called telomerase for telomere terminal transferase) that adds telomeric repeat sequences onto a single-stranded oligonucleotide primer (Greider and Blackburn, 1985). The abolishment of this activity upon treatment with nucleases and proteases led to the discovery that this polymerase activity is mediated by a ribonucleoprotein (RNP) complex that relies on an intrinsic RNA component as a

template for polymerization (Greider and Blackburn, 1987; Greider and Blackburn, 1989).

Following the identification of telomerase activity from *Tetrahymena*, similar telomere-specific polymerization activities were characterized in divergent eukaryotes. These includes other ciliated protozoa (Zahler and Prescott, 1988; Shippen-Lentz and Blackburn, 1989)), humans (Morin, 1989), mouse (Prowse *et al.*, 1993), yeast (Cohn and Blackburn, 1995), as well as plants (Fitzgerald *et al.*, 1996). These studies revealed several conserved *in vitro* biochemical properties of telomerase: (1) it is an RNP complex that uses an intrinsic RNA template sequence;(2) DNA synthesis occurs in a 5' to 3' direction; (3) synthesis of single-stranded telomeric repeats occurs on the TG-rich DNA strand; and (4) duplex telomeric blunt ends are not telomerase substrates. Functional evidence that telomerase was the RNA-templated polymerase that adds telomere repeats to the ends of eukaryotic chromosomes was provided by demonstrating that the expression of mutant telomerase RNAs causes the synthesis of the corresponding mutant telomeric repeats (Shippen-Lentz and Blackburn, 1990; Yu *et al.*, 1990; McEachern and Blackburn, 1995; Marusic *et al.*, 1997).

With several of the telomerase components now cloned from divergent organisms, the reconstitution of functional telomerase in heterologous systems suggest the requirement of only two minimal components for activity *in vitro*; a protein catalytic subunit containing motifs conserved in several viral reverse transcriptases, as well as an RNA component. In the following sections, I will discuss the current knowledge regarding the (1) telomerase RNA, (2) the telomerase protein catalytic subunit, (3) the synthesis of telomeric DNA by telomerase.

1.5.1 The Telomerase RNA

The discovery of a *Tetrahymena thermophila* RNP complex that synthesizes telomeric repeat sequences (Greider and Blackburn, 1985; Greider and Blackburn, 1987) led to the identification of the first telomerase RNA subunit (Greider and Blackburn, 1989). Telomerase catalytic activity is dependent on this RNA molecule that contains a sequence complementary to telomeric DNA (referred as telomerase RNA template). The telomerase RNA was identified based on its cofractionation with highly purified telomerase activity, its sensitivity to RNAse treatment, and the sensitivity of this RNA and telomerase activity to preincubation with RNAse H in the presence of a DNA oligonucleotide complementary to the putative template domain of the RNA (Greider and Blackburn, 1989).

The telomerase RNA has diverged significantly throughout eukaryotic evolution. In addition to the remarkable length diversity between telomerase RNAs from different organisms, the primary nucleotide (nt) sequence of telomerase RNAs shows no conservation among eukaryotic species. Because of the lack of sequence conservation, many different strategies were used for the identification of telomerase RNA subunits from divergent organisms. The 192-nt long telomerase RNA from the ciliated protozoa *Euplotes crassus* was identified based on its sensitivity to RNAse H-mediated cleavage when telomeric DNA oligonucleotides were used (Shippen-Lentz and Blackburn, 1989). The gene coding for the *Saccharomyces cerevisiae* 1351-nt long telomerase RNA was cloned as a result of a screen to find genes that altered proper telomere function when overexpressed (Singer and Gottschling, 1994). The 451-nt human telomerase RNA

(hTR) was identified by preparing cDNAs from RNA molecules purified with streptavidin-bound biotinylated oligonucleotides containing the predicted human telomerase template sequence (Feng *et al.*, 1995). Functional *in vitro* reconstitution assays in which recombinant RNA is added to micrococcal nuclease-treated telomerase extracts demonstrated that the identified *Tetrahymena* and human genes encoded telomerase RNA subunits (Autexier and Greider, 1994; Autexier *et al.*, 1996).

Eukaryotic telomerase RNAs are transcribed by different RNA polymerases. The finding that the telomerase RNA subunit from ciliated protozoa is (1) not 5' capped, (2) contains a 3' end poly (U) tract (Greider and Blackburn, 1989), (3) that it is bound by a La motif-containing protein (Aigner *et al.*, 2000), and (4) that its synthesis is sensitive to α-amanitin (Yu *et al.*, 1990) strongly suggest that it is transcribed by RNA polymerase III. Although direct evidence for the presence of a 3' polyadenylated telomerase RNA was only demonstrated for the yeast *S. cerevisiae*, experimental data suggest that both yeast and mammalian telomerase RNAs are products of RNA polymerase II (Chapon *et al.*, 1997; Hinkley *et al.*, 1998; Chen *et al.*, 2000). The significance of the different telomerase RNA transcription mechanisms in telomerase RNP biogenesis will be discussed in section 1.7.

The secondary structures of ciliate and vertebrate telomerase RNAs have been determined by phylogenetic sequence analysis (Romero and Blackburn, 1991; ten Dam *et al.*, 1991; McCormick-Graham and Romero, 1995; Chen *et al.*, 2000). Despite the weak primary sequence conservation within and between eukaryotic species, the mammalian telomerase RNA secondary structure displays topological features common to the structure of its ciliate counterpart, suggesting the evolutionary conservation of certain

functional structural domains. The secondary structure models of ciliate and vertebrate telomerase RNAs are shown in Figure 1-5.

Most ciliate telomerase RNAs share the evolutionary conservation of four stems, numbered from the 5' end to the 3' end (I, II, III, and IV). Stem I is a long-range helix that consist mostly of G-C base-pairing. Although stem I is present in most ciliate telomerase RNAs, this structure is not critical for the reconstitution of telomerase activity *in vitro* (Autexier and Greider, 1998) or for the binding of telomerase protein components present in *Tetrahymena* protein extracts (Autexier and Triki, 1999). Stem II is not found in all ciliated protozoa telomerase RNAs. Using *Tetrahymena* telomerase reconstitution in a rabbit reticulocyte lysate (RRL) system, the proximal portion of stem II was shown to be important for telomerase activity and the efficient association between the telomerase RNA and the protein catalytic subunit (Licht and Collins, 1999). More recently, binding of the *Tetrahymena* catalytic subunit to residues flanking the proximal region of stem II was suggested to play a role in template boundary definition by *Tetrahymena* telomerase (Lai *et al.*, 2002). The base-pairing present in stem IIIa and IIIb result in the formation of a pseudoknot structure. Although mutational disruption of the *T. thermophila* pseudoknot perturbs the efficient assembly of a functional telomerase RNP *in vivo* (Gilley and Blackburn, 1999), similar mutations do not affect TERT binding and telomerase activity in *in vitro* reconstitution systems (Autexier and Greider, 1998; Licht and Collins, 1999). The deletion of stem IV from the *T. thermophila* RNA results in weak telomerase activity levels *in vitro*, but does not seem to significantly affect the binding of the protein catalytic subunit (Autexier and Greider, 1998; Licht and Collins, 1999). Recently, the RNase footprinting analysis of the RRL-reconstituted *Tetrahymena* telomerase RNP suggest that

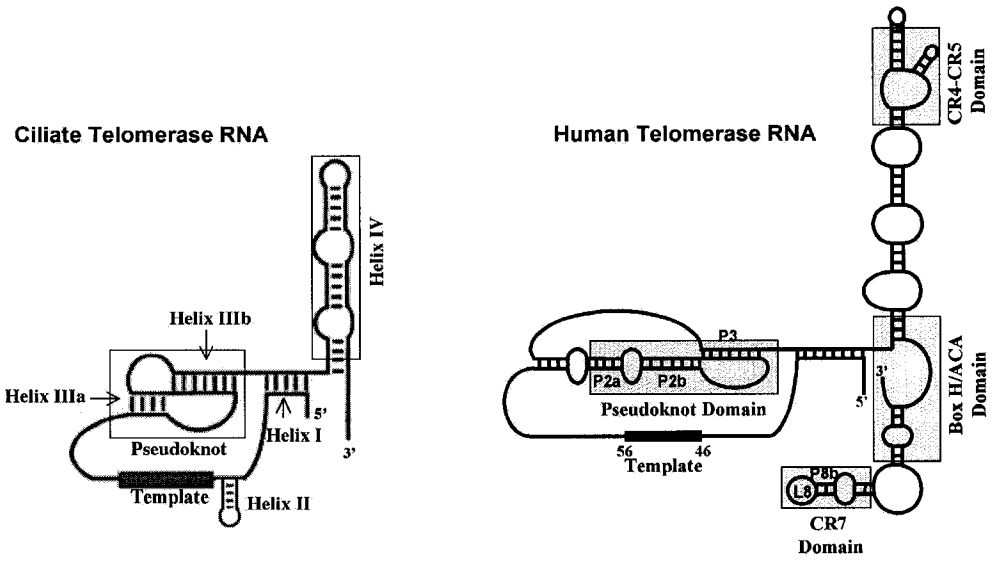

Figure 1-5. Secondary Structure of Ciliate and Vertebrate Telomerase RNA

TERT binding to the telomerase RNA induces a conformational change in the pseudoknot structure that is dependent on an intact stem-loop IV structure (Sperger and Cech, 2001). Thus, although the stem-loop IV from the ciliate telomerase RNA is not essential for efficient binding to the protein catalytic subunit, this structure seems to be critical for proper telomerase RNA folding and RNP catalysis.

A secondary structure for vertebrate telomerase RNA was proposed based on the phylogenetic comparative analysis of telomerase RNA sequences from more than 35 different vertebrate species (Chen *et al.*, 2000). In addition to a highly conserved RNA template sequence, vertebrate telomerase RNAs share four conserved structural elements: a pseudoknot, the CR4-CR5 domain, the box H/ACA domain, and the CR7 domain (see Figure 1-5). This secondary structure prediction was recently analyzed experimentally using *in vitro* and *in vivo* chemical as well as RNase mapping procedures (Antal *et al.*, 2002b). Although this study validates the overall proposed secondary structure for the human telomerase RNA (hTR), it was unable to confirm the formation of a stable pseudoknot; suggesting that the formation of the vertebrate telomerase RNA pseudoknot might be a transient event. Helices P2a, P2b, and P3 establish the vertebrate telomerase RNA pseudoknot (Figure 1-5). Using different *in vitro* reconstitution systems for mammalian telomerase, telomerase RNA mutations predicted to destabilize the pseudoknot indicate the essential function of this domain for catalytic activity, but not for TERT protein binding (Autexier *et al.*, 1996; Bachand and Autexier, 2001; Bachand *et al.*, 2001; Martin-Rivera and Blasco, 2001). The CR4-CR5 domain turns out to be one of the most interesting structures of the vertebrate telomerase RNA. This domain is a high-affinity binding site for the protein catalytic subunit (Mitchell and Collins, 2000; Bachand

and Autexier, 2001; Chen *et al.*, 2002). Furthermore, the ability to reconstitute mammalian telomerase activity in RRL via the expression of the protein catalytic subunit in the presence of different segments of the mammalian telomerase RNA permitted the demonstration that: (1) the mammalian telomerase RNA harbors two independent TERT-binding sites, and (2) that the CR4-CR5 domain is not only required for providing a TERT-binding domain, but is also essential for the catalytic activation of mammalian telomerase (Mitchell and Collins, 2000; Bachand and Autexier, 2001; Chen *et al.*, 2002).

Before the secondary structure of the vertebrate telomerase RNA was solved, Kathy Collins' group elegantly established that the 3' end of human and mouse telomerase RNAs folds into a box H/ACA small nucleolar (sno) RNA-like domain (Mitchell *et al.*, 1999a). The vertebrate telomerase RNA box H/ACA folds into an evolutionary conserved harpin-hinge-harpin-tail structure ((Chen *et al.*, 2000); see Figure 1-5). The hinge region contains the consensus H box sequence (5'-ANANNA-3') and the tail includes the ACA box consisting of an ACA trinucleotide located 3-nt upstream of the mature 3' end. The role of the box H/ACA snoRNA-like domain of hTR will be discussed in further detail in section 1.7.2; yet, this domain is clearly dispensable for mammalian telomerase activity *in vitro* (Autexier *et al.*, 1996; Tesmer *et al.*, 1999; Beattie *et al.*, 2000; Bachand and Autexier, 2001; Martin-Rivera and Blasco, 2001). Conversely, the integrity of the box H/ACA domain is essential for the accumulation of hTR *in vivo*, and consequently, for cellular telomerase activity (Mitchell *et al.*, 1999a). This suggests that the box H/ACA snoRNA-like domain is important for vertebrate telomerase RNA biogenesis, including RNA processing and/or RNP assembly. Helices P8a and P8b, as well as the L8 loop establish the CR7 domain (Figure 1-5). Similarly to

the box H/ACA domain, the CR7 domain is dispensable for telomerase activity *in vitro*, but not for mammalian telomerase accumulation *in vivo* (Autexier *et al.*, 1996; Tesmer *et al.*, 1999; Beattie *et al.*, 2000; Bachand and Autexier, 2001; Martin-Rivera and Blasco, 2001). These results, coupled to fact that the CR7 domain is not a conserved feature of known box H/ACA snoRNAs, suggest that the CR7 domain is likely to have a specific vertebrate telomerase function related to its biogenesis. Thus, the evolutionary conserved structure of the vertebrate telomerase RNA harbors a core region with structural similarity to the ciliate telomerase RNA, and three additional conserved vertebrate-specific domains: the CR4-CR5, the box H/ACA, and the CR7 domains.

The secondary structure of the long ~1.3 kb *S. cerevisiae* telomerase RNA (TLC1) has not yet been determined. Interestingly, more than half of the telomerase RNA from the yeast *S. cerevisiae* and *K. lactis* can be deleted without impairment on cell growth (Roy *et al.*, 1998; Livengood *et al.*, 2002). However, in addition to the template region, a few functional domains have been identified in the yeast telomerase RNA. A stem structure in the yeast telomerase RNA was shown to be critical for template boundary definition of *K. lactis* telomerase (Tzfati *et al.*, 2000). The *S. cerevisiae* TLC1 telomerase RNA contains a consensus-binding site for the Sm protein complex that is essential for TLC1 stability and telomerase-dependent telomere maintenance *in vivo* (Seto *et al.*, 1999). The functional role of the TLC1 Sm-binding site will be discussed in further detail in section 1.7.1.

1.5.2 The Protein Catalytic Subunit

A gene coding for a telomerase protein catalytic subunit was first identified using reverse genetics in *Euplotes aediculatus* (Lingner *et al.*, 1997b). The telomerase RNP from this ciliated protozoa had been previously purified to yield an active molecular complex of ~230-kDa, consisting of a 66 kDa RNA and two proteins of 123-kDa and 43-kDa (Lingner and Cech, 1996). The partial sequencing of the 123-kDa protein by nanoelectrospray tandem mass spectrometry led to the cloning of the corresponding gene from a *E. aediculatus* genomic library (Lingner *et al.*, 1997b). BLAST searches of protein databases for p123 homologs yielded the *Saccharomyces cerevisiae* Est2p. Interestingly, the *EST2* gene had previously been identified as part of a complementation group competent to rescue yeast mutants with reduced telomere lengths (Lendvay *et al.*, 1996). The presence of reverse transcriptase motifs in p123 and Est2p suggested that these proteins corresponded to the catalytic subunit of *E. aediculatus* and *S. cerevisiae* telomerase, respectively. At the same time, an independent group also reported the identification of the *S. cerevisiae* telomerase catalytic subunit based on an elegant genetic synthetic-lethal screen (Counter *et al.*, 1997). Using yeast genetics, several groups demonstrated that single amino acid substitutions in various Est2p reverse transcriptase motifs leads to a catalytically inactive telomerase *in vitro* and telomere shortening *in vivo* (Counter *et al.*, 1997; Lingner *et al.*, 1997b). Thus, the telomerase RNP is a specialized reverse transcriptase, referred to as the telomerase reverse transcriptase or TERT.

Based on the sequence of p123 and Est2p, TERT has now been identified in various organisms including fission yeast (Nakamura *et al.*, 1997), humans (Harrington *et al.*,

1997b; Kilian *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997), *Tetrahymena* (Bryan *et al.*, 1998; Collins and Gandhi, 1998), mice (Greenberg *et al.*, 1998; Martin-Rivera *et al.*, 1998), and *Xenopus* (Kuramoto *et al.*, 2001). In contrast to the lack of similarity in terms of length and nucleotide sequence between the telomerase RNA subunit of various organisms, the amino acid sequence of TERT proteins is relatively well conserved through evolution. TERT genes encode large (103-kDa to 134-kDa) proteins that are basically charged (pI > 10.0). The TERT protein family is most similar in sequence to the reverse transcriptases (RTs) of non-LTR retrotransposons and group II introns. These reverse trancriptases polymerize DNA by copying a RNA template sequence onto the 3' hydroxyl group of a DNA primer (Eickbush, 1997; Nakamura and Cech, 1998). This differs from the human immunodeficiency virus (HIV) RT that initially polymerizes DNA onto the 3' hydroxyl group of a tRNA primer (Barat *et al.*, 1989).

TERTs can be divided into three major regions (Figure 1-6). A large and basic N-terminal region that includes the highly conserved telomerase (T)-specific motif. A central region that consists of the seven characteristic RT motifs present within all the TERT subunits that have been identified so far. Carboxy-terminal to the RT motifs is the third and more variable region of TERT proteins. Much of the information acquired on the structure and function of the telomerase RNP result from the functional reconstitution of human and *Tetrahymena* telomerase activity *in vitro* in a rabbit reticulocytes lysate (RRL) system (Weinrich *et al.*, 1997; Beattie *et al.*, 1998; Collins and Gandhi, 1998). Functional reconstitution is accomplished by the coupled transcription and translation of TERT cDNA in the presence of *in vitro*-synthesized telomerase RNA. Human telomerase

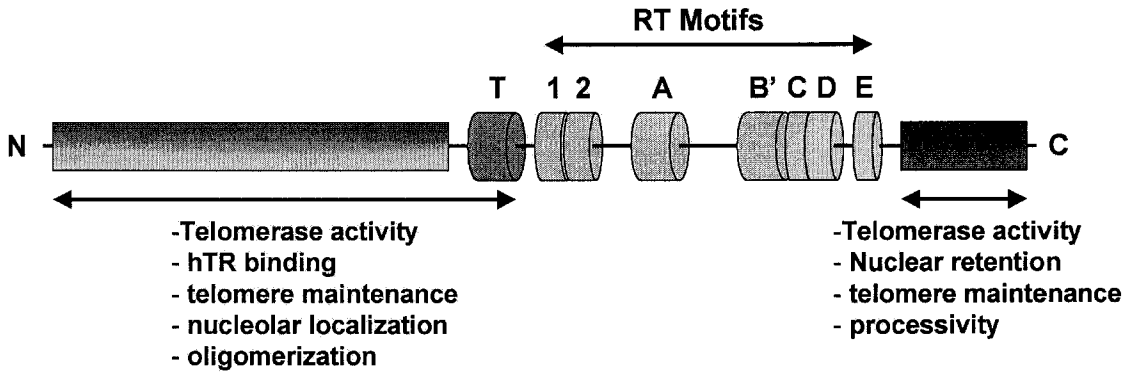

Figure 1-6. Telomerase Reverse Transcriptase

was also reconstituted by the coexpression of hTR and the human TERT (hTERT) subunit *in vivo* in *Saccharomyces cerevisiae* (Bachand and Autexier, 1999), and by the addition of *in vitro*-synthesized hTR to purified hTERT that was recombinantly expressed in insect cells (Masutomi *et al.*, 2000). Though these results suggest that the telomerase RNA and the TERT subunit are minimally required to reconstitute a functional telomerase RNP *in vitro*, it is still unclear whether these two components are sufficient for telomerase activity reconstitution. In the following paragraphs, I will summarize the current knowledge on the three different regions of TERT: (1) the RT domain, (2) the large N-terminal region, and (3) the C-terminal region.

Though TERT subunits contain the seven motifs common to viral RTs (1, 2, A, B', C, D, and E; see Figure 1-6), several features of the telomerase enzyme distinguish it from other RTs. Notably, telomerase is a RNP in which there is a stable association between the TERT subunit and the RNA component. Second, the TERT subunit only copies a limited sequence region within its associated RNA template. The relative spacing between the RT motifs of TERTs is different from that of known RTs; specifically the linker regions between motifs 2 and A and motifs A and B' are typically longer in TERT than in viral RTs (see Figure 1-6). Nonetheless, the early studies performed on TERTs clearly indicate that the previously identified residues demonstrated to be critical for the catalysis of viral RTs and conserved among TERTs are also essential for telomerase activity (Counter *et al.*, 1997; Harrington *et al.*, 1997b; Lingner *et al.*, 1997b; Weinrich *et al.*, 1997). Recent mutagenesis studies further suggest that the polymerization mechanism of telomerase may be similar to those of the viral RTs (Bryan *et al.*, 2000a; Miller *et al.*, 2000; Bosoy and Lue, 2001; Peng *et al.*, 2001). This implies

that the RT region of TERT is likely to fold into a similar ternary structure as the one solved by the crystallization of the HIV-1 RT (Kohlstaedt *et al.*, 1992; Ding *et al.*, 1998; Huang *et al.*, 1998), and that is reminiscent of the shape of a right hand. In this structure, the seven RT motifs are located in the palm and finger domains. The palm domain constitutes the active site pocket where three highly conserved aspartic acid residues are directly involved in the catalysis of dNTP addition.

Much information regarding the large and basic N-terminal region of TERT subunits has been acquired in the last two years. Though the similarity in amino acid sequence between the various TERT N-terminal regions is significantly less than for the RT motifs, recent sequence alignments have identified new blocks of conservation in their amino termini in addition to the highly conserved T-motif (Malik *et al.*, 2000; Miller *et al.*, 2000; Xia *et al.*, 2000; Moriarty *et al.*, 2002). Several independent functions have been ascribed to the N-terminal region of the TERT subunit based on extensive mutagenesis studies: (1) telomerase RNA binding (Friedman and Cech, 1999; Beattie *et al.*, 2000; Bachand and Autexier, 2001; Moriarty *et al.*, 2002) (2) TERT multimerization (Arai *et al.*, 2002), (3) a telomere maintenance function that is separable from telomerase activity (Armbruster *et al.*, 2001), and (4) nucleolar localization (Etheridge *et al.*, 2002). Though some residues in the TERT-specific T-motif clearly function in binding the telomerase RNA (Bryan *et al.*, 2000b; Moriarty *et al.*, 2002), it is obvious that other regions of the TERT N-terminus are involved in binding RNA (Friedman and Cech, 1999; Beattie *et al.*, 2000; Bachand and Autexier, 2001; Lai *et al.*, 2001). Indeed, the TERT region necessary and sufficient for *Tetrahymena* telomerase RNA binding was expressed independently in

a recombinant form (Lai *et al.*, 2001). This *Tetrahymena* TERT RNA-binding domain includes the T-motif and the 300 N-terminal residues.

Most retroviral RTs dimerize to form catalytically functional enzymes. Genetic and biochemical data suggest that *S. cerevisiae* telomerase contains at least two telomerase RNA components per functional active site (Prescott and Blackburn, 1997). Similar results were recently obtained demonstrating that the human telomerase RNP contains two functionally cooperating hTR molecules (Wenz *et al.*, 2001). In addition, two separate inactive TERT proteins can functionally complement each other in *trans* (Beattie *et al.*, 2001), suggesting that the telomerase complex contains at least two TERTs subunits. These results suggest that telomerase enzymes function as oligomers, most likely via the formation of dimers. Although further characterization is required, studies using deleted versions of hTERT suggest that N-terminal residues are involved in hTR-independent TERT protein oligomerization (Beattie *et al.*, 2001; Arai *et al.*, 2002). Recent mutational analyses of TERT subunits from *S. cerevisiae* and humans have also identified N-terminal mutants demonstrating close to wild-type telomerase activity *in vitro*, but that are unable to maintain telomere length *in vivo* (Xia *et al.*, 2000; Armbruster *et al.*, 2001). Interestingly, this phenotype has been previously observed for hTERT proteins harboring carboxy-terminal epitope tags derived from the influenza virus hemaglutinin (HA) surface protein (Counter *et al.*, 1998; Ouellette *et al.*, 1999). Though the molecular defects of these mutants have not yet been defined, the most likely explanation to the phenotype associated with these mutant TERT proteins is their inability to be recruited to telomeres.

Very few studies have investigated the functional role of the TERT C-terminal residues. This region is clearly not involved in telomerase RNA binding since human and *Tetrahymena* TERT subunits lacking C-terminal residues extending to motif E are not affected in their ability to associate with the RNA component *in vitro* (Beattie *et al.*, 2000; Bachand and Autexier, 2001; Lai *et al.*, 2001). Yet, the same deletions abolish the reconstitution of telomerase activity, suggesting an important role for C-terminal residues in the polymerization activity of telomerase. In most organisms studied, telomerase is processive (Greider, 1991). The processivity of a polymerase is defined as the probability of dissociating from the template per base added (von-Hippel *et al.*, 1983). Recent experiments using the yeast TERT subunits Est2p indicate that the C-terminal extension might contribute to telomerase processivity (Peng *et al.*, 2001). Certain isoforms of 14-3-3 proteins were identified as hTERT-binding partners when the C-terminal region of hTERT was used as bait in a yeast two-hybrid screen (Seimiya *et al.*, 2000). This study further demonstrated that (i) the hTERT C-terminal extension harbors a typical leucine-rich nuclear export signal (NES) that is recognized by the general nuclear export factor Crm1/exportin-1; and (ii) that binding of 14-3-3 proteins to hTERT is important for its nuclear retention most likely by inhibiting Crm1 association with the C-terminal NES.

1.5.3 The Synthesis of Telomeric DNA by Telomerase

Based on the experiments and evidence described in the two previous paragraphs, we conclude that the reconstitution of telomerase activity *in vitro* requires not only the RT motifs of the TERT subunit, but also (i) amino acid residues outside of the RT motifs and (ii) RNA sequences distal from the telomerase RNA template region. This suggests that

the specialized function of telomerase to use a defined internal template occurred during evolution by combining a polymerase module (RT motifs) with a RNA-binding module (N-terminal domain) that positions the RNA template sequence within the catalytic active site pocket. Despite the extensive knowledge on the molecular mechanism of polymerization by retroviral RTs, the mechanism by which telomerase polymerizes single-stranded telomeric DNA remains poorly understood. In contrast to retroviral RTs, telomerase needs to specify the RNA templating region and its borders, and also provide a mechanism to move along the template during elongation. Furthermore, telomerase needs to dissociate from the newly synthesized DNA and translocate prior to a subsequent round of polymerization.

As illustrated in Figure 1-7, four major steps are associated with telomeric DNA synthesis by telomerase: (1) primer/telomere recognition and binding, (2) catalytic polymerization, (3) translocation or dissociation, and (4) following translocation, a new polymerization step. To date, it is not yet clear to what extent the complementarity between the telomerase RNA template and the DNA primer plays a role in substrate recognition by telomerase. First, experiments using telomerase partially purified from *Tetrahymena* and human cells demonstrate that substrate primers containing 5' telomeric repeats followed by a nontelomeric 3' terminus are efficiently elongated (Harrington and Greider, 1991; Morin, 1991). Second, the substitution of the yeast telomeric template for the human telomeric template in the yeast telomerase RNA results in yeast cells that have short telomeres with human telomeric sequences *in vivo* (Henning *et al.*, 1998). These results support the presence of a telomerase anchor site: a functional DNA-binding domain with specificity for telomeric sequences. A telomerase anchor site would support

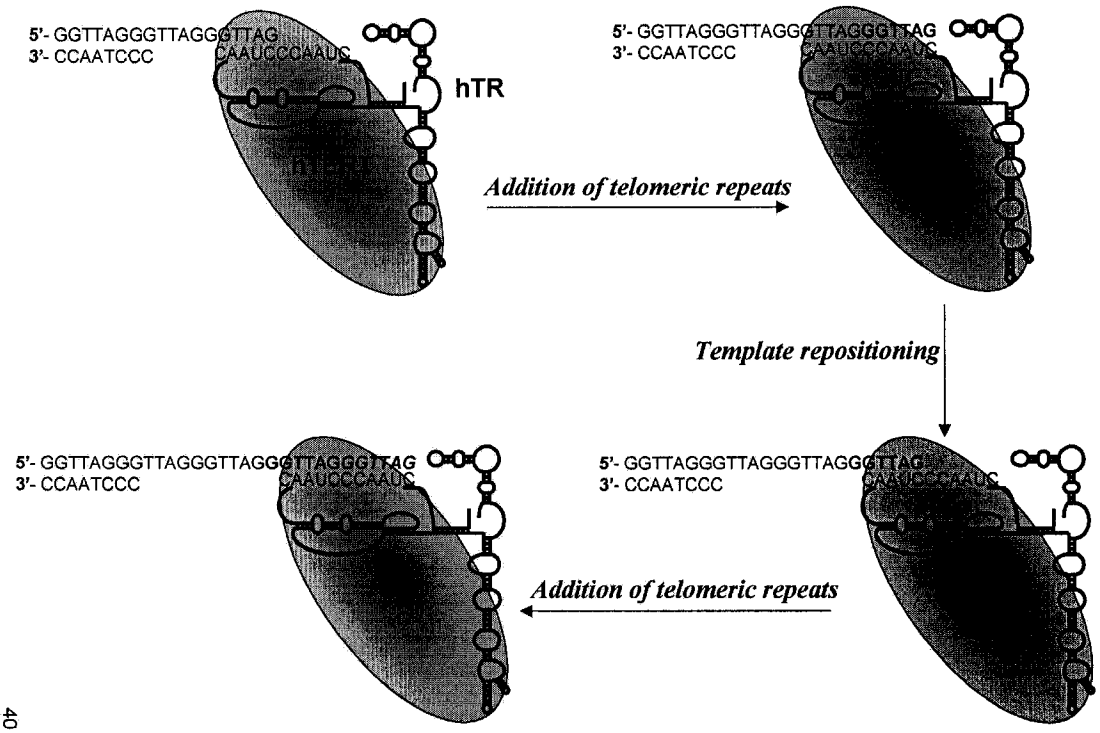

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Figure 1-7. The Synthesis of Telomeric DNA by Telomerase

telomerase processivity by the following mechanism: with the 5' end of a primer in the anchor site, the 3' end of the primer could translocate in the catalytic site without the complete dissociation of the primer from telomerase. Evidence for a telomerase anchor site was indeed demonstrated by the cross-linking between a DNA oligonucleotide and the *Euplotes* TERT subunit 20-22 residues upstream from the primer's 3' end (Hammond *et al.*, 1997).

Following substrate recognition and alignment (see Figure 1-7), telomerase elongates the DNA primer/telomere by copying specific templating residues from the RNA component. 5' template boundary definition by telomerase has been well studied, especially in ciliated protozoa where a conserved four-nucleotide sequence located upstream of the template is important (Lingner *et al.*, 1994; Autexier and Greider, 1995). Recently, it was demonstrated that telomerase RNA sequences essential for *Tetrahymena* telomerase template boundary definition were also important for high affinity TERT binding (Lai *et al.*, 2002). This suggests that in *Tetrahymena*, specific interactions between conserved nucleotides located upstream of the template and amino acids in the TERT RNA-binding domain are responsible for the definition of the 5' template boundary, most likely via steric hindrance of the active site pocket. Though elements involved in template boundary definition by human telomerase have not yet been defined, a stem-loop structure 5' to the template sequence of the yeast *K. lactis* telomerase RNA is necessary to define template boundary in this organism (Tzfati *et al.*, 2000).

Once the end of the telomerase RNA template is reached, the primer translocates to reposition for second-round synthesis (see Figure 1-7). Few studies have investigated the events that characterize the translocation step. Following the elongation of the DNA

primer to the end of the telomerase RNA template, up to 11 Watson-Crick base pairs of a RNA-DNA hybrid can potentially exist in the active site of human telomerase (Figure 1-7). Disrupting such a long helix for second-round synthesis would be energetically costly. However, a kinetic analysis of primer-telomerase complex stability in *Euplotes* suggests that the telomerase catalytic pocket maintains only a constant and small number of base pairs between the DNA primer and the RNA template (Hammond and Cech, 1998). Thus, in a mechanism analogous to DNA transcription by RNA polymerases, the addition of a new base to the 3' end of the DNA primer by telomerase would trigger the disruption of a base pair at the 3' end of the template. Alternatively, a helicase activity could be intrinsic to, or associated with telomerase.

The synthesis of telomeric DNA by telomerase *in vivo* is likely to be much more complex than the simple addition of telomeric repeats on a DNA primer *in vitro*. Such complexity was recently highlighted by elegant experiments in yeast that helped explain phenomena observed more than 10 years ago. It was originally shown that yeast harboring mutations in the gene coding for DNA polymerase α had very long telomeres compared to wild-type strains (Carson and Hartwell, 1985). Similarly, yeast that express mutant subunits of the replication factor C (RFC) complex exhibit elongated telomeres (Adams and Holm, 1996). This telomere elongation phenotype, which is specific for RFC and DNA pol α, requires catalytically active telomerase (Adams and Holm, 1996). Genetic evidence provided by the Gottschling and Gilson groups suggest that telomerase-mediated G-rich strand synthesis and lagging C-rich strand synthesis by the conventional DNA replication machinery act in a coordinate fashion (Diede and Gottschling, 1999; Marcand *et al.*, 2000). An artificial telomere end created via the generation of a

chromosomal double-stranded break in yeast cells is elongated by telomerase only when cells are (i) allowed to pass through S phase, and (ii) express a functional DNA pol α-primase complex and DNA pol δ (Diede and Gottschling, 1999). Using a sequence-specific recombination assay to effectively generate a genetically tractable short telomere, Marcand *et al.* similarly demonstrated that telomerase-mediated telomere addition occurs in late S phase, concomitant with telomere DNA replication. The demonstration of biochemical and genetic interactions between the ssDNA telomere-binding protein Cdc13p and (1) the telomerase complex (Evans and Lundblad, 1999; Pennock *et al.*, 2001), and (2) DNA polymerase α (Qi and Zakian, 2000) strongly support a model where Cdc13p recruits both telomerase and the conventional DNA replication machinery. This model would ensure proper telomere length control by coordinating the synthesis of the telomeric G-rich and C-rich strands. It will be interesting to determine whether such coincident telomeric strand synthesis occurs in divergent eukaryotes.

1.6 Telomerase-Associated Proteins

Telomerase activity can be reconstituted *in vitro* via the coexpression of the catalytic subunit and the telomerase RNA. This suggests that TERT and TR are sufficient for the generation of a catalytically active telomerase *in vitro*. However, it is likely that additional proteins associate with telomerase components or with the functional holoenzyme transiently or constitutively. These proteins could be involved in the maturation of telomerase components, telomerase assembly and regulation, subcellular localization, recruitment to telomeres, or other telomerase-associated events. In this

section, I will summarize the major telomerase-associated proteins of ciliates, yeast, and humans.

1.6.1 Ciliates

The first telomerase-associated proteins were initially isolated based on their copurification with the *Tetrahymena thermophila* telomerase RNP. The genes coding for the two isolated proteins of 80-kDa and 95-kDa were cloned and used to generate antiserums based on their deduced amino acid protein sequence (Collins *et al.*, 1995). Though a p80-specific antibody specifically coimmunoprecipitated telomerase activity from *T. thermophila* cell extracts, the presence of conserved polymerase motifs in both p80 and p95 remained uncharacterized (Collins *et al.*, 1995). Recombinant p80 and p95 purified from a bacterial expression system can form a complex and both independently bind the *T. thermophila* telomerase RNA *in vitro* (Gandhi and Collins, 1998). Yet, the p95 protein binds preferentially to telomeric ssDNA, whereas p80 binds the telomerase RNA with more affinity. Once the p133 telomerase catalytic subunit from *T. thermophila* was cloned (Bryan *et al.*, 1998; Collins and Gandhi, 1998), it was clear that p80 and p95 were not the catalytic constituents of telomerase. A recent study where the interactions between purified recombinant p80, p95, p133, and *in vitro*-synthesized telomerase RNA are examined, concludes that neither p80 nor p95 associate with telomerase components *in vitro* and *in vivo* (Mason *et al.*, 2001). *In vivo*, the absence of either p80 or p95 does not affect the levels of telomerase activity detected from *Tetrahymena* cell extracts (Miller and Collins, 2000), suggesting that these proteins are not essential for telomerase assembly and catalytic activity. However, elimination of the genes coding for p80 and

p95 results in telomere lengthening (Miller and Collins, 2000). The direct effect of the lack of p80 and p95 expression on *Tetrahymena* telomere length remains to be demonstrated, and consequently, the role of these two proteins in telomerase-mediated telomere maintenance is still undefined.

The purification of telomerase from the ciliated protozoa *Euplotes aediculatus* led to the identification of the first telomerase catalytic subunit (Lingner and Cech, 1996; Lingner *et al.*, 1997b). Interestingly, a 43-kDa protein copurified with the *Euplotes* telomerase RNP (Lingner and Cech, 1996). Partial sequencing of this 43-kDa protein led to the identification of the corresponding gene (Aigner *et al.*, 2000). BLAST searches based on the deduced amino acid sequence from the *p43* gene revealed homology to the La autoantigen protein family. La protein family members are present in evolutionary divergent organisms and function in the maturation and biogenesis of RNA polymerase III transcripts by associating with their 3'-terminal oligouridylate stretch. The telomerase RNA and more than 90% of the telomerase activity from *E. aediculatus* cell extracts are coimmunoprecipitated using antibodies specific to the p43 protein (Aigner *et al.*, 2000). The cofractionation of p43 with telomerase activity and its association with the vast majority of the mature telomerase *in vivo*, suggest that p43 plays an important role in telomerase function in *E. aediculatus*. The human La protein was also found to associate with telomerase activity (Ford *et al.*, 2001). This is surprising, since the La protein family members are known to associate primarily with RNA pol III transcripts, whereas the human telomerase RNA is transcribed by RNA polymerase II (Hinkley *et al.*, 1998; Chen *et al.*, 2000).

1.6.2 Yeast

The ease by which the yeast *Saccharomyces cerevisiae* can be genetically manipulated permitted the establishment of a genetic screen for mutants with telomere replication defects. This screen led to the identification of four essential *ever shorter telomeres* (*EST*) genes; *EST1, EST2, EST3,* and *EST4* (Lundblad and Szostak, 1989; Lendvay *et al.*, 1996). While the *EST2* gene was found to encode the *S. cerevisiae* telomerase protein catalytic subunit, *EST4* codes for the single-stranded telomeric DNA-binding protein Cdc13p. Yeast cells deleted for the *EST1* and *EST3* genes show progressive telomere shortening, but retain levels of telomerase activity comparable to wild-type cells (Cohn and Blackburn, 1995; Lingner *et al.*, 1997a). This suggested that Est1p and Est3p are not likely to be involved in the biogenesis and assembly of yeast telomerase, but rather in a downstream event.

Similarly to Cdc13p, the Est1 protein has affinity for single-stranded telomeric DNA *in vitro*, but unlike Cdc13p, Est1p requires a free 3'end terminus (Virta-Pearlman *et al.*, 1996). More recently, the yeast telomerase RNA TLC1 and Cdc13p were reported as physical partners of Est1p (Qi and Zakian, 2000; Zhou *et al.*, 2000; Livengood *et al.*, 2002)). These experimental results led to a model in which the telomere-associated Cdc13p recruits the telomerase holoenzyme via interactions with the telomerase-associated Est1 protein. A $CDC13_{DBD}$-EST2 fusion protein where the DNA-binding domain (DBD) of Cdc13p is fused to the yeast TERT subunit can function in telomere length maintenance in *EST1*-depleted yeast cells. These results support a model where Est1p and Cdc13p comediate telomerase access on telomeres *in vivo* (Evans and Lundblad, 1999). Whereas much information on the function of Est1p has been acquired

in the past few years, very little is known about the Est3 protein. Similarly to Est1p, the 20-kDa Est3p physically associates with the mature telomerase RNP (Hughes *et al.*, 2000). However, in *EST2*-deleted cells Est3p can no longer coimmunoprecipitate the TLC1 telomerase RNA, suggesting that Est3p is not binding RNA but rather Est2p or another telomerase-associated protein (Hughes *et al.*, 2000).

The Sm protein complex, involved in the biogenesis of small nuclear RNPs, also associates with the yeast telomerase RNP (Seto *et al.*, 1999). The functional role of this telomerase-associated complex will be discussed in section 1.7.1.

1.6.3 Mammals

In contrast to ciliates and yeast where only a few telomerase-associated partners have been described, the human telomerase RNP has at least ten reported associated proteins. This may reflect the increased complexity involved in regulating the human telomerase RNP or the more intensive research to which the human enzyme is subjected. The first mammalian telomerase-associated protein was identified based on sequence homology searches using the sequences from the *Tetrahymena* p80 and p95 subunits (Harrington *et al.*, 1997a; Nakayama *et al.*, 1997). The N-terminal region of the identified protein, TEP1, shows limited sequence conservation to *T. thermophila* p80. Antibodies specific to the ~240-kDa TEP1 protein coimmunoprecipitate the telomerase RNA and telomerase activity from cell extracts (Harrington *et al.*, 1997a). Surprisingly, *TEP1*$^{-/-}$ mice are viable and cells from various tissues show normal telomerase activity levels and telomere length (Liu *et al.*, 2000). Further studies will be required to determine the functional role of the association between TEP1 and the mammalian telomerase RNP.

Using a yeast two-hybrid screen with the TRF1 telomere-binding protein as bait, a protein was identified, PinX1, that interacts both with TRF1 and hTERT *in vitro* and in cells (Zhou and Lu, 2001). Overexpression of PinX1 or an N-terminus deleted mutant form of PinX1 in telomerase-positive cells causes (i) a significant reduction in the cellular levels of telomerase activity, (ii) progressive telomere shortening, and (iii) growth arrest. Addition of recombinantly-purified PinX1 or mutant PinX1 to cell extracts prepared from telomerase-positive cells shows a dose-dependent inhibition of telomerase activity *in vitro.* Lastly, injection of transformed cells stably overexpressing the cDNA of PinX1 and its antisense into mice causes tumor growth inhibition and increases tumorigenecity, respectively. These results suggest that PinX1 is a potent endogenous telomerase inhibitor, and thus, a possible tumor suppressor protein.

The identification of hTERT- and hTR- binding proteins was also possible by means of genetic screens performed in yeast that used hTERT or hTR subunits as baits. The human homolog of the *Drosophila* Staufen protein and the ribosome-associated L22 protein were identified using the pseudoknot domain of hTR (see Figure 1-4) as bait in the three-hybrid system (Le *et al.*, 2000). Human Staufen was also shown to be in a specific hTR-binding complex present in partially purified human telomerase extracts (Bachand *et al.*, 2001). Though Staufen is a well-established RNA-binding protein involved in the proper localization of specific mRNAs during *Drosophila* development (Roegiers and Jan, 2000), the role of its human homolog in telomerase function has not yet been determined. Using the first 195 amino acids of hTERT in the two-hybrid system, Holt *et al.* identified the chaperone protein p23 as an hTERT-binding partner (Holt *et al.*, 1999). Based on this information, they provided evidence that p23, together

with other proteins such as Hsp90, are key molecular chaperones required for the reconstitution of telomerase activity *in vitro* in RRL.

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are abundant, predominantly nuclear, RNA-binding proteins that primarily associate with RNA pol II transcripts, but that are also involved in many other cellular activities (Krecic and Swanson, 1999). Several hnRNPs can bind RNA and/or ssDNA, and have been reported to associate with mammalian telomerase. The N-terminal fragment of hnRNP A1, referred to as UP1, binds single-stranded telomeric DNA and interacts with telomerase *in vitro* (LaBranche *et al.*, 1998). In addition, a cell line deficient for hnRNP A1 expression has short telomeres (LaBranche *et al.*, 1998). The hnRNP C1 and C2 proteins can be cross-linked to radiolabeled hTR and antibodies specific to C1/C2 coimmunoprecipitate human telomerase activity from cell extracts (Ford *et al.*, 2000). The inability of hnRNP C1/C2 to associates with a catalytically active mutant of telomerase that is not competent in telomere elongation (presumably due to a defect in telomere recruitment-see Counter *et al.*, 1998; Ouellette *et al.*, 1999) suggests that C1/C2 may function in providing access of telomerase to the telomere. Similarly to the UP1 fragment from hnRNP A1, hnRNP D was also shown to associate with the human telomerase RNP and to bind single-stranded telomeric DNA (Eversole and Maizels, 2000). However, the roles of hnRNP A1, C1/C2, and D in telomere and/or telomerase functions remain to be determined.

Other proteins such as dyskerin, hGAR1, NOP10, NHP2, and the product of the *survival of motor neuron* (*SMN*) gene also associate with the human telomerase RNP. Since these proteins are likely involved in the assembly and the biogenesis of mammalian telomerase, they will be discussed in the next section.

1.7 The Biogenesis of Telomerase

The characterization of the telomerase RNP has taught us that evolution can guide divergent organisms to develop different means to generate a similar molecular complex. Indeed, evidence has accumulated over the past years suggesting that protozoa, yeast, and vertebrates use different pathways to assemble their respective telomerase RNP. As for many other RNPs, the pathway used for assembly mostly depends on the nature of the RNA components. Many steps are likely to occur following the synthesis of the telomerase RNA from its corresponding gene: 3' end processing events, addition of a 5' cap structure, nuclear retention or export/re-import, nucleotide modifications, assembly with the TERT subunits. Recent results suggest that spliceosomes and transcriptosomes are pre-assembled in a substrate-independent fashion (Gall *et al.*, 1999; Stevens *et al.*, 2002). Similarly, telomerase is likely pre-assembled into a functional RNP before recruitment to its site of action, the telomere. All these events are important regulatory steps essential for the reconstitution of a functional telomerase RNP *in vivo*.

1.7.1 Yeast

As mentioned previously, 5-10% of the yeast telomerase RNA harbors a ~80 nt-long poly (A) tail (Chapon *et al.*, 1997). Pulse chase-like experiments performed under the control of galactose-dependent TLC1 expression suggest that the deadenylated form of TLC1 is generated from the maturation of polyadenylated TLC1 transcripts (Chapon *et al.*, 1997). Furthermore, the biochemical purification of the yeast telomerase catalytic subunit, Est2p, indicates that deadelyated TLC1 RNAs are enriched in the mature yeast

telomerase RNPs *in vivo* (Friedman and Cech, 1999). Whether Est2p associates with the mature 3' end-processed TLC1 RNA or 3' end processing occurs after TLC1 association with Est2p is currently unknown.

A major breakthrough regarding yeast telomerase biogenesis came with the demonstration that the TLC1 RNA is bound by Sm proteins and acquires a 2,2,7-trimethylguanosine (TMG) cap structure on its 5' end (Seto *et al.*, 1999). Both of these events are hallmarks of spliceosomal Uridine (U)-rich small nuclear (sn) RNP assembly (Will and Luhrmann, 2001). The Sm core complex contains seven proteins (B/B', D1-D3, E, F, and G) predicted to form a closed ring structure around a conserved sequence motif within some of the UsnRNAs (Kambach *et al.*, 1999; Mura *et al.*, 2001; Will and Luhrmann, 2001). Following the identification of a putative Sm-binding site in the primary nucleotide sequence of the TLC1 RNA, the functional relevance of the Sm-TLC1 interaction was investigated. The introduction of mutations in the Sm-binding site of TLC1 resulted in low levels of telomerase RNA, short telomeres, but not in cell death (Seto *et al.*, 1999). Because these experiments were performed in a genetic background that prevents telomere maintenance via recombination-mediated pathways, the absence of cell death in these cells suggests either the use of a cryptic Sm-binding site or residual telomerase assembly by an Sm-independent pathway.

These findings suggest that yeast telomerase assembly proceeds through a similar pathway as for UsnRNP assembly. The biogenesis of UsnRNPs is best characterized in vertebrates due to the use of *Xenopus* oocytes (Will and Luhrmann, 2001). The current model for vertebrate UsnRNP assembly is the following: after RNA Pol II-dependent transcription of certain UsnRNAs and their polyadenylation, these UsnRNAs are exported

out of the nucleus via the utilization of specific export factors. In the cytoplasm, recognition of UsnRNAs by certain Sm proteins leads to the formation of a ring structure where the Sm-binding site in the RNA is surrounded by the Sm core complex. The Sm complex is required for 5' cap hypermethylation, 3' end processing and nuclear import of the UsnRNAs (Mattaj, 1986; Seipelt *et al.*, 1999). In the nucleus, the association of proteins specific to each UsnRNAs leads to the assembly of functional UsnRNPs via poorly understood events. It is likely that yeast telomerase follows these general steps for RNP assembly. However, there is no evidence for a cytoplasmic phase in the biogenesis of UsnRNPs in yeast.

1.7.2 Vertebrates

In contrast to yeast, no evidence is currently available suggesting a poly (A) tail for the mammalian telomerase RNA (Zaug *et al.*, 1996; Chen *et al.*, 2002). Rather, 3' end determination of the mammalian telomerase RNA *in vivo* indicates that it is discrete (Zaug *et al.*, 1996), suggesting a possible 3' end-processing event. A major clue regarding the pathway of mammalian telomerase biogenesis was provided by the identification of a small nucleolar (sno) RNA-like domain in the 3' region of human and mouse telomerase RNAs (Mitchell *et al.*, 1999a). SnoRNAs in the form of snoRNP complexes are involved in the post-transcriptional modifications of pre-ribosomal RNAs (rRNA) and are of two major classes: box C/D and box H/ACA. Whereas box C/D snoRNAs direct 2'-O-methylation of specific ribose sugars in pre-rRNA, box H/ACA snoRNAs direct the conversion of certain uridine to pseudouridine residues (Weinstein and Steitz, 1999). Vertebrate telomerase RNAs have a box H/ACA snoRNA-like domain

(Mitchell *et al.*, 1999a; Chen *et al.*, 2000). Mutations that perturb the folding of the H/ACA box of human and mouse telomerase RNAs prevent both their cellular accumulation (Mitchell *et al.*, 1999a; Martin-Rivera and Blasco, 2001) and their localization to the nucleolus of microinjected *Xenopus* oocytes (Narayanan *et al.*, 1999), but do not affect *in vitro* reconstitution of telomerase activity (Beattie *et al.*, 2000; Bachand and Autexier, 2001). Human and *Xenopus* telomerase RNAs were also shown to localize in Cajal (or coiled) bodies (Lukowiak *et al.*, 2001). The nucleolus and Cajal bodies are nuclear compartments rich in RNA-binding proteins where the assembly and maturation of many RNP particles are believed to occur (Matera, 1999).

The human telomerase RNA associates with the H/ACA box snoRNA-binding proteins dyskerin, hGAR1, NH2P, and NOP10 (Mitchell *et al.*, 1999b; Dragon *et al.*, 2000; Pogacic *et al.*, 2001). Though the specific role of these proteins in vertebrate telomerase function is unclear, studies support the physiological relevance of their association with telomerase RNAs. First, the X-linked form of the disease dyskeratosis congenita is caused by mutations in the gene that encodes the box H/ACA snoRNP protein dyskerin (Heiss *et al.*, 1998). Cells from individuals affected by this disease have low levels of hTR and telomerase activity as well as short telomeres (Mitchell *et al.*, 1999b). Second, mature hTR can accumulate in *S. cerevisiae* (Bachand and Autexier, 1999; Dez *et al.*, 2001) and this accumulation requires the presence of the yeast H/ACA proteins (Dez *et al.*, 2001). Thus, these proteins are likely involved in the maturation of vertebrate telomerase RNAs and in the assembly of functional telomerase RNPs.

Interestingly, evidence does not support a role for Sm proteins in vertebrate telomerase biogenesis ((Lukowiak *et al.*, 2001); see Chapter 4), and conversely, box

H/ACA snoRNP proteins are not required for TLC1 RNA accumulation in *S. cerevisiae* (Dez *et al.*, 2001). 5'-TMG cap-specific antibodies do not coimmunoprecipitate hTR from extracts of microinjected *Xenopus* oocytes and human cells, suggesting that in contrast to the *S. cerevisiae* telomerase RNA, vertebrate telomerase RNAs do not acquire a hypermethylated 5'cap structure ((Lukowiak *et al.*, 2001); see Chapter 4). Furthermore, microinjection experiments in *Xenopus* oocyte nuclei indicate that human and *Xenopus* telomerase RNAs are not exported into the cytoplasm, in contrast to the major spliceosomal UsnRNAs (Lukowiak *et al.*, 2001). Although much characterization is still needed, the current experimental results suggest that vertebrate telomerase assembly is nuclear, most likely requiring events in subnuclear compartments such as the nucleolus and Cajal bodies. Following transcription of the vertebrate telomerase RNA, it could be targeted to the nucleolus for maturation, processing, and/or stabilization by box H/ACA snoRNP proteins. As will be presented in Chapter 4, we found that the product of the *survival of motor neuron* (*SMN*) gene is a human telomerase-associated protein. The established function of SMN in the assembly of diverse RNPs and its localization to the nucleolus and Cajal bodies (Terns and Terns, 2001; Paushkin *et al.*, 2002) support a model where vertebrate telomerase biogenesis requires events performed in these subnuclear compartments.

1.8 Telomerase, Telomeres, and Cancer

The maintenance of functional telomeres at chromosome ends is essential for the prolonged, often immortal, survival of many single-celled eukaryotic organisms with linear genomes. In the past decade, many studies indicate that this rule also applies to

immortal and tumor mammalian cells. As the enzyme telomerase is responsible for telomere maintenance in most cells, the role of telomerase and telomeres in replicative senescence, cellular immortalization, and tumorigenic conversion is being intensively studied. In the following sections, I will summarize the major experimental findings that suggest the causal relationships between telomere dysfunction and replicative senescence, and telomerase activation and cellular immortalization.

1.8.1 Replicative Senescence

The process of replicative senescence is observed when primary cells are cultured *in vitro*. More that 40 years ago, Hayflick and Moorhead observed that human cultured primary cells stop growing after a finite number of cell divisions (Hayflick and Moorhead, 1961; Hayflick, 1965). For example, *in vitro*-cultured fibroblasts stop growing after 40 to 80 cell divisions. These early observations suggested the presence of a molecular mechanism capable of counting the number of accomplished cell divisions, and establishing a state of growth arrest when the number of divisions reached a maximum. This state of growth arrest is often referred to as replicative or cellular senescence. Though cells in replicative senescence are no longer dividing, they (1) are metabolically active, (2) have a characteristically enlarged morphology, (3) upregulate cellular markers such as a pH-dependent lysosomal β-galactosidase, p21, and p16.

What is the molecular mechanism that enables primary cells to evaluate the number of cell divisions accomplished? As discussed in section 1.4, the inability of the conventional DNA replication machinery to fully replicate the linear ends of eukaryotic chromosomes poses a problem. Most human somatic cells do not have telomerase

activity, and therefore should lose telomeric DNA with each cell division as predicted by the end replication problem. The analysis of *in vitro*-cultured cells and cells from donors of different ages clearly shows progressive telomere shortening with increasing number of cell divisions *in vitro* and *in vivo* (Harley *et al.*, 1990; Hastie *et al.*, 1990). This pioneering work mostly directed by Calvin Harley, coupled to additional experimental observations, led to a hypothesis in which replication-mediated telomere shortening plays an important role in the establishment of replicative senescence. This hypothesis commonly referred to as "telomere hypothesis of cellular aging or senescence" is illustrated in Figure 1-8. The presence of telomerase activity in most human germ cells enables the maintenance of telomeres and the indefinite proliferation of these cells. However, as most human somatic cells lack detectable telomerase activity, each DNA replication event leads to the loss of telomeric DNA. The telomere hypothesis of cellular aging originally suggested that after a certain number of cell divisions causing telomeres to reach a critically minimal length, telomere-dependent cellular signals would trigger the induction of growth arrest resulting in replicative senescence.

The identification of the human telomerase protein catalytic subunit permitted an experimental setup to test the hypothesis that cellular senescence is induced in a telomere-dependent fashion. As illustrated in Figure 1-8, the ectopic expression of hTERT in telomerase-negative primary fibroblasts and epithelials cells reconstitutes active telomerase, adds new DNA sequence repeats onto telomeres, prevents replicative senescence, and allows these cells to proliferate indefinitely (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998). These results strongly support a model in which "critically" short telomeres trigger a growth arrest phenotype resulting in cellular senescence. The

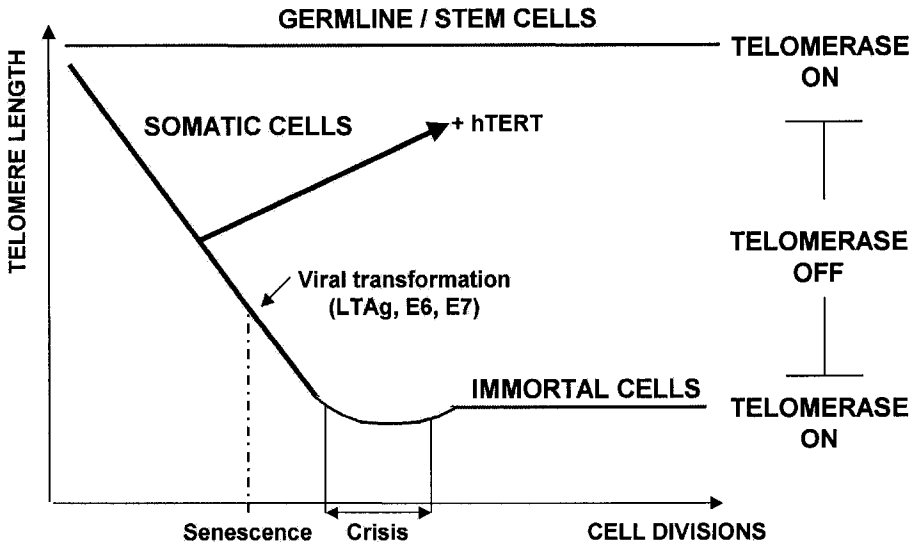


Figure 1-8. Telomere Hypothesis of Cellular Aging

57

induction of growth arrest with characteristics of replicative senescence upon the cellular expression of a dominant-negative mutant of the telomere-protecting protein TRF2 (van Steensel *et al.*, 1998), further supports the functional relationship between telomere homeostasis and the activation of senescence.

What cellular signaling pathways are involved in the telomere-dependent activation of replicative senescence? The infection of primary human cells with transforming viruses such as SV40 and HPV, or expression of the product of their viral oncogenes (*LTAg* and *E6/E7*, respectively), is sufficient to allow the bypass of cellular senescence (Shay *et al.*, 1991). Because LTAg/E6 and E7 respectively inactivate the action of the p53 and Rb tumor suppressor proteins, these results suggest that the cell cycle arrest imposed upon replicative senescence is activated by signals that go through the p53 and/or the Rb pathways (see Figure 1-8). These observations are supported by the proliferation of human primary cells beyond their normal replicative lifespan upon downregulation of p53 and Rb expression via the use of antisense oligonucleotides (Hara *et al.*, 1991). In contrast to the ectopic expression of hTERT in human primary cells however, the inactivation of the p53 and Rb pathways does not allow primary cells to divide indefinitely. Indeed, following an additional 20-30 population doublings depending on the cell type, the p53/Rb-inactived cells will enter another phase of growth arrest referred to as "crisis". The "crisis" state will be discussed later.

The molecular machinery responsible for the induction of telomere-dependent cellular senescence via the p53/Rb signaling pathways is incompletely understood. However, recent experiments have shed light on how these growth arrest pathways could sense telomere dysfunction. First, overexpression of a dominant-negative (DN) mutant of

TRF2 in telomerase-positive cells leads to either replicative senescence or apoptosis, depending of the cell type (van Steensel *et al.*, 1998; Karlseder *et al.*, 1999). Interestingly, the growth arrest (apoptosis or senescence) induced by the mutant TRF2 is very rapid and occurs without any detectable telomere shortening; yet chromosomal abnormalities including end-to-end fusions are readily observed (van Steensel *et al.*, 1998; Karlseder *et al.*, 1999). Since TRF2 promotes telomere-specific loop structures, the rapid onset of either senescence or apoptosis by mutant TRF2 is likely to be caused by the disruption of this protective structure and the recognition of 'uncapped' telomeres by DNA damage response pathways. Telomere dysfunction induced by mutant TRF2 indeed activates a damage pathway in response to dsDNA breaks. This response involves the action of the cell-cycle signaling kinase ATM and the activation of apoptosis in a p53-dependent manner (Karlseder *et al.*, 1999). Whether the molecular interaction of telomerase (Li *et al.*, 1999) and telomeres (Stansel *et al.*, 2002) with the tumor suppressor p53 plays a role in the induction of senescence or apoptosis is not known. Recently, the overexpression of wild-type TRF2 in telomerase-negative fibroblasts was shown to increase the rate of telomere shortening without accelerating the activation of senescence (Karlseder *et al.*, 2002). These findings support the concept that replicative senescence is induced by the disruption of a protective 'cap' structure at telomeres rather than by the complete loss of telomeric DNA. Furthermore, recent data acquired using cells from mice homozygote or heterozygote for a deletion in a gene encoding a telomerase component suggest that the essential function of telomerase is to replenish the ends of critically short telomeres, rather than maintaining a given average telomere length (Hathcock *et al.*, 2002; Liu *et al.*, 2002). These data provide additional evidence that

critically short telomeres, rather than the average telomere length, elicit the cellular response to telomere dysfunction (Hemann *et al.*, 2001).

Conversely, other cell types acquire a senescent phenotype even though they maintain their telomere lengths via telomerase. Human primary keratinocytes and mammary epithelial cells ectopically expressing hTERT have a finite replicative lifespan (Kiyono *et al.*, 1998). Similarly, most rodent cells senesce in culture even though they contain active telomerase (Shay and Wright, 2001). Several studies also report the establishment of a senescent phenotype upon the induction of stress signals such as DNA-damage (DiLeonardo *et al.*, 1994; Robles and Adami, 1998), oxidative stress (Chen *et al.*, 1995), or via inappropriate mitogenic signaling of the RAS pathway (Serrano *et al.*, 1997). Recent findings, however, question the existence of these telomere-independent senescence mechanisms. In the February 2001 issue of *Science*, two elegant studies provided evidence that some rodent telomerase-positive primary cells can replicate indefinitely in culture, yet a change in culture conditions could elicit entry into senescence (Mathon *et al.*, 2001; Tang *et al.*, 2001). Similarly, altering the growth conditions of the primary keratinocytes and epithelial cells used by Kiyono *et al.*, by growing them on feeder layers rather than on plastic, allowed these cells to proliferate indefinitely upon telomerase activation (Ramirez *et al.*, 2001). Thus, in the presence of telomerase activity there does not seem to be any intrinsic cellular mechanisms limiting the lifespan of primary cells, yet extrinsic factors (such as inadequate culture conditions, damage signals, and oxidative stress) can limit their proliferation capacity.

1.8.2 Cellular Immortalization and Cancer

In the early 1990s, the link between telomere shortening and the *in vitro* and *in vivo* replicative lifespan of telomerase-negative primary cells was well established. From this, it could be predicted that telomere maintenance was critical for cellular immortalization. One of the first pieces of evidence supporting this assumption was provided from collaborative work from the Bacchetti, Greider, and Harley labs. This study permitted to better define the steps occurring during 'crisis' and cellular immortalization. As mentioned above, primary cells in which the p53/Rb pathways have been altered bypass replicative senescence presumably by inactivating the cell-cycle checkpoints that sense telomere dysfunction. Yet, these cells are not immortal and following 20-30 population doublings where telomere length continues to decrease after each mitosis, they enter 'crisis' (see Figure 1-8). At crisis, extensive genomic instability and high rates of apoptosis kills most of the cells within the population, nevertheless rare cells (1 in 10^7) survive and become immortal (see Figure 1-8). One of the major finding from the collaboration of the Bacchetti, Greider, and Harley labs was that all of the surviving immortal cell lines had activated telomerase (Counter *et al.*, 1992), thus establishing telomere maintenance as a requirement for cellular immortality.

Although this study was one of the first demonstrating a correlation between telomerase activation and cellular immortalization, only a few immortal cell lines were studied (Counter *et al.*, 1992). The generality of this correlation was established by analyzing a wide variety of human cells and tissues with a highly sensitive PCR-based assay for the detection of telomerase activity (Kim *et al.*, 1994). This study revealed that,

whereas most human somatic cells and tissues are telomerase-negative, 85% of human immortal cell lines and tumor cells possess telomerase activity. It is now well accepted that telomere maintenance is a requirement for the indefinite proliferative capacity of immortal cells. Immortal cells avoid the massive genomic instability resulting from critically short telomeres by either activating endogenous telomerase or by using alternative means of telomere length maintenance (ALT) (Bryan *et al.*, 1995; Bryan *et al.*, 1997). All of the immortal cell lines and tumors cells that have been investigated to date have a telomere length maintenance mechanism, clearly establishing the link between telomere maintenance and cellular immortalization.

Although ectopic expression of telomerase in human primary fibroblasts is sufficient for immortalization (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998), these immortalized cells do not show any sign of malignancy such as growth transformation, bypass of cell-cycle-induced checkpoints, and genomic instability (Jiang *et al.*, 1999; Morales *et al.*, 1999; Vaziri *et al.*, 1999). Thus, the maintenance of the shortest telomeres via telomerase activation is sufficient to (i) immortalize primary cells by preventing the genomic instability associated with 'uncapped' telomeres, (ii) but is not sufficient to transform them into tumorigenic cells. However, human tumor cells are created *in vitro* when a constitutively active oncogenic allele of *ras* (H-*ras*), the SV40 large and small T antigens, and hTERT are introduced into human primary fibroblasts and epithelial cells (Hahn *et al.*, 1999). These findings established that telomere maintenance (via telomerase) is necessary but not sufficient for the development of tumors *in vitro*, and that additional genetic alterations are required.

Overexpression of hTERT proteins mutated in essential residues within the reverse transcriptase motifs abolish telomerase activity and act as dominant-negative mutants in telomerase-positive human cancer cell lines (Hahn *et al.*, 1999; Zhang *et al.*, 1999; Colgin *et al.*, 2000). In most of the cases, and especially in cancer cell lines with short telomere length, the expression of a dominant-negative version of hTERT causes progressive telomere shortening, followed by chromosome end-to-end fusions, activation of DNA damage responses and cell death by apoptosis (Hahn *et al.*, 1999; Zhang *et al.*, 1999; Colgin *et al.*, 2000) . The ability to kill certain cancer cell lines through the inhibition of telomerase is also supported by studies in which telomerase is inhibited by the use of modified oligonucleotides (Herbert et al., 1999), ribozymes (Ludwig et al., 2001), or specific inhibitors (Damm et al., 2002). Telomerase RNA mutated in the template sequence and that result in the incorporation of the mutant sequence into human telomeres also result in the loss of viability of human cancer cells (Marusic et al., 1997; Kim et al., 2001; Guiducci et al., 2001). Overall, these studies strongly support the model where telomere maintenance by telomerase is essential for cellular immortalization and cancer, and that rationally-designed drugs that specifically target telomerase may be useful in anticancer therapy

In conclusion: (1) the *in vitro* phenomena of replicative senescence and crisis may represent tumor suppressive mechanisms in the human organism, (2) telomere length maintenance by telomerase or ALT is essential for tumorigenesis by allowing cancer cells to proliferate indefinitely and acquire a sufficient number of mutations to generate a malignant growth, (3) telomerase biology is likely to provide promising targets for cancer treatment; as most tumor cells need telomerase activity, whereas normal cells do not.

1.8.3 Mouse Models

The information gained using cell culture systems established the critical role of telomere maintenance, mainly mediated by telomerase, in cellular immortalization and cancer. To address the role of telomere maintenance in cancer development and aging in a living organism, a mouse deficient for telomerase activity was developed via the deletion of the gene coding for the telomerase RNA component (Blasco *et al.*, 1997). Because of the long telomeres of the laboratory mouse strain used for the development of the original telomerase knockout mice, successive breeding of the telomerase-deficient animals was required to generate telomeres that approach the lengths observed in humans. By the fourth generation, the effects of telomere dysfunction were detectable in highly proliferative tissues such as the testis, the bone marrow, and the spleen (Lee *et al.*, 1998). As expected, defects associated with telomere dysfunction were detected in earlier-generation animals in a mouse strain with naturally shorter telomeres (Herrera *et al.*, 1999). One of the most surprising results from the development of telomerase-deficient mice was the increased frequency of spontaneous malignancies in late-generation mice, as compared to wild-type or early-generation animals (Rudolph *et al.*, 1999); suggesting that telomere shortening in these mice was increasing the frequency of cancer, rather than preventing its progression.

An explanation proposed for this anomalous finding is that short 'uncapped' telomeres can trigger dual outcomes: (1) the activation of growth arrest pathways via senescence or apoptosis or (2) genomic instability. Genomic instability in cultured cells containing chromosomes with critically short telomeres is well established (see above

discussion on 'crisis' state), but also in late-generation telomerase-null mice (Hande *et al.*, 1999) and plants (Riha *et al.*, 2001). Indeed, cells isolated from proliferative tissues of late-generation telomerase-null mice show extensive chromosomal abnormalities (Blasco *et al.*, 1997). In yeast, the chromosomal alterations induced by short and dysfunctional telomeres lead to an increase in the overall genomic mutation rate (Hackett *et al.*, 2001). Based on this observation in yeast, the fact that late-generation telomerase knockout mice have a higher incidence of spontaneous tumors in proliferative tissues could be explained by the more rapid accumulation of deleterious oncogenic mutations as a result of dysfunctional telomeres. Thus, depending on the capacity of a cell type to rapidly acquire oncogenic mutations, the protective effects of the proliferative checkpoints activated by 'uncapped' telomeres could be outweighed. Such a mechanism is supported by the accelerated carcinogenesis observed in mice deficient in both telomerase and the tumor suppressor p53, where the incidence of chromosomal instability is accentuated as compared to telomerase-null mice (Chin *et al.*, 1999; Artandi and DePinho, 2000). The ability of p53 to induce growth arrest in cells containing DNA damage and dysfunctional telomeres (Karlseder *et al.*, 1999), coupled with the likely higher mutation rate caused by unstable telomeres (Hackett *et al.*, 2001), suggest that the increase tumor frequency in $mTR^{-/-}/p53^{-/-}$ mice results from the improved survival of genetically damaged cells.

However, the story is not that simple. Crossing telomerase-null mice with mice deleted for tumor suppressor genes other than p53 results in reduced carcinogenesis. Mice deficient for the *CDKN2A* locus (a locus responsible for the expression of the two potent tumor suppressors p16 and $p19^{ARF}$) are highly prone to cancer when compared to

wild-type mice (Serrano *et al.*, 1996). Yet, when crossed to late-generation telomerase-null mice with short telomeres, *CDKN2A*$^{-/-}$ mice have a reduce tumor frequency compared to when they are crossed with early-generation telomerase-null mice with long telomeres (Greenberg *et al.*, 1999a). A similar situation is observed when carcinogens are applied to the skin of telomerase-knockout mice; short telomeres seem to protect against the development of epithelial tumors (Gonzalez-Suarez *et al.*, 2000). Thus, telomerase may play a paradoxical role by either promoting or inhibiting tumor progression depending on the genetic context. Clearly, further characterization of the genetic backgrounds responsible for promoting or inhibiting cancer formation in the presence of short telomeres will be required, particularly regarding pathways involved in response to DNA damage that are p53-dependent and independent.

1.8.4 From Mice to Humans: Dyskeratosis Congenita

Dyskeratosis congenita (DKC) is a genetic disorder with variable modes of inheritance, X-linked and autosomal dominant. X-linked DKC patients are diagnosed early in life and usually succumb to the disease by the age of 16. This disease is characterized primarily by defects in cellular proliferative capacity, such as cutaneous symptoms and bone marrow failure. Whereas bone marrow failure is the major cause of mortality in DKC individuals, predisposition to malignancies is also observed in older DKC patients.

The X-linked form of DKC is caused by mutations in the gene coding for the nucleolar protein dyskerin, the homolog of the yeast *CBF5* gene (Heiss *et al.*, 1998). Dyskerin and Cbf5p are putative pseudo-uridine synthases. In association with specific

H/ACA snoRNAs, pseudo-uridine synthases post-transcriptionally modify ribosomal RNA via the isomerization of uridine residues. Deletion of *CBF5* in *S. cerevisiae* causes defects in pseudo-uridylation and processing of pre-rRNAs (Lafontaine *et al.*, 1998). In 1999, the Collins lab reported that the X-linked form of DKC results from a telomerase deficiency (Mitchell *et al.*, 1999b). Whereas cells from DKC individuals have no detectable defects in snoRNA, snRNA, and rRNA accumulation and pseudo-uridylation, they have low levels of hTR and telomerase activity as well as short telomeres (Mitchell *et al.*, 1999b). Strong additional evidence that telomerase deficiency is a causative factor in DKC came from the finding that the RNA component of telomerase is mutated in the autosomal dominant form of DKC (Vulliamy *et al.*, 2001). Three different types of mutations were identified from independent families with autosomal dominant DKC: (1) a mutation in one allele of the *hTR* gene causing the deletion of the H/ACA box and the CR7 domain, (2) a single nucleotide mutation within the P3 helix of the pseudoknot, and (3) a two-nucleotide mutation in the P8b helix of the CR7 domain (see Figure 1-5). It is not yet clear whether these mutations cause DKC via a dominant-negative effect or haplo-insufficiency.

1.9 Telomerase Regulation

As discussed above, telomerase activity is present in most immortal and tumor cells, whereas it is not detectable in the majority of somatic diploid cells. Notably, there is a strong correlation between the presence of hTERT mRNA and telomerase activity (Kilian *et al.*, 1997; Meyerson *et al.*, 1997). The lack of hTERT mRNA in mortal primary cells and its detection in immortal and tumor cells suggest that *hTERT* gene expression is

required for the telomerase activation that occurs during cellular immortalization and tumorigenesis. Similar observations have been reported during development; telomerase activity detectable at the blastocyst stage and in most embryonic tissues before 20 weeks of developmental gestation is subsequently lost (Wright *et al.*, 1996). Thus, telomerase is active in development to compensate for telomere sequence loss during this stage where extensive proliferation is required to sustain tissue growth and differentiation. The human telomerase RNA is expressed in most cells and tissues (Feng *et al.*, 1995). Yet, variations in the levels of hTR expression have been reported (Yashima *et al.*, 1998). However, based on the strikingly long cellular half-life of hTR (Yi *et al.*, 1999), telomerase regulation via hTR accumulation would require significant proliferative time in order to have a measurable influence.

The cloning of the promoter region from the *hTERT* gene identified putative binding sites for several transcription activators as well as repressors (Cong *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999). Several *hTERT* transcriptional activators have been identified using a variety of techniques, but mostly by the ectopic expression of putative positive regulators (Ducrest *et al.*, 2002). The best characterized of the activators has been the effect of the c-Myc proto-oncogene on *hTERT* gene expression (Wang *et al.*, 1998; Falchetti *et al.*, 1999; Greenberg *et al.*, 1999b; Wu *et al.*, 1999). Experimental evidence suggest that c-Myc directly activates the expression of hTERT mRNA: (i) c-Myc-induced *hTERT* expression is rapid, independent of cell proliferation and of additional protein synthesis, (ii) c-Myc associates with *hTERT* promoter sequences in extracts from telomerase-positive HL60 cells as assayed by chromatin immunoprecipitation. Estrogen also activates *hTERT* gene expression via the activation

of estrogen response elements (ERE) located several hundred nucleotides upstream from the hTERT initiation codon (Misiti *et al.*, 2000). Conversely, the transcriptional repressor Mad was shown to negatively regulate *hTERT* expression (Oh *et al.*, 2000). The switch from Myc to Mad at the *hTERT* promoter has been suggested to play a role in the downregulation of hTERT mRNA and telomerase activity during the differentiation of the promyelocytic leukemic cell line HL60 (Xu *et al.*, 2001).

Alternative splicing of hTERT pre-mRNA is also likely involved in the regulation of cellular telomerase activity levels. Indeed, alternative splicing was suggested to explain the isolation of six additional hTERT cDNAs following the identification of the *hTERT* gene (Kilian *et al.*, 1997). Though the endogenous protein products encoded by these alternatively spliced forms of the hTERT pre-mRNA have not yet been detected, the mRNA deletion variants have been detected by RT-PCR in immortalized cells, normal tissues, and during embryonic development (Kilian *et al.*, 1997; Ulaner *et al.*, 1998; Ulaner *et al.*, 2000). Overexpression of one variant (deleting 36 bp within the RT motif A of hTERT-see Figure 1-6) in telomerase-positive cells negatively affects endogenous telomerase activity and causes telomere shortening that eventually leads to cell death (Colgin *et al.*, 2000; Yi *et al.*, 2000).

Studies have also reported that the protein catalytic subunit of human telomerase is post-translationally regulated via phosphorylation. Protein kinase Cα (PKCα), Akt kinase, and c-Abl modulate the activity of telomerase (Li *et al.*, 1998; Kang *et al.*, 1999; Kharbanda *et al.*, 2000). Whereas the studies reporting hTERT as a substrate for the PKC and Akt kinases do not provide data supporting the physiological relevance of the hTERT-PKC and hTERT-Akt interactions (Li *et al.*, 1998; Kang *et al.*, 1999), the

evidence that hTERT is a physiological substrate of c-Abl is more convincing (Kharbanda *et al.*, 2000). c-Abl is a protein tyrosine kinase that binds to proline rich-containing substrates via a conserved SH3 domain (Feller *et al.*, 1994). Anti-HA immunoprecipitates prepared from extracts of human 293 cells cotransfected with HA-tagged hTERT and c-Abl constructs recover lower level of telomerase activity as compared to cells in which HA-hTERT was cotransfected with vector control or catalytically inactive c-Abl kinase. Similarly, expression of HA-hTERT in mouse embryonic fibroblasts (MEF) from c-Abl$^{-/-}$ mice results in lower levels of telomerase activity as compared to c-Abl$^{+/+}$ mice, and the telomeres from c-Abl$^{-/-}$ mice MEF are shorter than wild-type mice. These findings support c-Abl as a negative regulator of telomerase activity.

Recruitment of telomerase to human telomeres is likely to be regulated. The description of catalytically active telomerase mutants not functional in telomere elongation and cellular immortalization (Counter *et al.*, 1998; Ouellette *et al.*, 1999; Armbruster *et al.*, 2001), as well as the identification of proteins involved in telomerase recruitment to yeast telomeres (Evans and Lundblad, 1999; Pennock *et al.*, 2001) support this type of regulation in human as well. As discussed above, it is well established using *in vitro*-cultured cells that telomerase activation during 'crisis' is necessary for most cellular immortalization events. Though telomerase is active in more that 85% of human tumor cells, whether telomerase activation occurs during the tumorigenic conversion of cells in the organism is less clear. Indeed, the idea that tumors *in vivo* result from the malignant transformation of non-terminally differentiated telomerase-positive cells remains an open possibility.

1.10 Telomere maintenance without telomerase

Telomerase is the enzyme responsible for the maintenance of telomeric sequences in most organisms as well as in most immortalized human and tumor cells. Yet, a subset of immortalized and tumor cells do not display detectable levels of telomerase activity (Bryan *et al.*, 1995, 1997; Henson *et al.*, 2002). These cells maintain the length of their telomeres by one or more mechanisms referred to as alternative lengthening of telomeres (ALT). The pathways used for telomere maintenance without telomerase are best characterized in the budding yeast *Saccharomyces cerevisiae*. Initial studies by Lundblad and Blackburn demonstrate that yeast in which the *EST1* gene is disrupted exhibit progressive telomere shortening that lead to a senescence-like phenotype (Lundblad and Blackburn, 1993). Though most of the cells within the population eventually senesce, rare survivors escape the lethal consequences of the telomere defects. These surviving cells exhibit dramatic rearrangements at the ends of their chromosomes: amplification of telomeric and/or sub-telomeric repeat sequences as detected by telomere restriction fragment analysis. The lack of survivors in yeast cells genetically disrupted for both the *EST1* and the *RAD52* genes suggested that homologous recombination events are involved in the telomerase-independent maintenance of telomeres in yeast (Lundblad and Blackburn, 1993). Two independent recombination pathways have been characterized in yeast that involves the *RAD50* and *RAD51* genes (Le *et al.*, 1999). Experimental evidence also suggest that recombination is involved in the maintenance of telomeric sequences in telomerase-negative immortalized human cells (Dunham *et al.*, 2000). Notably, recent data in both yeast and mammalian cells indicate that reduction in DNA mismatch repair pathways may influence telomere maintenance in the absence of

telomerase by contributing to telomere instability (Rizki and Lundblad, 2001; Varley et al., 2002). Further studies in yeast and mammalian cells will be required to better understand the molecular mechanisms that underlie ALT pathways.

REFERENCES: See page 199

OBJECTIVES

The goal of the present thesis was to characterize the human telomerase ribonucleoprotein both structurally and functionally. I was also interested in telomerase-associated proteins involved in the biogenesis and assembly of the human telomerase RNP. To fully understand the role of telomerase in telomere synthesis and in cell survival, it is essential to dissect the minimal and associated components of telomerase. To address this, we developed a heterologous expression and reconstitution system using the budding yeast *Saccharomyces cerevisiae* (Chapter 2) and ultimately generated recombinant enzyme. Using an expression and reconstitution system, it was possible to dissect the minimal components of human telomerase (hTERT and hTR; Chapter 3). To better understand the biogenesis and assembly of the human telomerase RNP, it is essential to first identify associated proteins. Chapter 4 described the identification of a telomerase-associated protein (SMN) that is likely to be involved in the biogenesis of a functional human telomerase enzyme.

Chapter 2

FUNCTIONAL RECONSTITUTION OF HUMAN TELOMERASE EXPRESSED IN *SACCHAROMYCES CEREVISIAE*

PREFACE

Human telomerase activity is reconstituted by the addition of *in vitro*-synthesized human telomerase RNA to a rabbit reticulocyte lysate (RRL) programmed to express the telomerase catalytic subunit. This raised the possibility that RRL-associated proteins may be involved in human telomerase assembly *in vitro*. However, the scarcity of the protein levels expressed using RRL makes it difficult to analyze the biochemical composition of the telomerase RNP reconstituted using this system. To establish a human telomerase reconstitution system more easily amenable to biochemical analysis, we investigated whether the genetically tractable yeast *S. cerevisiae* could reconstitute a functional human telomerase RNP *in vivo*.

SUMMARY

Telomerase is a ribonucleoprotein enzyme complex that adds DNA repeats at the end of chromosomes. In an effort to establish an *in vivo* heterologous expression system for active human telomerase, we expressed hTERT (*h*uman *te*lomerase *r*everse *t*ranscriptase) in *Saccharomyces cerevisiae* and affinity-purified the protein as a fusion with glutathione-S-transferase (GST). Addition of the GST moiety to the N-terminus of hTERT did not interfere with telomerase activity when GST-hTERT was expressed in rabbit reticulocyte lysate (RRL) in the presence of the human telomerase RNA (hTR).

Active human telomerase was immunoprecipitated from yeast lysates that co-expressed GST-hTERT and hTR. In addition, telomerase activity could be reconstituted *in vitro* by the addition of hTR to GST-hTERT that was immunoprecipitated from either RRL or *S. cerevisiae* lysates. The expression and reconstitution of human telomerase activity in yeast will provide powerful biochemical and genetic tools to study the various components required for the assembly and function of this enzyme.

INTRODUCTION

Eukaryotic cells possess linear chromosomes which are predicted to lose terminal sequences after every DNA replication event (Autexier and Greider, 1996; Reddel, 1998). In order to circumvent this end replication problem, most eukaryotic cells possess a ribonucleoprotein (RNP) enzyme, telomerase, which uses its RNA template to synthesize DNA repeats at the ends of chromosomes (Colgin and Reddel, 1999; O'Reilly *et al.*, 1999; Pardue and DeBaryshe, 1999). The protein and RNA components of this enzyme complex have been identified in several organisms from yeast to humans (Nugent and Lundblad, 1998; O'Reilly *et al.*, 1999). In humans, the catalytic subunit of telomerase (hTERT, for *h*uman *te*lomerase *r*everse *t*ranscriptase) is a 125 kDa protein with distinctive motifs common to reverse transcriptases (RT), as well as a telomerase-specific (T) motif (Harrington *et al.*, 1997b; Kilian *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997).

The first *in vitro* reconstitution system used to study human telomerase consisted of adding recombinantly synthesized human telomerase RNA (hTR) to partially purified, micrococcal-nuclease-treated 293 cell extracts (Autexier *et al.*, 1996). More recently, human and *Tetrahymena thermophila* telomerase reconstitution has been accomplished by the *in vitro* transcription and translation of the protein catalytic component (hTERT and p133, respectively) in the presence of telomerase RNA in rabbit reticulocyte lysates (RRL) (Weinrich *et al.*, 1997; Beattie *et al.*, 1998; Collins and Gandhi, 1998). Studies using RRL-reconstituted telomerase suggest that hTERT and hTR are the only two components necessary to reconstitute human telomerase activity *in vitro*. Interaction of

the telomerase-associated protein (TEP1) with hTERT is observed in mouse cell extracts and with hTERT produced in RRL (Harrington *et al.*, 1997a; Beattie *et al.*, 2000). However, several lines of evidence suggest that proteins in the RRL are necessary for assembly of a functional telomerase enzyme. Using the yeast two-hybrid system, Holt and coworkers identified a chaperone protein, p23, that interacts with hTERT and that could be associated with a complex called "the foldosome" important for ribonucleoprotein assembly (Holt *et al.*, 1999). In addition, a recent study on the role of the *T. thermophila* telomerase RNA in telomerase function suggests that proteins other than the catalytic subunit are necessary for the functional assembly of this RNP in rabbit reticulocyte lysates (Licht and Collins, 1999).

Telomerase is active in most transformed and tumor cell lines, whereas the majority of normal diploid cells demonstrate no detectable telomerase activity (Kim *et al.*, 1994; Autexier and Greider, 1996; Shay and Bacchetti, 1997). Consequently, the telomerase enzyme has become an attractive target for chemotherapy. A better understanding of the biogenesis and structure of this RNP complex will therefore be necessary to evaluate the role of telomerase in cellular immortalization and cancer. Reconstitution of the telomerase RNP by overexpression of its components in heterologous organisms will be crucial to study the molecular mechanisms of this enzyme. In this study, we investigated whether human telomerase activity could be reconstituted by expressing hTERT and hTR in *S. cerevisiae*. We describe the expression and glutathione-sepharose affinity purification of the human telomerase catalytic subunit as a fusion to GST in yeast. In addition, we demonstrate that *S. cerevisiae* is capable of

reconstituting a functional human telomerase enzyme when GST-hTERT and hTR are co-expressed.

MATERIALS AND METHODS

Yeast strain -Yeast strain cIABYS86 (*MAT*α, *leu*2, *Ura*3-52, *his*3, *pra*1, *prb*2, *prc*1, *cps*) was used as a host strain for hTERT protein expression and also as a source of yeast cell lysate (Dubois *et al.*, 1998).

Construction of plasmids-Clone 712562, containing bases 1624-3399 of the hTERT coding sequence and 3' downstream sequences, was obtained from the I.M.A.G.E. (Integrated Molecular Analysis of Genomes and their Expression) Consortium through Genome System Inc., and was used for construction of an hTERT mutation using the Quick Change Site-Directed Mutagenesis kit from Stratagene (Lennon *et al.*, 1996). The oligonucleotides 5'-CTCCTGCGTTTGGTTAACGATTTCTTGTTG-'3 and 5'-CAACAAGAAATCGTTAACCAAACGCAGGAG-'3 were used to generate the D868N mutation and to create a *Hinc* II restriction site. This new construct (phTRTDNC) was sequenced using an Applied Biosystems automatic sequencer to confirm the presence of the mutation.

To generate the hTERT expression plasmids, the *htert* gene from pGRN121 (Nakamura *et al.*, 1997) was amplified by PCR using the 5' primer 5'-TGCTCTAGACCCGCGCGCTCCCCGC-'3 and the 3' primer 5'-CCCAAGCTTGGCGGGTGGCCATCAGTC-'3 containing *Xba*I and *Hin*dIII sites, respectively, as well as with the 5' primer 5'-CCGGAATTCTATGCCGCGCGCTCCCC-'3 and the 3' primer 5'-GAATGCGGCGCGTCAGTCCAGGATGGTCTTG-'3 containing *Eco*RI and *Not*I sites, respectively. The *Xba*I-*Hin*dIII and the *Eco*RI-*Not*I-digested PCR fragments were then cloned in pEGKT (Mitchell *et al.*, 1993) digested with *Xba*I-*Hin*dIII and in pET-28b (Novagen) digested with *Eco*RI-*Not*I, respectively. To generate the

hTERT D868N yeast expression construct, a 2.5 Kb *Mlu*I-*Hin*dIII fragment of pEGKT-hTERT was replaced with a *Mlu*I-*Hin*dIII fragment from a derivative of phTRTDNC containing the D868N mutation. To generate the pET-28a-GST-hTERT construct, the pEGKT-hTERT plasmid was linearized by digestion with *Hin*dIII. This linearized construct was subjected to partial digestion by *Sac*I restriction enzyme using a standard method (Ausubel *et al.*, 1998). This resulted in a 4150 bp GST-hTERT DNA fragment, which was gel purified and cloned into the *Sac*I-*Hin*dIII sites of pET-28a (Novagen). To construct the human telomerase RNA (hTR) yeast expression vector, the *hTR* gene was amplified by PCR from pGRN33 (Feng *et al.*, 1995) using the 5' primer 5'-CGCGGATCCCGGCAGCGCACCGGGTTGCGG-'3 and the 3' primer 5'-CGCGGATCCGCATGTGTGAGCCGAGTCCTGGGT-'3, both containing *Bam*HI restriction sites. The *Bam*HI-digested PCR fragment was cloned into the *Bam*HI site of the p413-GAL1 vector (Mumberg *et al.*, 1994).

Protein Expression and affinity purification-Yeast containing the different constructs were grown in selective medium containing 2% raffinose to an optical density at 600 nm of 0.6-0.8. To induce transcription from the *GAL1* promoter, galactose was added to a final concentration of 4%, and growth continued for 12-16 h. Cell were harvested, washed with sterile water and resuspended in either RIPA or TMG buffer (Cohn and Blackburn, 1995). Due to the inefficiency of the yeast protein extraction, 25 ml of a yeast culture was lysed by vortexing with glass beads for 6 pulses of 30 sec with at least 1 min interval (4°C) between each pulse. After removal of the glass beads and cell debris by two centrifugations (10,000 X g) at 4°C, the specific proteins in the lysates were affinity purified by the addition of glutathione-sepharose (Amersham Pharmacia Biotech) for 2 h

at 4°C, washed four times with lysis buffer (supplemented with NaCl to a final concentration of 0.5M) and subjected to SDS-PAGE for either coomassie blue (BioRad) staining or nitrocellulose transfer.

In vitro transcription and translation-pET-28b-hTERT and pET-28a-GST-hTERT plasmids were included in coupled transcription/translation (Promega) reactions (10-15 μl) at a final concentration of 25 ng/μl, with or without 10ng/μl of gel-purified hTR telomerase RNA. Gel purified human and *T. thermophila* telomerase RNA was generated as described previously (Autexier *et al.*, 1996; Autexier and Triki, 1999).

Immunoprecipitations-Immunoprecipitations were performed using yeast protein lysates (in TMG buffer) or 2-3 μl of reticulocyte lysate previously diluted into 500 μl of Buffer A (Autexier *et al.*, 1996). After a 1 h incubation at 4°C with preimmune serum, lysates were subjected to immunoprecipitation with specific antibodies (anti-GST from Amersham Pharmacia Biotech; anti-T7 from Novagen; anti-MYC from Invitrogen) for 1 h at 4°C. This was followed by incubation with pre-washed and lysis buffer-pre-equilibrated protein-A-sepharose (Amersham Pharmacia Biotech) for an additional 2 h. Antibody-coated beads were then washed four times with the respective lysis buffer and subjected to SDS-PAGE/Western blot analysis, TRAP assay, and RT-PCR.

Telomerase activity assays-Telomerase activity was assayed by a two-tube telomerase assay (modified TRAP) as described previously (Autexier *et al.*, 1996), with minor modifications. PCR reactions were performed for 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 m 30 s, using Taq polymerase from MBI Fermentas. ACX, TSNT and NT primers (Kim and Wu, 1997) were used at a concentration of 20 pmol/μl, 1 x 10^{-23} mol/μl and 2 pmol/μl, respectively. Amplification of TSNT by TS and NT primers

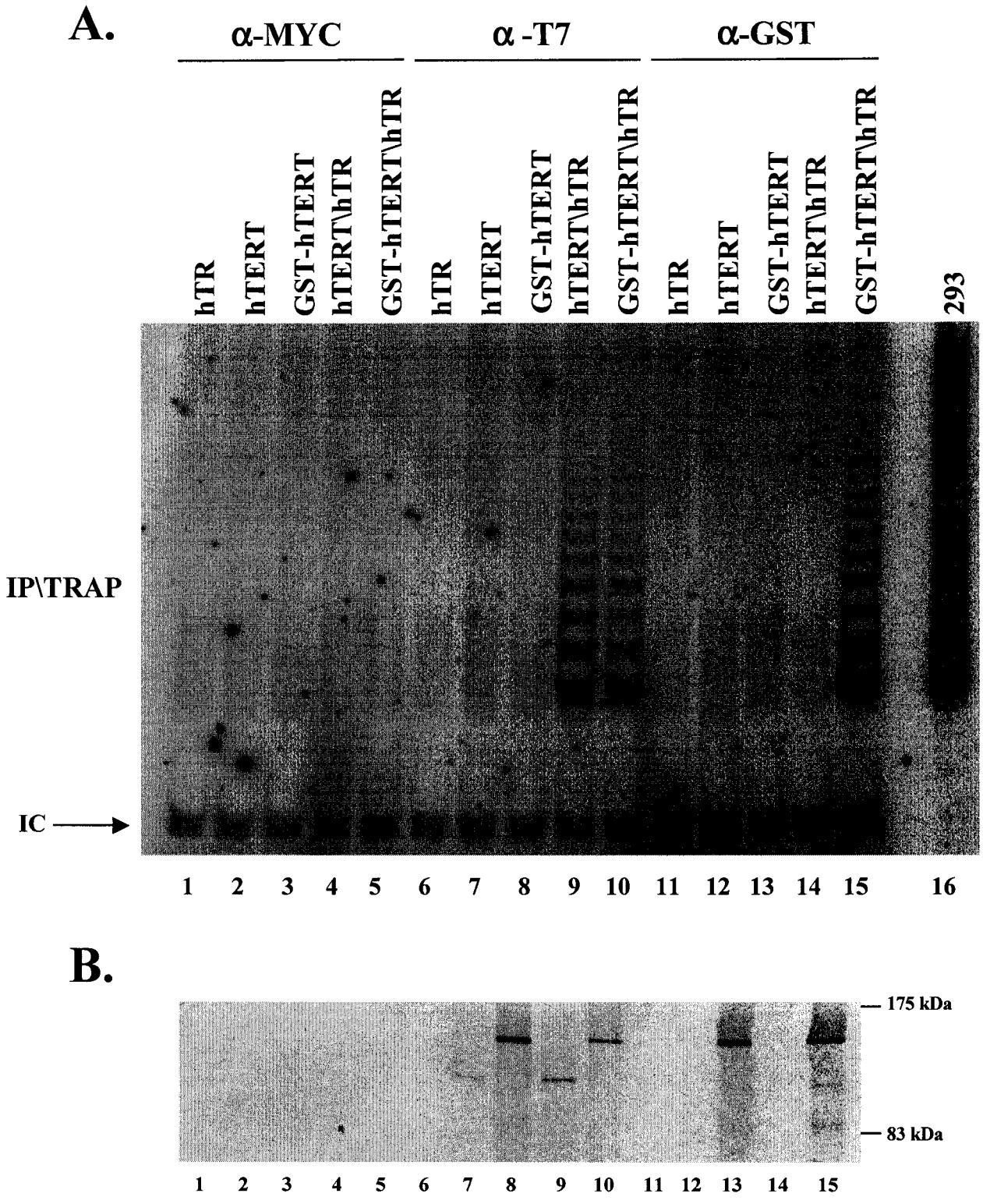
generated a 36 bp PCR internal control (IC). The positive control used in TRAP assays was partially purified 293 cell extracts prepared as described previously (Autexier *et al.*, 1996).

RESULTS AND DISCUSSION

Expression and Activity of a GST-hTERT fusion in rabbit reticulocyte lysates

Since the cloning of the gene encoding the catalytic subunit of the human telomerase enzyme, reconstitution of enzymatic activity has been achieved by expressing hTERT in the presence of hTR in rabbit reticulocyte lysates (Weinrich *et al.*, 1997; Beattie *et al.*, 1998). In order to extend the study of human telomerase, we expressed a glutathione-S-transferase (GST)-tagged hTERT in yeast (Fig. 2-2). To first determine whether the GST moiety fused to the N-terminus of hTERT interfered with telomerase function, the entire GST-hTERT coding sequence from pEGKT/hTERT was cloned into a T7-expression vector (see Materials and Methods). This GST-hTERT construct was transcribed and translated in a rabbit reticulocyte lysate (RRL) in the presence of hTR, and was immunoprecipitated using a goat anti-GST antibody (Fig. 2-1B, lane 15). The immunoprecipitate was then assayed for telomerase activity using a modified TRAP assay (see Materials and Methods). The GST-hTERT/hTR complex was active in the telomerase assay (Fig. 2-1A, lane 15), while no telomerase activity was detected when a T7-tagged hTERT/hTR complex was subjected to immunoprecipitation using the same anti-GST antibody (Fig. 2-1A, lane 14). As expected, immunoprecipitation of hTERT or GST-hTERT proteins synthesized in the absence of hTR did not reconstitute telomerase activity (Fig. 2-1A, lanes 7, 8, and 13), although these proteins were expressed (Fig. 2-1B). Immunoprecipitates of hTERT/hTR or GST-hTERT/hTR complexes were also prepared with a monoclonal antibody recognizing a small tag (T7 tag) fused to the N-terminus of both proteins (Fig. 2-1B, lanes 9 and 10). Both T7-tagged immunoprecipitated complexes demonstrated telomerase activity (Fig. 2-1A, lanes 9 and

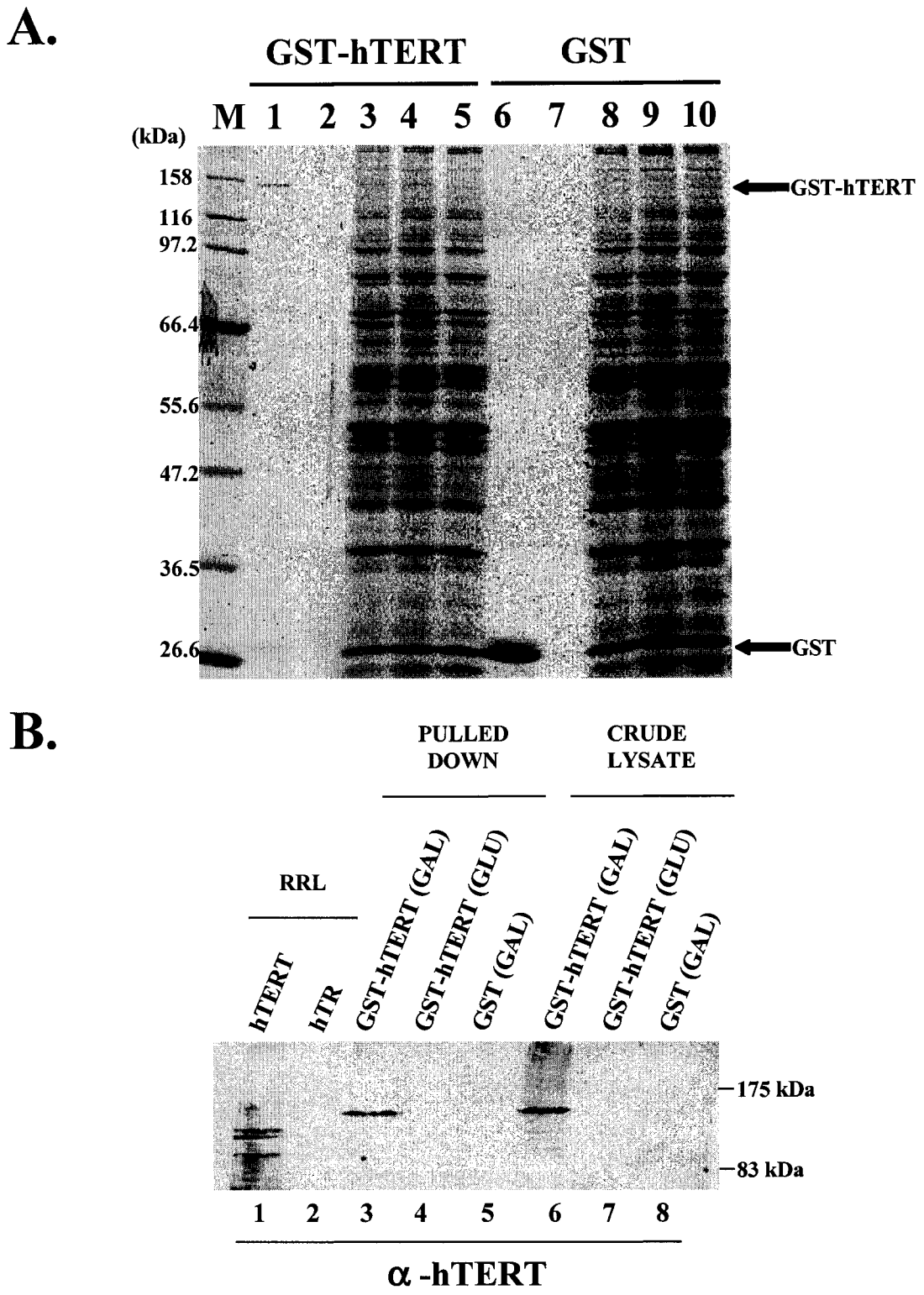
10). Immunoprecipitates prepared with a control anti-MYC antibody did not possess detectable telomerase activity (Fig. 2-1A, lanes 1 to 5). The results shown in Figure 2-1 indicate that addition of the 25 kDa GST protein to the N-terminus of the human telomerase catalytic subunit does not prevent the functional reconstitution of telomerase activity by hTERT in RRL.



3. 2-1. **Telomerase activity of the GST-hTERT fusion in rabbit reticulocyte lysates.** (A) [^{35}S]-methionine-eled hTERT and GST-hTERT were synthesized in a rabbit reticulocyte lysate in the presence or absence of the nan telomerase RNA component (hTR). As a control, RRL synthesis was performed with hTR RNA only nes 1, 6, and 11). Aliquots from each reaction were diluted with buffer (see Materials and Methods) and munoprecipitated using either anti-MYC (lanes 1 to 5), anti-T7 (lanes 6 to 10), or anti-GST (lanes 11 to 15) ibodies. After extensive washing, 1/10 of the immunoprecipitate was assayed for (A) telomerase activity, and remnant of the beads were (B) subjected to SDS-PAGE and autoradiography. 293, 0.1 μg of partially purified 3 cell extract; IC, internal PCR control for the TRAP assay.

Expression and affinity purification of the human telomerase catalytic component from *S. cerevisiae*

The GST-hTERT fusion protein was expressed in *S. cerevisiae* under the control of the galactose-inducible *GAL1* promoter. Lysates were prepared from yeast grown in glucose (repressed) or galactose (induced), and subjected to glutathione-sepharose batch affinity chromatography. The GST-hTERT fusion produced by yeast grown in galactose (Fig. 2-2A, lane 4) was affinity purified with glutathione-sepharose and migrated with a molecular weight of approximately 150 kDa (Fig. 2-2A, lane 1), consistent with hTERT and GST components of 125 and 25 kDa (lane 6), respectively. This fusion protein was not detected in extracts from uninduced yeast grown in glucose (Fig. 2-2A, lanes 2 and 5). The 150 kDa fusion was confirmed to be GST-hTERT in a Western blot (Fig. 2-2B, lanes 3 and 6) probed with an affinity purified anti-hTERT antibody (Harrington *et al.*, 1997). No protein was detected with the anti-hTERT antibody in: (i) glutathione-sepharose-purified proteins or crude lysate isolated from uninduced yeast (lanes 4 and 7, respectively); or (ii) lysates from yeast expressing a GST control (lanes 5 and 8). T7-tagged hTERT synthesized in RRL (Fig. 2-2B, lane 1) was used as a positive control. Species smaller than the full-length hTERT (which may be nonspecific cleavage products, products from initiation at downstream AUG, or premature translation termination) are routinely detected from RRL expressing hTERT with this antibody (Beattie *et al.*, 1998).



3. 2-2. **Expression and affinity purification of the human telomerase catalytic subunit from *S. cerevisiae.***) GST-hTERT was expressed and purified from yeast by affinity chromatography of crude extracts using tathione-sepharose. Lanes 5 and 10, uninduced soluble fractions; lanes 4 and 9, induced soluble fractions; es 3 and 8, unbound fractions from glutathione-sepharose; lanes 2 and 7, bound fractions from uninduced yeast ate; lanes 1 and 6, bound fractions from galactose-induced yeast lysate. (B) Glutathione-sepharose purified l crude lysate fractions from galactose-induced GST (lanes 5 and 8, respectively), uninduced (lanes 4 and 7, pectively) and galactose-induced (lanes 3 and 6, respectively) GST-hTERT, were loaded onto a 7.5% SDS-GE, transferred to a nitrocellulose membrane, and immunoblotted with an affinity-purified hTERT antibody. controls, T7-hTERT (lane 1) and hTR (lane 2) synthesized and added, respectively, in a rabbit reticulocyte ate (RRL) were included. GLU, Glucose; GAL, Galactose.

Reconstitution of human telomerase activity by co-expression of hTERT and hTR in *S. cerevisiae*

As the GST-hTERT fusion expressed in RRL was functional and reconstituted human telomerase activity (Fig. 2-1), we examined whether telomerase activity could be reconstituted in *Saccharomyces cerevisiae* by co-expressing the catalytic and the RNA components. The gene encoding the *hTR* RNA was also cloned under the control of a *GAL1* promoter. This construct was transformed into yeast cells expressing either GST, wild-type GST-hTERT, or GST-hTERT with a point mutation at amino acid 868 that changes a conserved Asp^{868} residue to an asparagine in motif C of hTERT. Following selection of the double transformants, we examined the expression of hTR in yeast that were grown in media containing galactose. Reverse-transcription and polymerase chain reaction (RT-PCR) on total yeast RNA using hTR-specific primers confirmed the presence of hTR in yeast transformed with the hTR-expressing construct; this specific RNA was not detected in control yeast transformed with the vector alone (data not shown).

Once an inducible system for co-expression of hTERT and hTR in *S. cerevisiae* was established, cell lysates were prepared from yeast grown in selective media containing galactose or glucose. Yeast cell lysates were subjected to immunoprecipitation using an anti-GST serum and the immunoprecipitates analyzed for telomerase activity (Fig. 2-3A). Immunoprecipitates from two independent yeast clones co-expressing the wild-type GST-hTERT and hTR were positive for telomerase activity as analyzed by the TRAP assay (Fig. 2-3A, lanes 3 and 4). To ensure that the activity was due to hTERT rather than a co-immunoprecipitating protein, the D868N hTERT

mutant was also immunoprecipitated and shown to lack telomerase activity (Fig. 2-3A, lane 6). The lack of telomerase activity observed for this active site point mutant suggests that the activity observed in lanes 3 and 4 is attributable to hTERT, and also confirms that the integrity of motif C is essential for activity (Counter *et al.*, 1997; Lingner *et al.*, 1997; Weinrich *et al.*, 1997; Beattie *et al.*, 1998). RT-PCR analysis of the immunoprecipitates revealed that hTR was specifically associated with the reconstituted telomerase (Fig. 2-3C, lanes 3 and 4), as well as with the D868N GST-hTERT point mutant (lane 6). RT-PCR analysis of immunoprecipitates from control yeast that expressed GST alone did not detect hTR RNA (Fig. 2-3C, lane 7).

Reconstitution of telomerase activity as well as expression of the protein and RNA components of human telomerase were only detected from yeast grown in galactose-containing medium. Growth under repressing conditions (glucose) did not induce the expression of hTERT (Fig. 2-3B, lane 5) or hTR (data not shown), demonstrating the specificity of the expression system. Human telomerase activity could not be detected using immunoprecipitates from yeast transformed with control vectors (Fig. 2-3A, lanes 1 and 7), confirming that both hTERT and hTR components were required to reconstitute human telomerase activity. The results shown in Figure 2-3 demonstrate that co-expression of hTERT and hTR in *S. cerevisiae* specifically reconstituted human telomerase activity.

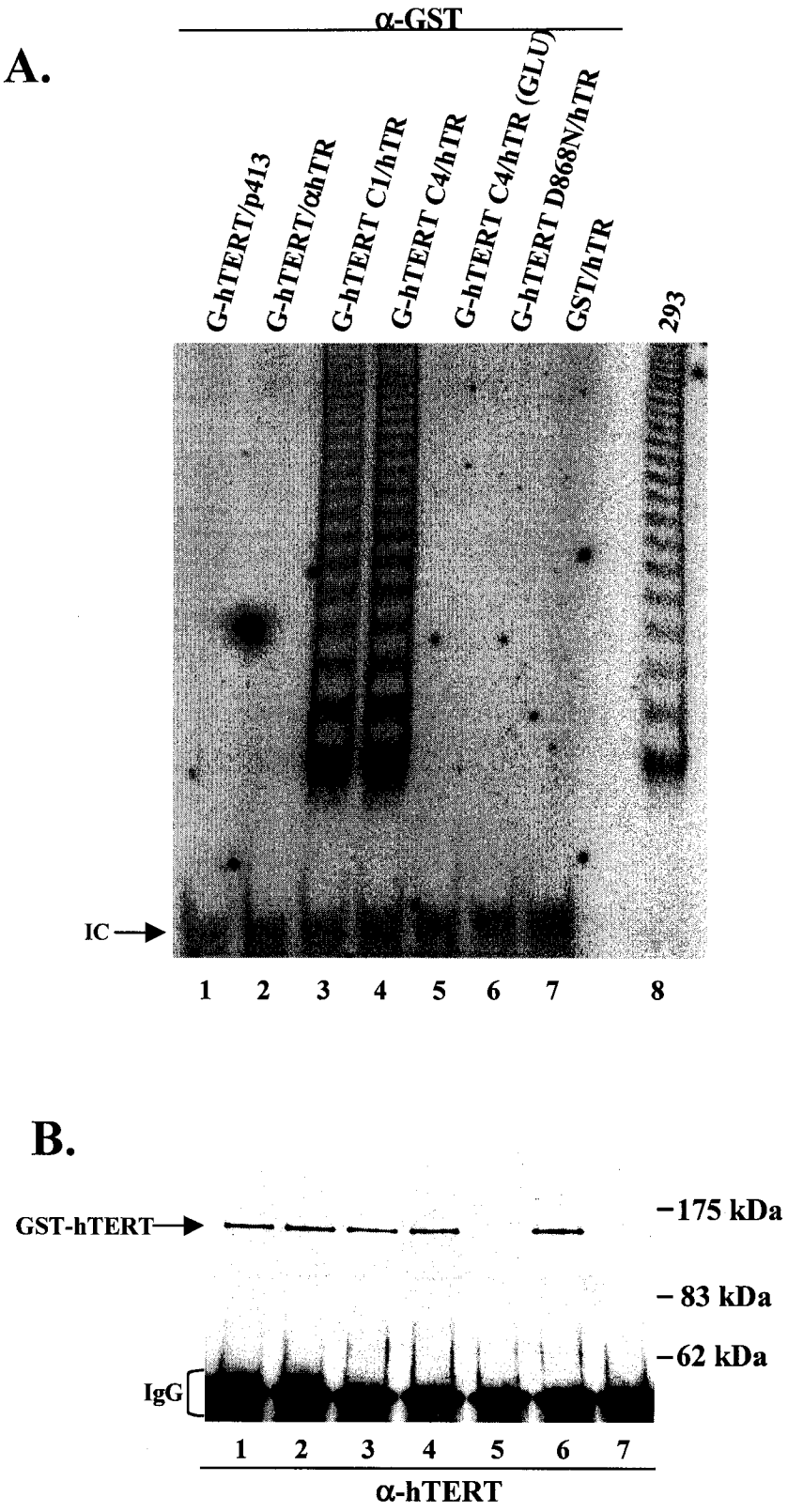


FIG. 2-3. **See following page for legend.**

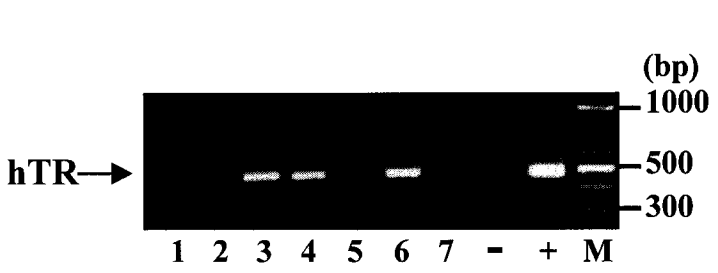


FIG. 2-3. **Reconstitution of human telomerase activity in *S. cerevisiae* by co-expression of hTERT and hTR.** (A) Yeast transformed with the following constructs: pEGKT/hTERT and p413 vector (lane 1); pEGKT/hTERT and p413/antisense hTR (αhTR) (lane 2); pEGKT/hTERT clone 1 (C1) and p413/hTR (lane 3); pEGKT/hTERT clone 4 (C4) and p413/hTR (lane 4); pEGKT/hTERT clone 4 (C4) and p413/hTR grown in the presence of glucose (GLU) (lane 5); pEGKT/hTERT D868N and p413/hTR (lane 6); pEGKT vector and p413/hTR (lane 7), were grown and induced with galactose (except for lane 5). Yeast lysates were subjected to immunoprecipitation using a goat anti-GST serum and the immunoprecipitates analyzed for (A) telomerase activity, (B), expression of GST-hTERT by Western blot, and (C), expression and association of hTR RNA with hTERT by RT-PCR using hTR-specific primers. For panels (A), (B), and (C), lanes 1 to 7 correspond to the constructs described above. In (A), telomerase activity was analyzed by the TRAP assay and 50 ng of partially purified 293 cell extract was used as a positive control (lane 8). IC represents the internal PCR control. G-hTERT represents GST-hTERT. In (B), a Western blot was performed using an affinity-purified hTERT antibody. IgG represents the immunoglobulins used during the immunoprecipitation. The position of the protein markers are indicated on the right in kDa. In (C), RT-PCR was performed in the absence (-) or presence (+) of *in vitro* synthesized hTR RNA as controls reactions. Lane M was loaded with a 100 bp ladder.

S. cerevisiae lysate does not stimulate assembly of the GST-hTERT/hTR complex *in vitro*

Recent reports suggest that *in vitro* reconstitution of human and *T. thermophila* telomerase RNPs requires proteins present in rabbit reticulocyte lysates (Holt *et al.*, 1999; Licht and Collins, 1999). Similarly, we asked whether the reconstitution of telomerase activity by the addition of hTR to immunoprecipitated hTERT may be dependent on proteins present in *S. cerevisiae* lysate. We used anti-GST serum to immunoprecipitate RRL-expressed GST-hTERT synthesized in the absence of the human telomerase RNA, as well as *S. cerevisiae*-expressed wild-type and D868N mutant GST-hTERT, which were expressed in the absence of the hTR-expressing construct. Following extensive washing, the immunoprecipitated proteins were incubated with either human or *T. thermophila* telomerase RNA in the presence or absence of a fresh yeast protein extract. Reconstituted reactions were assayed, after a 45 min incubation at 30°C, for telomerase activity. The addition of fresh yeast protein extract was neither required for nor stimulated the telomerase activity reconstituted by the assembly of hTR with immunoprecipitated RRL- or *S. cerevisiae*-expressed GST-hTERT (Fig. 2-4, lanes 1 vs. 2 and 7 vs. 8, respectively). Addition of *T. thermophila* telomerase RNA did not reconstitute human telomerase activity (lanes 3 and 9), highlighting the specificity of the reconstitution reaction for human telomerase RNA. Incubation of human telomerase RNA with the *S. cerevisiae*-expressed and immunoprecipitated GST-hTERT D868N mutant did not reconstitute activity (Fig. 2-4, lanes 4 and 5), confirming the requirement for a catalytically active hTERT component. In addition to using immunoprecipitated GST-tagged hTERT in

reconstitution reactions, we also expressed a T7-tagged hTERT in RRL, immunoprecipitated the protein with a monoclonal anti-T7 antibody, and functionally reconstituted telomerase activity by only adding hTR (data not shown). The results presented in Figure 2-4 suggest that the *in vitro* assembly of a functional hTERT/hTR telomerase RNP using immunoprecipitated hTERT does not require factors from the yeast protein lysate.

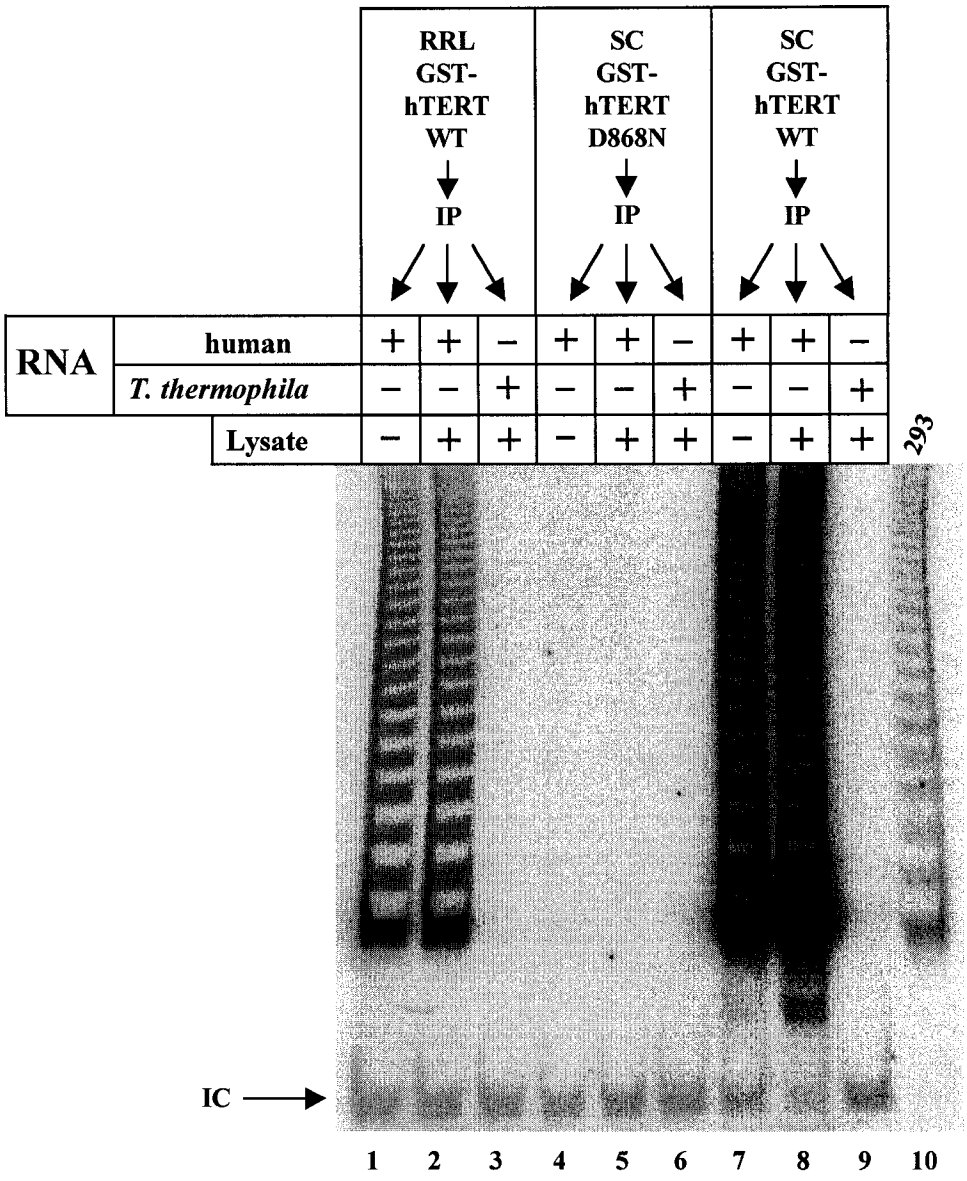


FIG. 2-4. ***S. cerevisiae* lysate does not stimulate *in vitro* reconstitution of human telomerase RNP.** Rabbit Reticulocyte Lysate (RRL)-expressed GST-hTERT (no hTR) and *S. cerevisiae*-expressed (no hTR) GST-hTERT wild-type and D868N mutant were immunoprecipitated (IP) using anti-GST serum. Immunoprecipitated proteins were washed extensively (five times) and incubated with 200 ng of either human or *T. thermophila* telomerase RNA in the presence or absence of fresh yeast protein extract. After 45 min at 30°C, reconstitution reactions were diluted to 40 µl and assayed for telomerase activity by TRAP analysis. IC, internal PCR control; SC, *Saccharomyces cerevisiae*. As a positive control, 50 ng of partially purified 293 cell extract was used (lane 10).

We have expressed and affinity purified the catalytic subunit of human telomerase as a GST fusion in the yeast *Saccharomyces cerevisiae*. To our knowledge, this is the first report that describes the heterologous expression of a catalytically active, full-length, recombinant hTERT in a system other than rabbit reticulocyte lysate. We demonstrated that the GST-hTERT fusion functions to reconstitute human telomerase activity in RRL when synthesized in the presence of recombinant human telomerase RNA. We also showed that co-expression of GST-hTERT and hTR in *S. cerevisiae* produced a telomerase enzyme complex that was catalytically active *in vitro*.

Quantification of the relative telomerase activity reconstituted by the addition of in *vitro*-synthesized hTR to affinity purified GST-hTERT generated from yeast or RRL indicate that comparable amounts (as determined by silver staining) of GST-hTERT immunoprecipitated from yeast or RRL yield similar levels of telomerase activity (Fig. 2-4 and data not shown). However, the amount of active hTERT that can be generated in yeast is limited only by the amount of yeast cultured and the efficiency of the protein extraction, making the yeast system a more abundant and practical source of active human telomerase than RRL. Preliminary attempts to reconstitute human telomerase activity using the soluble recombinant GST-hTERT fusion expressed from yeast and *in vitro*-synthesized hTR were unsuccessful (data not shown). We found that the GST-hTERT/hTR complex synthesized in RRL and precipitated with glutathione-sepharose had significantly less telomerase activity than the same complex precipitated with an anti-GST antibody (data not shown; Fig. 2-1*A*, lane 15), suggesting that the active conformation of the GST-hTERT fusion may be altered upon binding to glutathione.

Recent *in vitro* studies indicate that the assembly of a functional telomerase RNP enzyme requires specific protein components (Holt *et al.*, 1999; Licht and Collins, 1999). Two chaperone proteins, p23 and Hsp90, were suggested to be important in the assembly of the human telomerase enzyme (Holt *et al.*, 1999). The ability to reconstitute a functional human telomerase RNP in the yeast *Saccharomyces cerevisiae* (Fig. 2-3) suggests that the cellular machinery required for the folding and/or assembly of the human telomerase catalytic subunit with its specific hTR may have been conserved through evolution. However, reconstitution of telomerase activity in the absence of yeast protein extracts using recombinantly synthesized hTR and immunoprecipitated GST-hTERT expressed from either RRL or *S. cerevisiae* (Fig. 2-4), suggests that yeast proteins may not be essential for human telomerase RNP assembly and activity *in vitro*. It is possible that proteins important for the assembly of the telomerase RNP *in vivo* were co-immunoprecipitated with the GST-hTERT protein. It is also conceivable that non-physiological *in vitro* levels of both hTERT and hTR can overcome an *in vivo* requirement for factors that assemble the human telomerase RNP. *In vitro* reconstitution of *T. thermophila* telomerase using immunoprecipitated p133 (catalytic subunit of *T. thermophila*) expressed in RRL and *Tetrahymena* telomerase RNA is dependent on proteins from the rabbit reticulocyte lysate, suggesting that human and *T. thermophila* telomerase may require different factors for RNP assembly (Licht and Collins, 1999).

We have shown that active human telomerase can be extracted from yeast co-expressing the hTERT and hTR components. Others have changed the endogenous yeast telomerase RNA template to a human telomerase RNA template to dictate the synthesis of TTAGGG repeats at yeast telomeres without growth impairment (Henning *et al.*,

1998). Whether the functional replacement of yeast telomerase by human telomerase is possible *in vivo* is presently under investigation. The *in vivo* expression of a catalytically active recombinant hTERT in *S. cerevisieae* will be useful for future genetic and biochemical studies.

ACKNOWLEDGMENT

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Chapter 3

IDENTIFICATION OF FUNCTIONAL REGIONS OF hTERT AND hTR REQUIRED FOR TELOMERASE ACTIVITY AND RNA-PROTEIN INTERACTIONS

PREFACE

Previous studies have identified functional regions within the human telomerase RNA required for the reconstitution of human telomerase activity *in vitro*. However, it is unclear whether these identified regions are required for the association with the protein catalytic subunit or involved in another step of telomerase catalysis. In this study, we delineated the functional regions of hTR required for its association with hTERT *in vitro*. Furthermore, we identified the functional domains of hTERT necessary for efficient association with the telomerase RNA and telomerase activity.

SUMMARY

Telomerase is a specialized reverse transcriptase (RT) that is minimally composed of a protein catalytic subunit and an RNA component. The RNA subunit contains a short template sequence that directs the synthesis of DNA repeats at the ends of chromosomes. Human telomerase activity can be reconstituted *in vitro* by the expression of the telomerase protein catalytic subunit (hTERT) in the presence of recombinant human telomerase RNA (hTR) in a rabbit reticulocyte lysate (RRL) system. We analyzed telomerase activity and binding of hTR to hTERT in RRL by expressing different hTERT and hTR variants. Human telomerase RNAs containing nucleotide substitutions that are

predicted to disrupt base pairing in the P3 helix of the pseudoknot weakly reconstituted human telomerase activity, yet retained their ability to bind hTERT. Our results also identified two distinct regions of hTR that can independently bind hTERT *in vitro*. Furthermore, sequences or structures between nucleotides 208 and 330 of hTR (which include the conserved CR4-CR5 domain) were found to be important for hTERT-hTR interactions and for telomerase activity reconstitution. Human TERT carboxy-terminal amino acid deletions extending to motif E or the deletion of the first 280 amino acids abolished human telomerase activity without affecting the ability of hTERT to associate with hTR, suggesting that the RT and RNA binding functions of hTERT are separable. These results indicate that the reconstitution of human telomerase activity *in vitro* requires regions of hTERT that (i) are distinct from the conserved RT motifs; and (ii) bind nucleotides distal to the hTR template sequence.

INTRODUCTION

The physical end of each eukaryotic chromosome consists of a nucleoprotein complex known as the telomere (Blackburn and Greider, 1995). The termini of telomeric DNA cannot be fully replicated by the conventional DNA replication machinery, and consequently, chromosomes shorten at each cell division (Lingner *et al.*, 1995; Lingner and Cech, 1998). One solution to this end replication problem is the ribonucleoprotein (RNP) complex, telomerase. Telomerase compensates for telomere erosion by replenishing the sequence repeats (TTAGGG in humans) at the 3' end of telomeric DNA (Collins, 2000).

Telomerase activity was initially identified in the ciliated protozoan *Tetrahymena thermophila* (Greider and Blackburn, 1985). The gene encoding the telomerase protein catalytic subunit was first identified by reverse genetics in the protozoan, *Euplotes aediculatus* (Lingner *et al.*, 1997). Catalytic subunits of telomerase, the telomerase reverse transcriptases (TERTs), contain conserved reverse transcriptase (RT) motifs and a telomerase-specific (T) motif (Lingner *et al.*, 1997; Nakamura *et al.*, 1997). In addition to the RT and T motifs, the amino-terminus of TERTs from ciliated protozoa share a common motif (CP motif) that is weakly conserved in the catalytic subunit of other organisms (Bryan *et al.*, 1998). TERT has now been identified in several organisms including budding and fission yeast, mouse, humans, and plants (Counter *et al.*, 1997; Harrington *et al.*, 1997; Kilian *et al.*, 1997; Lingner *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997; Greenberg *et al.*, 1998; Martin-Rivera *et al.*, 1998; Fitzgerald *et al.*, 1999). Telomerase catalytic activity is dependent on an intrinsic RNA molecule that

contains a short template sequence (Greider and Blackburn, 1989). The gene coding for the RNA subunit of telomerase has also been cloned from several organisms (Nugent and Lundblad, 1998). Contrary to the strong homology between the RT-like motifs of the TERTs (O'Reilly *et al.*, 1999), the telomerase RNA subunit from various organisms differs significantly in terms of size and nucleotide sequence (Nugent and Lundblad, 1998). Recently, a secondary structure for the human telomerase RNA (hTR) was proposed based on a phylogenetic comparison of telomerase RNA components identified from a variety of vertebrate species ((Chen *et al.*, 2000); illustrated in Fig. 3-1). Four conserved structural elements are universally present in the predicted secondary structure of vertebrate telomerase RNA: these are the pseudoknot domain, the CR4-CR5 domain, the H/ACA box, and the CR7 domain. Interestingly, the predicted vertebrate telomerase RNA secondary structure displays a structural topology similar to the ciliate telomerase RNA (Romero and Blackburn, 1991).

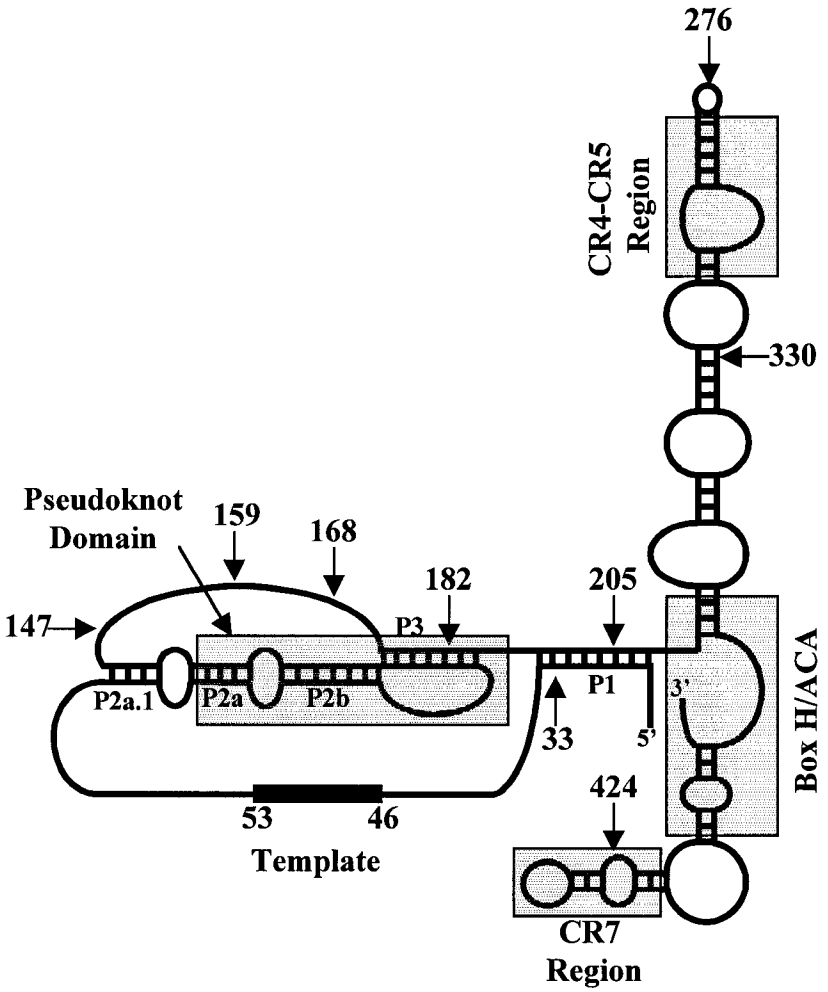

FIG. 3-1. **The human telomerase RNA (hTR).** Secondary structure of hTR with its telomeric template sequence (nt 46-53) and its 5' and 3' ends (adapted from Chen *et al.* 2000). Four universally conserved structural elements among vertebrate telomerase RNAs including the pseudoknot, the CR4-CR5 region, the H/ACA box, and the CR7 region are boxed in gray. The P1, P2a.1, P2a, P2b, and P3 helices are indicated. Arrows indicate the nucleotide position of the 5' and 3' ends of the different hTR truncations used in this study.

Human telomerase activity was initially reconstituted *in vitro* by the addition of recombinant human telomerase RNA to micrococcal nuclease-treated, partially purified 293 cell extracts (Autexier *et al.*, 1996). Using this reconstitution assay, a minimal functional region of hTR was identified between nucleotides 44-205. Reconstitution of human telomerase by the addition of recombinant hTR to *in vitro*-translated human TERT (hTERT) in a rabbit reticulocyte lysate (RRL) determined that residues 10-159 of hTR are sufficient to generate weak human telomerase activity *in vitro* (Beattie *et al.*, 1998). More recently, *in vitro* assembly reactions using different hTR truncations and hTERT synthesized separately in RRL or human cells, identified nucleotides 33 and 325 as the 5' and 3' functional boundaries of hTR, respectively (Tesmer *et al.*, 1999). This latter study also demonstrated that two inactive fragments of hTR (33-147 and 164-325) can cooperate to reconstitute a human telomerase RNP that is catalytically active *in vitro*. However, these studies have not investigated the molecular interactions between hTERT and hTR.

Structure and function studies of telomerase components from other organisms have also been performed. Evidence that specific RNA structures and sequences distinct from the template region contribute to the enzymatic action of telomerase has been reported (Bhattacharyya and Blackburn, 1997; Roy *et al.*, 1998). Telomerase RNA mutations predicted to perturb the pseudoknot structure of the *Tetrahymena* telomerase RNA reconstituted a catalytically active enzyme *in vitro* (Autexier and Greider, 1998; Licht and Collins, 1999), but not *in vivo* (Gilley and Blackburn, 1999). RRL-reconstitution of the *Tetrahymena* telomerase using RNA subunit variants, identified

catalytically inactive mutants that can bind to the protein catalytic component p133 (Licht and Collins, 1999) and suggest that telomerase RNA sequences or structures implicated in binding and catalysis are functionally distinct. Establishment of the template boundary by yeast telomerase is determined, at least in part, by a phylogenetically conserved secondary structure within the yeast telomerase RNA subunit (Tzfati *et al.*, 2000). A similar function for a conserved sequence upstream of the template region in the telomerase RNA from ciliated protozoa was also reported (Lingner *et al.*, 1994; Autexier and Greider, 1995).

Point mutations in catalytically important residues within the RT motifs of telomerase have clearly established an RT-like mechanism of action for telomerase, both *in vitro* and *in vivo* (Counter *et al.*, 1997; Harrington *et al.*, 1997; Lingner *et al.*, 1997; Weinrich *et al.*, 1997; Beattie *et al.*, 1998; Bryan *et al.*, 2000a; Haering *et al.*, 2000). However, the functional role of TERT domains beyond the RT motifs has not been extensively studied. Recent reports identified functionally important regions within the amino-terminal domain of the protein catalytic subunit of the *Tetrahymena* and *Saccharomyces cerevisiae* telomerases (Friedman and Cech, 1999; Bryan *et al.*, 2000b; Xia *et al.*, 2000). The data from these studies suggest a role for the amino-terminus of TERT in telomerase RNA binding. An extensive mutational analysis of the *Tetrahymena* TERT has also demonstrated a specific role for certain residues outside the RT motifs in template definition by *Tetrahymena* telomerase (Miller *et al.*, 2000).

Human telomerase activity is detected in more than 85% of transformed and tumor cell lines, yet is not observed in most normal human diploid cells (Shay and

Bacchetti, 1997; Artandi and DePinho, 2000; Oulton and Harrington, 2000). The inhibition of human telomerase in immortal and cancer cell lines leads to progressive telomere shortening and, in some cell types, cell death (Hahn *et al.*, 1999; Herbert *et al.*, 1999; Zhang *et al.*, 1999). A better understanding of the role of telomerase in cancer should validate the use of this enzyme as a target for anticancer therapy (Autexier, 1999). Consequently, it is important to clearly understand how telomerase is regulated and to dissect its mechanism of action. One step essential to the mechanism of action of telomerase is the interaction of the telomerase RNA with TERT.

We investigated the functional regions of both hTERT and hTR required for telomerase activity and RNA-protein interactions using human telomerase reconstituted in RRL. Our results suggest the presence, in hTR, of at least two independent hTERT binding sites, located between nucleotides 33-147 and nucleotides 164-330. We also identified sequences and possible structures distant from the template region of hTR that are essential for the reconstitution of human telomerase activity *in vitro*. Expression of amino- and carboxy-terminal hTERT deletions in RRL identified catalytically inactive mutants that retained their ability to associate with hTR, suggesting that the polymerase and RNA binding functions of hTERT are distinct.

MATERIALS AND METHODS

hTERT and hTR Plasmid Constructs-Cloning of nucleotides 1 to 451 of the human telomerase RNA into the pUC119 plasmid (phTR+1) has been described previously (Autexier *et al.*, 1996). Similarly, the construction of hTR substitution mutations: hTR170, hTR180, and hTR190 has also been described (Autexier *et al.*, 1996). The plasmids expressing human telomerase RNAs hTR33-147, hTR164-330, hTR164-208, and hTRACA-TGT, were generated by polymerase chain reaction (PCR) from the template vector pGRN33 (Feng *et al.*, 1995) using *Pfu* polymerase (Stratagene). To generate phTR33-147, nucleotides 33-147 of hTR were amplified by PCR using the 5' primer 5'-GGGGAAGCTTTAATACGACTCACTATAGGGCCATTTTTTGTCTAACCCTAACTG-3' and the 3' primer 5'-CGCGGATCCTCCGGAAGGCGGCAGGCCGAGGC-3', containing *Hin*d III and *Bsp*EI sites, respectively. phTR164-208 and phTR164-330 were constructed using the same 5' primer, 5'-GGGGAAGCTTTAATACGACTCACTATAGGGAGCAAACAAAAAATGTCAGCTGCTGGC-3', containing a *Hin*dIII site. The 3' primer was 5'-CGCGGATCCCGGGAGGCGAACGGGCCAG-3' for phTR164-208 and 5'-CGCGGATCCCTCGAGACCCGCGGCTGACAGAGCC-3' for phTR164-330, both containing an *Xho*I site. The substitution of the ACA trinucleotides (nt 446-448) for TGT in the ACA box of the human telomerase RNA was introduced by PCR using the 5' primer hTR+1 (5'-GGGGAAGCTTTAATACGACTCACTATAGGGTTGCGGAGGGTGGGCCTG-3') (Autexier *et al.*,

1996) and the 3' primer 5'-CGCGGATCCTGCGCAACAGTGAGCCGAGTCCTGGGTG-3', containing *Hin*dIII and *Bam*HI sites, respectively. The 5' primers used for these constructs all contain the phage T7 promoter immediately upstream of hTR sequences. The digested PCR pruducts were cloned into the pUC119 plasmid previously digested with the appropriate restriction enzymes. The sequence of the different hTR plasmid constructs was confirmed by dideoxy sequencing following the manufacturer's instructions (Amersham Pharmacia Biotech).

The construction of the pET28b-hTERT expression plasmid has been described previously (Bachand and Autexier, 1999). The ΔCT135 hTERT C-terminal truncation was expressed using pET28b-hTERT digested with *Apa*LI (cleaves at position 2992 within hTERT cDNA), generating a protein product lacking the last 135 amino acids. The other N- and C-terminal derivatives were generated by PCR amplification using pET28b-hTERT as a template and the appropriate pairs of primers. After digestion of the different PCR products with the appropriate restriction enzymes, the hTERT cDNA derivatives were cloned into the pET28 vectors (Novagen).

Preparation of Gel-Purified Human Telomerase RNAs-The different RNAs used in the coupled *in vitro* transcription/translation reactions in rabbit reticulocyte lysates, were transcribed *in vitro* using T7 RNA polymerase (New England Biolabs). Full-length hTR (1-451) and hTR 3' truncations 1-424, 1-276, 1-205, 1-182, 1-168, and 1-159 were *in vitro*-transcribed from the phTR+1 construct (Autexier *et al.*, 1996) previously digested with *Fsp*I, *Apa*LI, *Bsp*EI, *Sma*I, *Pvu*II, *Bbv*I, and *Xba*I, respectively. The plasmids

phTR170, phTR180, phTR190, and phTR(ACA-TGT) were digested with *Fsp*I, whereas phTR33-147, phTR164-208, and phTR164-330 were digested with *Bsp*EI, *Bam*HI, and *Xho*I, respectively. Following *in vitro* transcription, the reactions were DNAse-treated and purified on denaturing acrylamide gels as described previously (Autexier and Triki, 1999). The concentrations of the gel-purified RNAs were determined by spectrophotometry and their integrity/size was confirmed by either ethidium bromide staining of denaturing acrylamide gels or by Northern blot.

In vitro Transcription and Translation-Wild-type and hTERT derivatives engineered to have a T7-epitope tag at the amino-terminus were synthesized in rabbit reticulocyte lysates by incubating pET28b-hTERT or the different pET28-hTERT derivatives in coupled transcription/translation (Promega) reactions (20-25 μl) at a final concentration of 25 ng/μl, in the presence or absence of 2.5-5.0 ng/μl full-length or mutant gel-purified hTR derivatives. Expression in RRL followed instructions recommended by the manufacturer (Promega) using [S^{35}]-methionine (NEN) to radiolabel hTERT during protein synthesis.

Immunoprecipitations-Immunoprecipitations were performed by incubating 10-13.5 μl of reticulocyte lysate with protein-A-sepharose beads that were previously coated with the T7 monoclonal antibody (Novagen) in lysis buffer (10 mM Tris-HCl pH 7.5; 2.0 mM $MgCl_2$; 1.0 mM EGTA; 5.0 mM β-mercaptoethanol; 20% glycerol; 150 mM NaCl; 1% NP-40; 0.25 mM sodium deoxycholate; 0.2 mM AEBSF; 0.5 μg/ml leupeptin; 1 μg/ml pepstatin; 38 U/ml RNAguard (Amersham Pharmacia Biotech)). After a 2-3 h incubation

at 4°C, protein-A-sepharose-coated beads were washed four times using 1 ml of lysis buffer and subjected to SDS-PAGE, TRAP assay, or Northern analysis.

Northern Analysis-Total RNA was extracted from rabbit reticulocyte lysates by diluting 5 µl of RRL reactions with 45 µl of H_2O and by the addition of 450 µl of TRIZOL reagent (GIBCO BRL). To prepare RNA from antibody-coated beads, 75-90% of the immunoprecipitates were treated once with phenol, once with chloroform/isoamyl alcohol (24:1), and precipitated with 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol in the presence of 10 µg *E. coli* tRNA (Sigma). RNAs were separated by electrophoresis on either 4% or 6% acrylamide/7M Urea/0.6X TBE gels and then electrophoretically transferred to nylon membranes (Hybond+; Amersham Pharmacia Biotech) in 6 mM trisodium citrate/8 mM dibasic sodium phosphate for 2h at 350 mA. Blots were probed with random-primed *hTR* cDNA (Bachand *et al.*, 2000) and hybridizations performed at 55 C.

Telomerase activity assays-Telomerase activity was assayed by a two-tube modified TRAP as described previously (Bachand and Autexier, 1999). The positive control used in TRAP assays consisted of partially purified 293 cell extracts prepared as previously described (Autexier *et al.*, 1996).

RESULTS

Nucleotides 1-159 of hTR are sufficient for a stable interaction with hTERT *in vitro*

Reconstitution of human telomerase activity with recombinant hTR has been accomplished in systems such as the rabbit reticulocyte lysate (Weinrich *et al.*, 1997; Beattie *et al.*, 1998), the yeast *Saccharomyces cerevisiae* (Bachand and Autexier, 1999), and using purified recombinant hTERT expressed from baculovirus-infected cells (Masutomi *et al.*, 2000). These results suggest that the minimal components required to reconstitute human telomerase *in vitro* are hTERT and hTR. The ability to reconstitute human telomerase with only these two components *in vitro* and the mechanistic similarity of hTERT to reverse transcriptases (Lingner *et al.*, 1997; Weinrich *et al.*, 1997; Bryan *et al.*, 2000a; Miller *et al.*, 2000), suggest a stable interaction between the protein catalytic subunit and the RNA component of telomerase.

Modified human telomerase RNAs that had different 3' truncations or the hTR(ACA-TGT) substitution were added to RRL reactions during hTERT synthesis to identify hTR sequences or structures that bind the protein catalytic subunit *in vitro* (Fig. 3-2). The predicted secondary structures altered in the different hTR variants are described in Table 3-1 and shown in Fig. 3-1. Northern blot analysis using an hTR-specific probe was performed on the total RNA extracted from these RRL reactions to demonstrate that the levels and stability of the various hTRs in the lysates were not grossly different (Fig. 3-2A). These lysates were subjected to immunoprecipitation using an antibody to the T7-tag located at the amino-terminus of hTERT. The ability of the different RNAs to reconstitute human telomerase activity (Fig. 3-2B) and to associate

TABLE 3-1. **Summary of human telomerase RNA (hTR) mutation analyzed in this study and their telomerase activity and extent of binding to hTERT**

Telomerase RNA	Size in nucleotides (nt)	Sequence/structure alterations[a]	Telomerase activity		hTERT binding[c]
			293[b]	RRL[c]	
hTR1-451	451	none	+++	+++	+++
hTR1-424	424	delete 3' terminal 27 nt/delete ACA box; partly disrupt the CR7 domain	+++	+++	+++
hTR1-276	276	delete 3' terminal 175 nt/delete H/ACA box and CR7 domains; disrupt CR4-CR5 domain	+++	-	++
hTR1-205	205	delete 3' terminal 246 nt/delete H/ACA box, CR7, and CR4-CR5 domains	++	-	++
hTR1-182	182	delete 3' terminal 269 nt/delete H/ACA box, CR7, and CR4-CR5 domains; partly disrupt pseudoknot	+/-	-	++
hTR1-168	168	delete 3' terminal 283 nt/delete H/ACA box, CR7, and CR4-CR5 domains, disrupt pseudoknot domain	+/-	-	++
hTR1-159	159	delete 3' terminal 292 nt/delete H/ACA box, CR7, and CR4-CR5 domains, disrupt pseudoknot domain	-	-	++
hTR170	451	substitute nt 170-179/disrupt part of P3 helix of pseudoknot	+/-	+/-	+++
hTR180	451	substitute nt 180-189/disrupt part of P3 helix of pseudoknot	+/-	-	+++
hTR190	451	substitute nt 190-199/disrupt part of P1 helix	+	+	+++
hTR(ACA-TGT)	451	substitute ACA with TGT (nt 446-448)	n.d.	+++	+++
hTR33-147	114	span nt 33 to 147/lacks P1 helix, complete pseudoknot, CR4-CR5 domain, H/ACA box, and CR7 domains	n.d.	-	+
hTR164-208	42	span nt 164-208/lacks template sequence, pseudoknot, CR4-CR5, H/ACA, and CR7 domains	n.d.	-	-
hTR164-330	166	span nt 164-330/lacks template sequence, pseudoknot, H/ACA box, and CR7 domains	n.d.	-	++
hTR33-147; hTR164-208	114 ; 42	see hTR33-147 and hTR164-208	n.d.	-	+ ; -
hTR33-147; hTR164-330	114 ; 166	see hTR33-147 and hTR164-330	n.d.	+++	+ ; ++

[a] Structural alterations of hTR variants are based on the predicted secondary structure of hTR by Chen *et al.* which is shown in Fig. 1.
[b] The ability of various mutated hTRs to reconstitute telomerase activity from micrococcal nuclease-treated, partially purified 293 extracts was previously determined (Autexier *et al.*, 1996).
[c] Telomerase activity and hTERT binding of immunoprecipitates from rabbit reticulocyte lysates (RRL) that expressed hTERT in the presence of the different hTR derivatives.
[b,c] The telomerase activity and hTERT binding ability of the different hTR mutations are scored relative to wild-type hTR.
n.d.: not determined

with hTERT (as determined by their co-immunoprecipitation with hTERT; Fig. 3-2C) was analyzed. Immunoprecipitates prepared from lysates that expressed hTERT in the presence of wild-type hTR reconstituted human telomerase activity (Fig. 3-2B, lane 11) and contained hTR1-451 (Fig. 3-2C, lane 21). Human telomerase activity and hTR were not recovered from protein-A-sepharose if the T7-tag antibody was omitted (data not shown). In addition, an immunoprecipitate from a lysate in which hTERT was synthesized in the absence of hTR (Fig. 3-2A, lane 1) did not reconstitute human telomerase activity (Fig. 3-2B, lane 10) nor contain the human telomerase RNA (Fig. 3-2C, lane 20). All of the tested hTR variants were capable of associating with hTERT (Fig. 3-2C, lanes 22-28). However, only hTR1-451, hTR(ACA-TGT), and hTR1-424 reconstituted a catalytically active human telomerase RNP *in vitro* (Fig. 3-2B). These results suggest that the first 159 nucleotides of hTR are sufficient for a stable interaction with hTERT, but that sequences or structures between nucleotides 276 and 424 of hTR are necessary for the enzymatic function of telomerase *in vitro*.

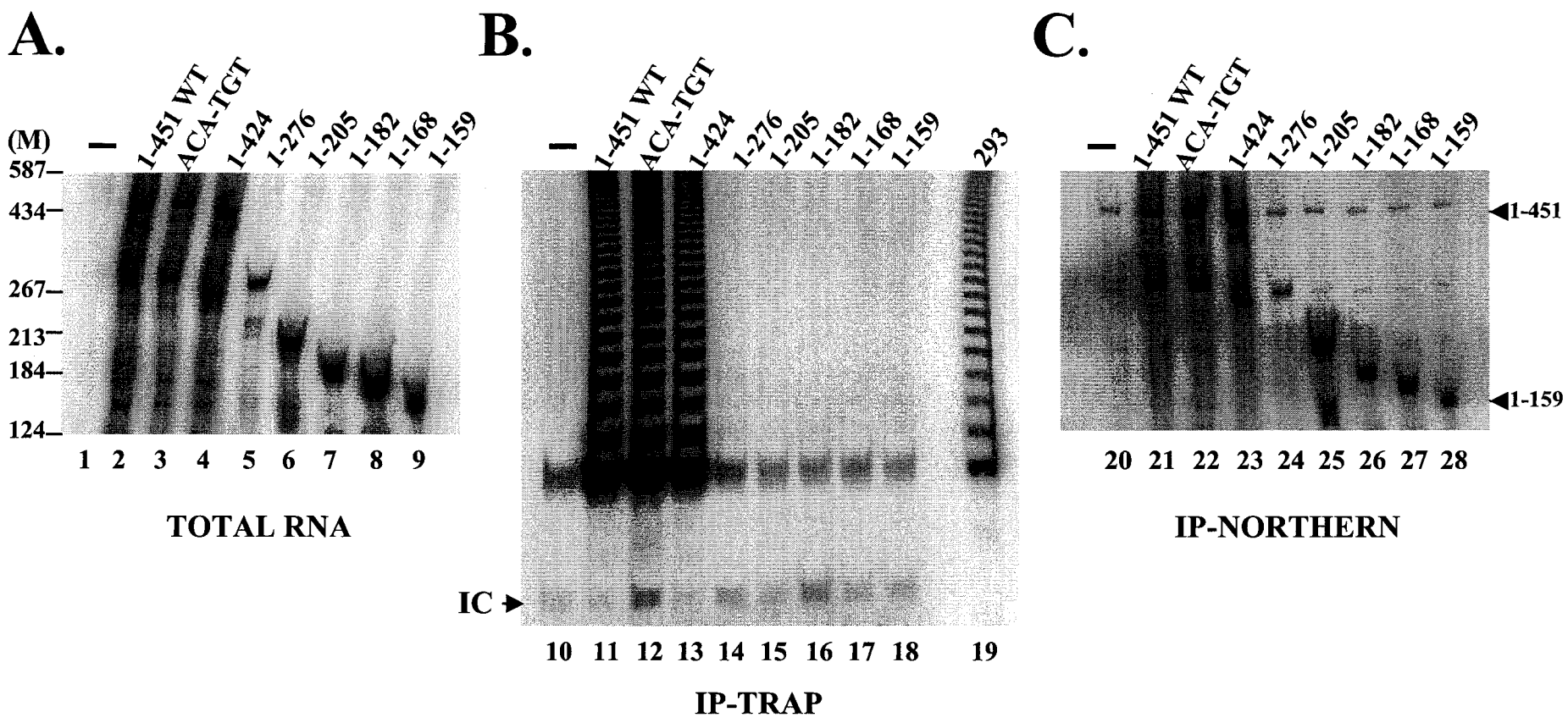

FIG. 3-2. **Sequences or structures located between nucleotides 276 and 424 of hTR are necessary for telomerase activity reconstitution *in vitro*.** (A) Northern blot of total RNA harvested from rabbit reticulocyte lysates (RRL) in which hTERT was synthesized in the absence (lane 1) or presence of wild-type hTR (lane 2), hTR(ACA-TGT) (lane 3) or 3' truncated (lanes 4-9) human telomerase RNAs. The Northern blot was probed for hTR-specific sequences. (M): DNA markers (in base pairs) are indicated on the left. RNAs were separated by electrophoresis on a 4% acrylamide/7M urea gel. (B) and (C) Equal volumes of RRL reactions generated in the absence (lanes 10 and 20) or presence of the different hTR variants [labeled above (B) and (C)] were subjected to immunoprecipitation (IP) with an antibody to the T7-tag. The washed beads were analyzed for (B) telomerase activity and (C) hTERT-hTR co-immunoprecipitation. In (B), telomerase activity was analyzed by the TRAP assay, and 100 ng of partially purified 293 cell extracts were used as a positive control (293, lane 19). IC: internal PCR control; WT: wild-type. In (C), hTERT-hTR co-immunoprecipitation was analyzed by Northern blot using an hTR-specific probe. The arrowheads indicate the position at which the hTR1-451 and hTR1-159 RNAs migrate.

Sequences or structures located between nucleotides 170-200 of hTR are required for a catalytic function in the telomerase RNP

Substitution of nucleotides 170 to 179 (hTR170), 180 to 189 (hTR180), and 190 to 199 (hTR190) of hTR greatly impairs the ability of hTR to reconstitute human telomerase activity *in vitro* ((Autexier *et al.*, 1996); see Table 3-1). Nucleotides 174-183, which are predicted to be part of the P3 helix in the hTR pseudoknot ((Chen *et al.*, 2000); see Fig. 3-1), are substituted in the hTR170 and hTR180 variants. To investigate whether these mutated hTRs are defective in binding to hTERT, we used the RRL expression system to examine the ability of hTR170, hTR180, and hTR190 to reconstitute human telomerase activity and to specifically associate with hTERT *in vitro*.

Human TERT was synthesized in RRL in the absence or presence of gel-purified wild-type hTR, hTR170, hTR180, and hTR190 (Fig. 3-3A, lanes 1-5). Using the T7-tag antibody, immunoprecipitates were prepared from these lysates and analyzed for: (i) the ability to reconstitute telomerase activity (Fig. 3-3B); and (ii) hTR-binding by Northern blot analysis of the co-immunoprecipitated RNAs (Fig. 3-3C). hTR190 reconstituted weak levels of human telomerase activity (Fig. 3-3B, lane 15). Immunoprecipitates prepared from RRL reactions that expressed hTERT in the presence of either hTR170 or hTR180 contained low (hTR170, lane 13) and undetectable (hTR180, lane 14) levels of telomerase activity. Northern blot analysis of co-immunoprecipitated RNAs demonstrated that all three mutated hTRs were capable of associating with hTERT as efficiently as wild-type hTR (Fig. 3-3C, compare lanes 24-26 to lane 23). These results

indicate that sequences or structures located between nucleotides 170-200 of hTR are required for the enzymatic action of the telomerase RNP rather than hTERT binding.

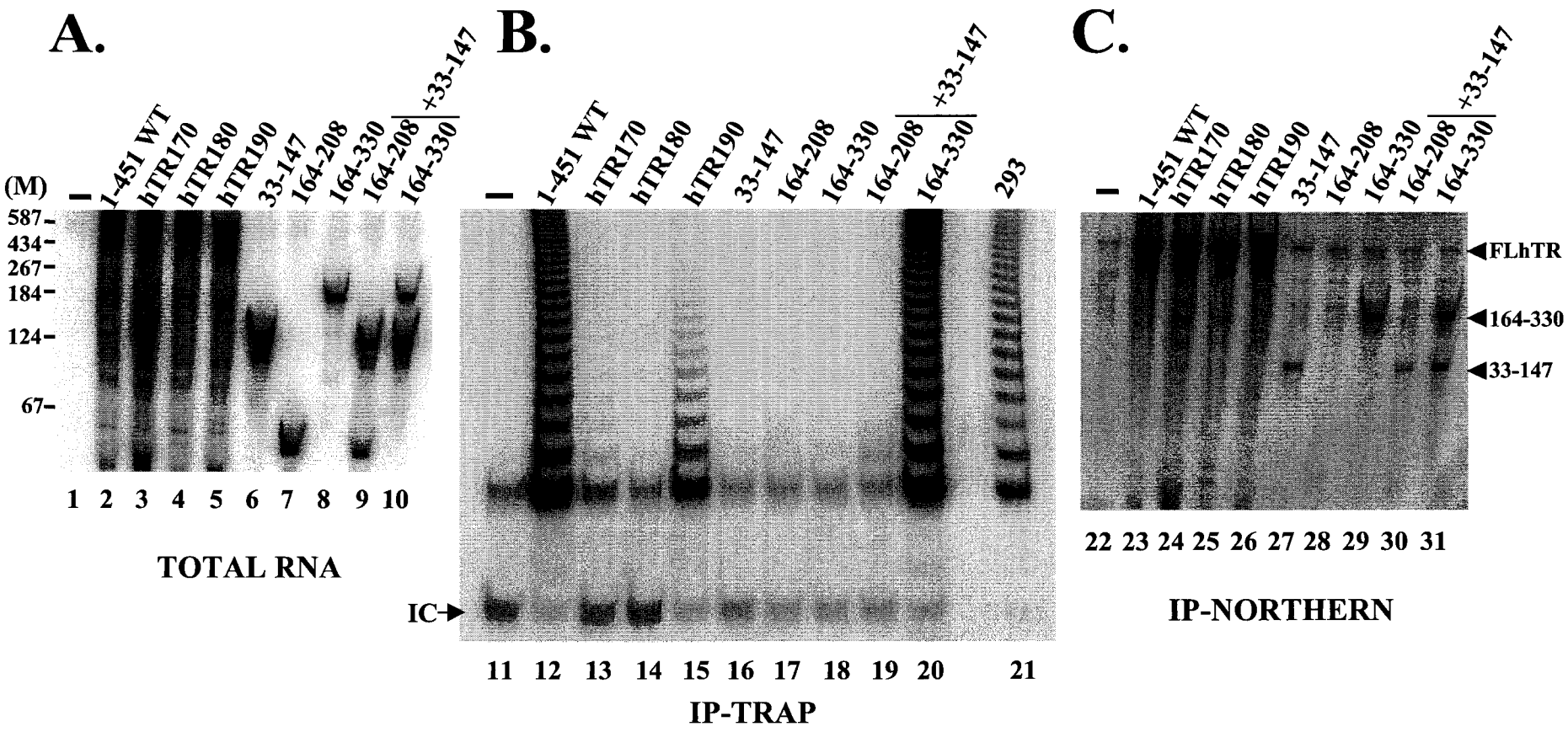

FIG. 3-3. **Role of the P3 helix of the hTR pseudoknot in telomerase function and two independent hTERT binding sites within the human telomerase RNA.** (A) Northern blot of total RNA harvested from rabbit reticulocyte lysates (RRL) in which hTERT was synthesized in the absence (lane 1) or presence of wild-type hTR (lane 2) and hTR variants [labeled above (A), lanes 3-10]. The Northern blot was probed for hTR-specific sequences. (M): DNA markers (in base pairs) are indicated on the left. RNAs were separated by electrophoresis on a 6% acrylamide/7M urea gel. (B) and (C) Equal volumes of RRL reactions generated in the absence (lanes 11 and 22) or presence of the different hTR variants [labeled above (B) and (C)] were subjected to immunoprecipitation (IP) with an antibody to the T7-tag. The washed beads were analyzed for (B) telomerase activity and (C) hTERT-hTR co-immunoprecipitation. In (B), telomerase activity was analyzed by the TRAP assay, and 100 ng of partially purified 293 cell extracts were used as a positive control (293, lane 21). IC: internal PCR control; WT: wild-type. In (C), hTERT-hTR co-immunoprecipitation was analyzed by Northern blot using an hTR-specific probe. The arrowheads indicate the position at which full-length hTR (FLhTR), hTR164-330, and hTR33-147 migrate.

Two independent hTERT binding sites exist within the human telomerase RNA

Human telomerase can be reconstituted in RRL by expressing hTERT in the presence of two inactive, non-overlapping segments of hTR (33-147 and 164-325) (Tesmer *et al.*, 1999). To determine whether hTR fragments spanning nucleotides 33-147, 164-208, and 164-330 bind the protein catalytic subunit, hTERT was expressed in RRL in the presence of distinct hTR segments: hTR33-147, hTR164-208, hTR164-330, individually or pair-wise (Fig. 3-3A, lanes 6-10; see also Table 3-1 and Fig. 3-1). Immunoprecipitates were prepared from these lysates using the T7-tag antibody, and analyzed for telomerase activity (Fig. 3-3B) and hTR co-immunoprecipitation (Fig. 3-3C).

Human telomerase activity was detected from the immunoprecipitate of a lysate that expressed hTERT in the presence of a mixture of hTR33-147 and hTR164-330 (Fig. 3-3B, lane 20) (Tesmer *et al.*, 1999). Northern blot analysis of RNAs extracted from the immunoprecipitates demonstrated that both hTR fragments (33-147 and 164-330) associated with hTERT (Fig. 3-3C, lane 31). Immunoprecipitates prepared from RRL extracts in which hTERT was synthesized in the presence of either hTR33-147 or hTR164-330 did not reconstitute human telomerase activity (Fig. 3-3B, lanes 16 and 18, respectively). This result is consistent with the observation by Tesmer *et al.* (Tesmer *et al.*, 1999) that these two hTR segments, separately, are unable to reconstitute a catalytically active telomerase RNP *in vitro*. Nonetheless, both hTR33-147 and hTR164-330 were independently capable of binding hTERT, albeit with different efficiencies (Fig. 3-3C, lanes 27 and 29, respectively). An RNA composed of nucleotides 164-330 of hTR

was co-immunoprecipitated with hTERT (compare the amount of input RNA [Fig. 3-3A, lane 8] to bound RNA [Fig. 3-3C, lane 29]), whereas significantly less of the hTR33-147 was co-immunoprecipitated (compare Fig. 3-3A and Fig. 3-3C for hTR33-147). These results suggest that two different regions, one located between nucleotides 33-147 and the other between nucleotides 164-330 of hTR, independently interact with the protein catalytic subunit with qualitatively different efficiencies.

Immunopurified hTERT expressed in the presence of hTR164-208 alone, or in combination with hTR33-147, did not reconstitute human telomerase activity (Fig. 3-3B, lanes 17 and 19). Northern blot analysis of co-immunoprecipitated RNAs indicated that the levels of hTR164-208 that associated with hTERT varied from undetectable (Fig. 3-3C, lanes 28 and 30) to extremely low (data not shown). In constrast, hTR164-330 formed a stable complex with hTERT (Fig. 3-3C, lanes 29 and 31), suggesting that sequences or structures located between nucleotides 208 and 330 of hTR may be important for hTERT-hTR interactions as well as for the activation of telomerase *in vitro* (see lanes 19 and 20 of Fig. 3-3B).

Regions within the amino and carboxyl terminus of hTERT play functionally different roles in telomerase catalysis and hTR binding *in vitro*

One of the features that distinguishes telomerase from conventional reverse transcriptases is that the telomerase RNA is an intrinsic component of the enzyme (Greider and Blackburn, 1989; Nugent and Lundblad, 1998). Recent evidence suggests a functional role for the amino terminal domain of the yeast and *Tetrahymena* TERTs in

telomerase RNA binding (Friedman and Cech, 1999; Bryan *et al.*, 2000b). In order to investigate the function of regions outside the RT motifs of the human telomerase protein catalytic subunit, we generated amino (N)- and carboxy (C)-terminal deletions of hTERT (Fig. 3-4) and used the RRL expression system to assay the ability of each truncation to reconstitute telomerase activity and bind hTR *in vitro*. Full-length hTERT, as well as N- and C-terminal deletions, were synthesized in RRL in the presence of gel-purified wild-type hTR and [S^{35}]-methionine (Fig. 3-5A). Immunoprecipitates were prepared from these lysates using the T7-tag antibody (Fig. 3-5B) and subjected to telomerase activity assays (Fig. 3-5C). None of the N- and C-terminal hTERT deletions reconstituted human telomerase activity (Fig. 3-5C, lanes 14-22), whereas full-length hTERT efficiently reconstituted activity (Fig. 3-5C, lane 13).

Co-immunoprecipitation of the RNAs was analyzed by Northern blot using an hTR-specific probe (Fig. 3-5D) to determine if the N- and C-terminal hTERT truncations affected hTR binding. The two catalytically inactive hTERT C-terminal deletions bound the human telomerase RNA (Fig. 3-5D, lanes 26-27). An immunoprecipitate from a lysate that expressed the ΔCT186 truncation contained hTR levels similar to an immunoprecipitate from a lysate that expressed full-length hTERT (compare lanes 27 and 25); yet the amount of hTR co-immunoprecipitated by the ΔCT135 C-terminal deletion was significantly less (lane 26). However, this result is consistent with the lower levels of the ΔCT135 protein that was expressed and immunoprecipitated from the lysate in this particular experiment (Fig. 3-5A and 5B, lane 3). These results indicate that the defect in

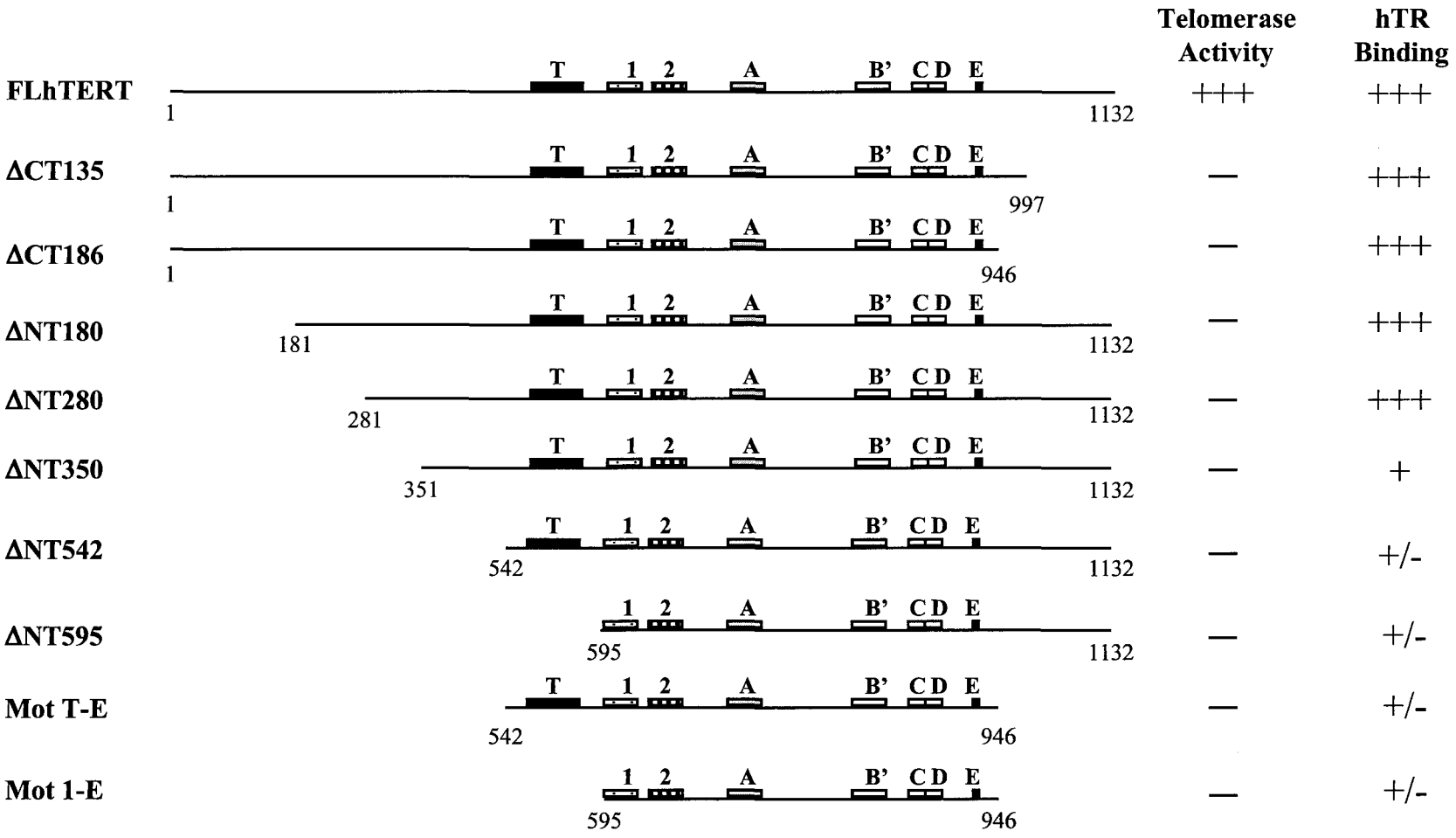

FIG. 3-4. **Summary of hTERT truncations analyzed and their telomerase activity and extent of binding to hTR.** Schematic representation of the human telomerase reverse transcriptase with the seven conserved reverse transcriptase (RT)-like motifs (1, 2, A, B', C, D, and E) as well as the telomerase-specific (T) motif (Nakamura *et al.*, 1997). Amino- and carboxy-terminal deletions in hTERT (including amino acid position) are indicated along with the relative telomerase activities and hTR binding abilities these proteins demonstrated after their immunoprecipitation from rabbit reticulocyte lysates (RRL) in which they were expressed in the presence of recombinant hTR. All these proteins were engineered to express an N-terminal epitope tag (T7-tag).

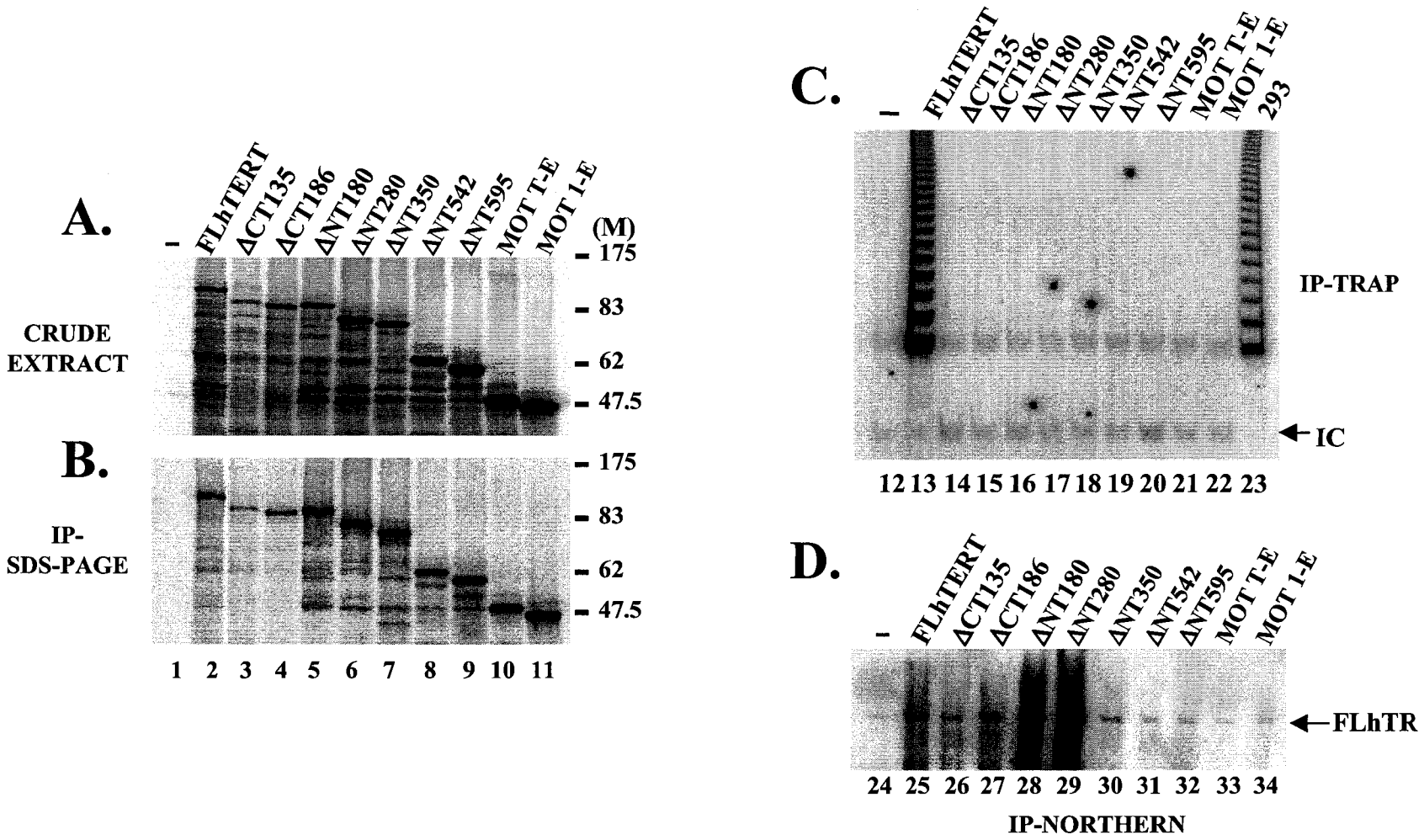

FIG. 3-5. **The polymerization and RNA binding functions of hTERT are independent *in vitro*.** (A) An equal volume of lysate in which the different hTERT proteins (labeled above the figure) were synthesized in the presence of recombinant hTR and [S^{35}]-methionine was analyzed for protein expression on a 7.5% SDS-PAGE. The gel was dried and exposed to phosphorimager screen. FLhTERT: Full-length hTERT; CT: C-terminal; NT: N-terminal; MOT: motif. (M): The position of the protein markers are indicated on the right in kilodalton (kDa). Equal volumes of the different RRL reactions were subjected to immunopurification using the T7-tag antibody. The washed beads were analyzed for (B) protein levels using a 7.5% SDS-PAGE, (C) telomerase activity, and (D) hTERT-hTR co-immunoprecipitation. (C) Telomerase activity was analyzed by the TRAP assay, and 100 ng of partially purified 293 cell extracts were used as a positive control (293, lane 23). IC: internal PCR control. (D) hTERT-hTR co-immunoprecipitation was analyzed by Northern blot using an hTR-specific probe. The arrow indicates full-length hTR (FLhTR).

the enzymatic activity of the two C-terminal hTERT deletions is not caused by a deficiency in hTR binding.

Immunoprecipitates from RRL extracts that expressed the ΔNT180 and ΔNT280 hTERT N-terminal truncations contained higher levels of hTR than an immunoprecipitate from a lysate expressing full-length hTERT (Fig. 3-5D, compare lanes 28-29 to lane 25). However, higher levels of these two N-terminal deletions were observed after immunoprecipitation of the proteins from RRL extracts (Fig. 3-5B, compare lanes 5-6 to lane 2). Deletion of the first 350 amino acids of hTERT significantly compromised its ability to associate with hTR *in vitro* (Fig. 3-5D, lane 30). Two larger N-terminal truncations of hTERT (ΔNT542 and ΔNT595; Fig. 3-5D, lanes 31-32) and human TERT containing the RT motifs in the presence or absence of the telomerase-specific (T) motif (MOT T-E and MOT 1-E; Fig. 3-5D, lanes 33-34) weakly co-immunoprecipitated hTR from RRL extracts. These results indicate that the loss of enzymatic activity of certain hTERT truncations (specifically the two C-terminal, the ΔNT180, and the ΔNT280 hTERT deletions) is not caused by an altered ability of the mutant proteins to associate with the human telomerase RNA *in vitro*.

DISCUSSION

We demonstrated evidence for the existence of two distinct hTERT binding regions within hTR, located between nucleotides 33-147 and 164-330. Our data also suggest a catalytic role for nucleotides 170-190, involved in the formation of the predicted pseudoknot in hTR, rather than a role in hTERT binding *in vitro*. The function of regions outside hTERT reverse transcriptase motifs was also examined by expressing a set of amino- and carboxy-terminal deletions in RRL. Our data support a role for N-terminal regions of hTERT in binding hTR. Furthermore, we defined domains of hTERT essential for telomerase activity that are not involved in binding hTR *in vitro*.

The telomerase RNA variants described in this study were assayed for hTERT binding and reconstitution of human telomerase activity in rabbit reticulocyte lysates. The effects of the RNA mutations on telomerase activity and hTERT binding may be indirect if the mutated RNAs differentially fold and alternatively base pair during *in vitro* transcription. Secondary structure analysis of these mutated RNAs will be required to assess the different roles of RNA folding, binding, and catalysis. However, all of the hTR variants analyzed in this study (except hTR164-208) retained either telomerase activity and/or hTERT-binding ability, suggesting that the mutated RNAs are not grossly misfolded.

The evolutionary conserved CR7 and Box H/ACA domains of hTR are dispensable for the reconstitution of human telomerase activity *in vitro*

In addition to a highly conserved template region, vertebrate telomerase RNAs contain four predicted structural elements: a pseudoknot, the CR4-CR5 domain, the Box H/ACA, and the CR7 domain ((Chen *et al.*, 2000); Fig. 3-1). The evolutionary conservation of these domains suggests important roles for these regions in vertebrate telomerase RNA stability, assembly or function. The ability of hTR1-424 and hTR(ACA-TGT) to bind hTERT (Fig. 3-2C) and to reconstitute human telomerase activity (Fig. 3-2B) suggest that the H/ACA box and the CR7 domain are dispensable *in vitro*. These observations are consistent with previous reports that examined the functional regions of hTR (Autexier *et al.*, 1996; Beattie *et al.*, 1998; Tesmer *et al.*, 1999; Mitchell and Collins, 2000). The CR7 domain is not conserved in small nucleolar RNAs (Chen *et al.*, 2000), and may play a specific role in vertebrate telomerase RNA function *in vivo* rather than *in vitro*, as demonstrated for the H/ACA box of hTR (Mitchell *et al.*, 1999; Mitchell and Collins, 2000).

Role of the hTR pseudoknot in the enzymatic function of human telomerase

Ciliate and vertebrate telomerase RNAs possess pseudoknots that are relatively similar in structural topology (Romero and Blackburn, 1991; ten Dam *et al.*, 1991; Chen *et al.*, 2000). The human telomerase RNA pseudoknot is established by helices P2a, P2b, and P3 (Fig. 3-1). The P3 helix is formed by base pairing between nucleotides 107-115 and nucleotides 174-183 of hTR (Chen *et al.*, 2000). An hTR variant with a 17-nt insertion at position 176 of hTR is unable to reconstitute human telomerase activity both *in vivo* (Feng *et al.*, 1995) and *in vitro* (Autexier *et al.*, 1996). This insertion was

suggested to disrupt the pseudoknot of hTR (Chen *et al.*, 2000). Similarly, two of the hTR variants with 10-nt substitutions, hTR170 and hTR180, likely perturb the P3 helix within the hTR pseudoknot (see Fig. 3-1 and Table 3-1). Our results using the RRL reconstitution system demonstrated that hTR170 and hTR180 bound hTERT (Fig. 3-3C). However, the hTR170 and hTR180 substitutions significantly altered the ability of hTR to reconstitute a catalytically active telomerase RNP *in vitro*, suggesting a role for the P3 helix of the hTR pseudoknot in a specific enzymatic action of telomerase, but not in hTERT binding. The hTR190 substitution, which is predicted to affect the P1 rather than the P3 helix ((Chen *et al.*, 2000); see Fig. 3-1), reconstituted human telomerase activity more efficiently than hTR170 and hTR180 (this study, (Autexier *et al.*, 1996); see Table 3-1). This result suggests that the P1 helix of hTR is not as critical as the P3 helix and is consistent with the observation that nucleotides 5' of the template that are involved in the formation of the P1 helix are dispensable for telomerase activity *in vitro* (Autexier *et al.*, 1996; Beattie *et al.*, 1998; Tesmer *et al.*, 1999). Furthermore, telomerase RNAs from mouse and some other rodents do not contain the P1 helix (Hinkley *et al.*, 1998; Chen *et al.*, 2000). In *Tetrahymena*, the pseudoknot of the telomerase RNA is essential for telomerase RNP assembly and activity *in vivo* (Gilley and Blackburn, 1999). However, mutations predicted to destabilize the pseudoknot structure of the *Tetrahymena* telomerase RNA do not significantly perturb telomerase activity *in vitro* (Autexier and Greider, 1998; Licht and Collins, 1999), indicating that the conditions required for the assembly of a functional telomerase RNP may differ *in vitro* and *in vivo*. A more detailed

analysis of the hTR pseudoknot will be required to establish the specific role of this structure in the enzymatic function of human telomerase both *in vitro* and *in vivo*.

Two independent hTERT binding sites within hTR

The hTERT-hTR interaction studies suggest that two segments of the human telomerase RNA can independently associate with hTERT *in vitro*. One hTR fragment, containing the template sequence, spans nucleotides 33-147. The association of hTR33-147 with hTERT is supported by results from Beattie and colleagues that demonstrate that nucleotides 10-91 of hTR are sufficient for associating with hTERT (Beattie *et al.*, 2000). The other hTR fragment contains the conserved CR4-CR5 domain and consists of nucleotides 164-330 of hTR. Base pairing interactions through helix P3 may, however, bring these two regions together into one structure *in vitro*. An RNA composed of nucleotides 33-147 of hTR is not predicted to form a complete pseudoknot (Fig. 3-1). The single-stranded template region of hTR alone is unlikely to provide specific and high affinity binding to hTERT, since the template must be accessible to hybridize to the substrate DNA during telomere synthesis. *In vivo* chemical modification of the *Tetrahymena* telomerase RNA indicates that nucleotides within the template region are not constantly associated with proteins (Zaug and Cech, 1995). In addition, the template sequence of yeast and *Tetrahymena* telomerase RNAs have been completely replaced by heterologous sequences without affecting RNP assembly and catalytic activity (Henning *et al.*, 1998; Ware *et al.*, 2000).

Our data and recent work by others (Tesmer *et al.*, 1999; Mitchell and Collins, 2000) suggest that hTERT binding to the evolutionary conserved CR4-CR5 domain of hTR is necessary for the formation of a fully active human telomerase RNP. First, the hTR co-immunoprecipitation experiments suggest that sequences or structures located between nucleotides 208 and 330 of hTR (which contain the CR4-CR5 domain; see Fig. 3-1) are critical for the efficient binding of the human telomerase RNA to hTERT (Fig. 3-3C). Secondly, hTR1-276 is unable to reconstitute human telomerase activity (Fig. 3-2), whereas the combination of hTR33-147 and hTR164-330 reconstitutes a catalytically active telomerase (Fig. 3-3). Therefore, nucleotides 276 to 330 of hTR are required for the reconstitution of a fully active telomerase RNP *in vitro*. hTR164-330 forms the CR4-CR5 domain in hTR, whereas nucleotides 1-276 are not sufficient for the formation of the CR4-CR5 stem-loop structure (see Fig. 3-1). Similarly, a 151-nt deletion at the 3'-end of hTR (1-300), which deletes part of the CR4-CR5 domain, drastically alters the ability of hTR to reconstitute human telomerase activity *in vitro* using hTERT previously synthesized in RRL (Tesmer *et al.*, 1999). However, nucleotides 1-325 of hTR, which include the conserved CR5 sequences necessary for the formation of the CR4-CR5 stem-loop structure, are sufficient to produce levels of telomerase activity similar to wild-type hTR (Tesmer *et al.*, 1999).

Our data suggesting that the CR4-CR5 domain of hTR is required for the formation of a fully active human telomerase RNP *in vitro* is, however, not entirely supported by previous studies. Human telomerase RNA truncations as short as 1-205 (Autexier *et al.*, 1996) and 10-159 (Beattie *et al.*, 1998) are sufficient to reconstitute low

levels of human telomerase activity *in vitro*. In this regard, we occasionally detected human telomerase activity from crude RRL extracts that expressed hTERT in the presence of hTR1-276 (data not shown), but not from the immunoprecipitates (Fig. 3-2). The immunopurification procedure may select telomerase RNPs that can maintain a stable catalytically active conformation *in vitro*.

Mitchell and Collins (Mitchell and Collins, 2000) recently reported that hTR harbors two independent hTERT binding sites, one located within nucleotides 1-209 and the other between nucleotides 241-330 of hTR. Nucleotides 241-330 of the human telomerase RNA contain the conserved CR4-CR5 domain and are necessary for the reconstitution of telomerase activity *in vivo*. Furthermore, this latter region is necessary and sufficient for the reconstitution of human telomerase activity *in vitro* when added in *trans* with hTR1-209 to hTERT previously synthesized in RRL (Mitchell and Collins, 2000). However, this study and the one by Mitchell and Collins (Mitchell and Collins, 2000) have not distinguished between a binding and a catalytic function for the CR4-CR5 domain. The requirement of the CR4-CR5 domain of hTR to reconstitute a fully active human telomerase RNP may reflect: (i) essential physical contacts between this domain and hTERT; and/or (ii) a direct role in catalytic functions, as was recently demonstrated for a specific structure within the yeast telomerase RNA (Tzfati *et al.*, 2000). Similarly, binding to a specific stem-loop structure within the pregenomic RNA of the Hepatitis B virus is also critical for the catalytic activation of this viral reverse transcriptase (Wang *et al.*, 1994).

Domains outside hTERT RT motifs are important for human telomerase activity *in vitro*

We investigated the functional role of regions outside the hTERT RT motifs by analyzing the catalytic activity of different amino- and carboxy-terminal hTERT deletions *in vitro.* Human telomerase activity was completely abolished by deleting 180 and 135 amino acids at the N- and C-terminal ends, respectively (Fig. 3-5). This is in contrast to data in which hTERT N-terminal deletions up to 300 amino acids reconstituted weak levels of telomerase activity in RRL (Beattie *et al.*, 2000). This discrepancy may result from differently tagged version of hTERT used in the different studies: N-terminal HIS-T7-tagged hTERT in the present study, whereas N- and C-terminal FLAG-tagged hTERT was used by Beattie and colleagues. The two C-terminal hTERT deletions, as well as the ΔNT180 and ΔNT280 truncations, bound hTR though they did not reconstitute a catalytically active enzyme, suggesting that these proteins are not grossly misfolded. Human TERT lacking the first 350 amino acids did not associate efficiently with the human telomerase RNA *in vitro*, whereas hTERT lacking C-terminal amino acids up to motif E bound hTR. These observations support data that indicate that an hTERT deletion derivative spanning amino acids 301-927 retains the ability to bind the human telomerase RNA *in vitro* (Beattie *et al.*, 2000). These results suggest that a binding domain for the human telomerase RNA may be located within the amino-terminus of hTERT. Consistent with this prediction is the finding that the first 300 amino acids of hTERT are required for the reconstitution of human telomerase activity in the presence of an RNA molecule spanning nucleotides 40-451 of hTR. Evidence for an RNA binding

function in the amino-terminus of TERT from *Saccharomyces cerevisiae* (Friedman and Cech, 1999), *Tetrahymena* (Bryan *et al.*, 2000b) and humans (Beattie *et al.*, 2000) further support this proposal. Site-directed mutagenesis of *Tetrahymena* TERT (tTERT) established a critical role for both the ciliate-specific (CP) and the telomerase-specific (T) motifs in RNA binding, whereas mutations in tTERT RT motifs had little effect on telomerase RNA binding (Bryan *et al.*, 2000b). However, the CP motif is not highly conserved in TERTs of non-ciliates, and it is likely that the RNA binding domain of TERTs from different organisms will vary.

Sequence alignments between TERTs have identified new blocks of conservation in their amino-terminus in addition to the previously described RT and T motifs (Lingner *et al.*, 1997; Nakamura *et al.*, 1997; Malik *et al.*, 2000; Miller *et al.*, 2000; Xia *et al.*, 2000). One of these regions was independently found by three groups (Malik *et al.*, 2000; Miller *et al.*, 2000; Xia *et al.*, 2000) and spans amino acids 134-175 of hTERT. This region is absent in the ΔNT180 N-terminal deletion which was catalytically inactive *in vitro* (Fig. 3-4). This result, together with data from the mutational analysis of conserved residues within this new motif (Miller *et al.*, 2000; Xia *et al.*, 2000), suggest an important role for this region in telomerase function.

We have identified functional regions within the human telomerase RNA and the human protein catalytic subunit that are essential for the reconstitution of telomerase activity *in vitro*. We demonstrated that two non-overlapping regions of hTR can independently associate with hTERT *in vitro*, and suggest a role for the hTR pseudoknot in the enzymatic action of telomerase. Our data also establish a role for the amino-

terminal region of hTERT in binding hTR *in vitro*, and demonstrate that domains outside the conserved RT motifs of hTERT are essential for telomerase reconstitution. A better knowledge of the interactions between the RNA and protein subunits of telomerase, as well as with telomerase-associated proteins, will help to elucidate the molecular mechanisms involved in the assembly, regulation, and mechanism of action of the telomerase RNP.

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Chapter 4

THE PRODUCT OF THE *SURVIVAL OF MOTOR NEURON* (*SMN*) GENE IS A HUMAN TELOMERASE-ASSOCIATED PROTEIN

PREFACE

The 3' end of vertebrate telomerase RNAs fold into a structure similar to the H/ACA box family of small nucleolar RNAs (snoRNAs), suggesting that mammalian telomerase is assembled via a snoRNP pathway. Previous work implicates SMN in the biogenesis of Sm small nuclear RNPs and more recently in the assembly of snoRNPs. In this study, we investigate the possibility that SMN associates with the human telomerase RNP and functions in its biogenesis.

SUMMARY

Telomerase is a ribonucleoprotein (RNP) complex that is minimally composed of a protein catalytic subunit, the telomerase reverse transcriptase (TERT), and an RNA component, the telomerase RNA (TR). The *survival of motor neuron* (*SMN*) gene codes for a protein involved in the biogenesis of certain RNPs. Here we report that SMN is a telomerase-associated protein. Using *in vitro* binding assays and immunoprecipitation experiments, we demonstrate an association between SMN and the telomerase RNP *in vitro* and in human cells. The specific immunopurification of SMN from human 293 cells copurified telomerase activity, suggesting that SMN associates with a subset of the functional telomerase holoenzyme. Our results also indicate that the human TR and the human TERT are not associated with Sm proteins, in contrast to *Saccharomyces*

cerevisiae telomerase. Immunofluorescence analysis showed that hTERT does not specifically colocalize with wild-type SMN in gems or Cajal bodies. However, a dominant-negative mutant of SMN (SMNΔN27) previously characterized to elicit the cellular reorganization of small nuclear RNPs caused the accumulation of hTERT in specific SMNΔN27-induced cellular bodies. Furthermore, coexpression of SMNΔN27 and hTERT in rabbit reticulocyte lysates decreased the efficiency of human telomerase reconstitution *in vitro*. Our results establish SMN as a novel telomerase-associated protein that is likely to function in human telomerase biogenesis.

INTRODUCTION

Telomere maintenance in most eukaryotic cells is established by the ribonucleoprotein (RNP) enzyme telomerase. The telomerase RNP minimally consists of an RNA molecule and a protein catalytic subunit, the telomerase reverse transcriptase (TERT). Using an internal template sequence in the telomerase RNA subunit, this specialized reverse transcriptase synthesizes simple guanine-rich sequences at the 3'-end of chromosomal DNA. The telomeric DNA repeats and associated telomere-binding proteins protect chromosomes from nuclease digestion, end-to-end fusions, and other DNA rearrangement events (Lundblad, 2000).

The length and nucleotide sequence of the telomerase RNA subunits are highly divergent (Nugent and Lundblad, 1998; Chen *et al.*, 2000). Secondary structure prediction suggests the presence of a small nucleolar (sno) RNA H/ACA box motif in the 3'-end of vertebrate telomerase RNAs (Mitchell *et al.*, 1999a; Chen *et al.*, 2000). Mutations that perturb the folding of the H/ACA box of human and mouse telomerase RNAs prevent both their cellular accumulation (Mitchell *et al.*, 1999a; Martin-Rivera and Blasco, 2001) and their ability to properly localize to the nucleolus of microinjected *Xenopus* oocytes (Narayanan *et al.*, 1999). Notably, the human telomerase RNA (hTR) associates with the H/ACA box snoRNA-binding proteins dyskerin, hGAR1, NH2P, and NOP10 (Mitchell *et al.*, 1999b; Pogacic *et al.*, 2001). The role of these proteins in vertebrate telomerase function is unclear. The X-linked form of the disease dyskeratosis congenita is caused by mutations in the gene that encodes the protein dyskerin (Heiss *et al.*, 1998). Cells from individuals affected by this disease have low levels of hTR and

telomerase activity as well as short telomeres (Mitchell *et al.*, 1999b), supporting an important role for snoRNP proteins in telomerase biogenesis.

Human telomerase activity is detected in over 85% of cancers and transformed cell lines, whereas it is absent from most normal human cells (Oulton and Harrington, 2000). Inhibition of human telomerase from immortal and cancer cell lines results in telomere shortening and, in certain cell types, cell death or senescence (Damm *et al.*, 2001; Harrington and Robinson, 2002). Consequently, a better understanding of the mechanisms involved in the assembly and regulation of the human telomerase RNP will be important for the rational design of telomerase inhibitors. In *S. cerevisiae*, the telomerase RNA (TLC1) associates with the heptameric Sm protein complex and acquires a 5' 2,2,7-trimethylguanosine (TMG) cap structure (Seto *et al.*, 1999); both of these events are hallmarks of small nuclear (sn) RNP assembly. Yet, little is known about the molecular machinery involved in the localization and assembly of vertebrate telomerase.

The product encoded by the *survival of motor neuron* (*SMN*) gene is present both in the cytoplasm and in the nucleus, where it localizes in different nuclear structures: gems, Cajal bodies (CB) and the nucleolus (Liu and Dreyfuss, 1996; Hebert *et al.*, 2001; Young *et al.*, 2001). SMN, and a set of associated proteins (Gemins), form a complex involved in the biogenesis of at least four Uridine (U)-rich snRNPs, U1, U2, U4, and U5, all major constituents of the splicing machinery (Fischer *et al.*, 1997; Liu *et al.*, 1997; Meister *et al.*, 2001). Biochemical data demonstrates that SMN directly interacts with the arginine-glycine rich domain of a subset of Sm proteins (Pellizzoni *et al.*, 1999; Brahms *et al.*, 2001; Friesen *et al.*, 2001). The Sm core complex contains seven proteins (B/B', D1-D3, E, F, and G) predicted to form a closed ring structure around a conserved

sequence motif within some of the UsnRNAs (Kambach *et al.*, 1999; Mura *et al.*, 2001; Will and Luhrmann, 2001). The precise function of the SMN complex is not completely characterized; however, evidence strongly suggests that the SMN complex facilitates or stabilizes the association of Sm proteins with U1, U2, U4, and U5 snRNAs, and the functional maturation of UsnRNPs (Fischer *et al.*, 1997; Meister *et al.*, 2001; Will and Luhrmann, 2001). Furthermore, using antibody addition experiments and a dominant-negative version of SMN that lacks the first 27 amino acids (SMNΔN27), Pellizzoni and colleagues demonstrated that SMN might also have a more direct role in pre-mRNA splicing (Pellizzoni *et al.*, 1998). As expected from the wide range of cellular pathways in which SMN is implicated (Terns and Terns, 2001), disruption of the gene encoding SMN in different organisms is lethal (Schrank *et al.*, 1997; Miguel-Aliaga *et al.*, 1999; Paushkin *et al.*, 2000).

The role of the Sm protein complex in *S. cerevisiae* telomerase biogenesis and the recent observation that SMN associates with snoRNP proteins (Jones *et al.*, 2001; Pellizzoni *et al.*, 2001a), prompted us to examine if SMN and/or Sm proteins are involved in human telomerase function. We report that SMN is a novel telomerase-associated protein. The human Sm protein complex does not interact with hTERT, hTR, or catalytically active telomerase, suggesting that the association of SMN with human telomerase is independent of Sm proteins. A previously characterized dominant-negative SMN protein (SMNΔN27) has the ability to perturb the normal subcellular localization of hTERT and decrease the efficiency of *in vitro* reconstitution of telomerase in rabbit reticulocyte lysates. Based on these and other recent results (Jones *et al.*, 2001; Pellizzoni

et al., 2001a), we suggest that SMN is involved in human telomerase biogenesis as an H/ACA snoRNP.

MATERIALS AND METHODS

Construction of plasmids-The cDNAs encoding human SMN and SMNΔN27 were amplified by RT-PCR using total cellular RNA extracted from HeLa cells. SmB, SmD1, and SmD3 cDNAs were amplified by PCR from IMAGE clones. Expression of the Myc-tagged version of these proteins in cultured human cells or rabbit reticulocyte lysates was performed by cloning the DNA fragments corresponding to the above-mentioned cDNAs into a modified pcDNA3.1 vector (InVitrogen) containing the sequence for the Myc-tag epitope (Chen and Richard, 1998). The expression construct for Flag-hTERT was a gift from Dr Lea Harrington (Amgen, University of Toronto). For Subcellular localization experiments, hTERT cDNA was subcloned into the pEGFP-C1 vector (Clonetech).

Antibodies-The antibodies used were as follows: mouse monoclonal Flag antibody (Sigma); affinity-purified goat anti-GST serum (Amersham Pharmacia Biotech); affinity-purified rabbit anti-hTERT serum generated using the following peptide DEAEVRQHREARPALLTSRLRFIPKC (Moriarty *et al.*, 2002); mouse anti-Sm (Lerner *et al.*, 1981); affinity-purified rabbit anti-hTERT serum generated using the following peptide DEAEVRQHREARPALLTSRLRFIPKC (Harrington *et al.*, 1997a); mouse monoclonal anti-SMN (clone 8; Transduction Laboratories); mouse monoclonal anti-Myc (9E10; ATCC hybridoma); mouse monoclonal 2,2,7-Trimethylguanosine-specific antibody (Oncogene Research Products).

Cell culture and manipulations-Human embryonic kidney cells (293) and HeLa cells were grown in DMEM with 10% Fetal Bovine Serum and antibiotics. Transient

transfections of 293 and HeLa cells were performed using Lipofectamine 2000 (Invitrogen) with 1-2 μg expression constructs combined per 35-mm dish.

In vitro binding assays- Reconstitution of active human telomerase by co-expression of GST-hTERT and the human telomerase RNA (hTR) in yeast was previously described (Bachand and Autexier, 1999). To investigate SMN and telomerase interaction *in vitro*, we generated [^{35}S]methionine-labeled Myc-SMN and luciferase using a rabbit reticulocyte lysate (RRL) kit as described per the manufacturer's instructions (Promega). Equal amounts of labeled proteins were first precleared overnight in 1ml of *in vitro* binding buffer (50 mM Tris, pH 7.5; 200 mM NaCl, 2 mM EDTA; 0.1% NP-40; protease inhibitors). Immunopurified human telomerase RNP was prepared by incubating protein extracts from 50 ml yeast pellets with GST-specific antibody (Amersham Pharmacia Biotech) and protein-A-sepharose (Sigma) in yeast lysis buffer (10 mM Tris pH 7.5; 2 mM $MgCl_2$; 5.0 mM β-mercaptoethanol; 20% glycerol; 1% NP-40; 0.25 mM Sodium Deoxycholate; 1.0 mM EGTA; 150 mM NaCl; plus protease and RNase inhibitors). Following a one-hour inucubation at 4° C, beads were washed 5 times in yeast lysis buffer supplemented to 500 mM NaCl. The precleared RRL-synthesized labeled proteins were then incubated with the immunopurified human telomerase RNP for an additional 2 hr at 4° C. After washing 5 times with 1 ml of *in vitro* binding buffer, bound proteins were eluted by boiling in SDS-PAGE loading dye and subjected to electrophoresis.

Immunoprecipitation, telomerase activity, and Northern blotting-20-24 hr post-transfection, cells were washed two times with PBS and resuspended in 500 μl of lysis

buffer (20 mM HEPES, pH 7.9; 2 mM $MgCl_2$; 0.2 mM EGTA; 10% glycerol; 1 mM DTT; 150 mM NaCl; 1.0% NP-40; plus protease and RNase inhibitors). Following homogenization by forcing the cells 5 times through a 25-gauge needle, the cell suspension was left rotating at 4° C for 30 min before clearing the lysate in a microcentrifuge for 15-20 min. For protein coimmunoprecipitations experiments, cell lysates were incubated with antibodies for 30 min, before the addition of protein-A-sepharose for an additional one-hour incubation at 4° C. After washing the beads four times with 1 ml of lysis buffer, the bound proteins were eluted and subjected to SDS-PAGE and immunoblotting. 5-10% of immunoprecipitates were assayed for telomerase activity by TRAP as previously described (Bachand and Autexier, 2001). The preparation of HeLa nucleolar-enriched nuclear extracts was based on a previously described protocol (Jordan *et al.*, 1996).

To analyze immunoprecipitated RNAs, HeLa total cells lysates were prepared from 70-80% confluent 10 cm dish in lysis buffer (20 mM HEPES pH 7.9; 300 mM KCl; 10% glycerol; 0.5 mM DTT; 1 mM EDTA; 2 mM $MgCl_2$; 1% NP-40; protease and RNase inhibitors) and subjected to immunoprecipitation as previously described (Bachand and Autexier, 2001). Probes used for Northern blotting were DNA oligonucleotides complementary to human U1, U2, and U6 snRNAs and the human telomerase RNA.

Indirect immunofluorescence-HeLa cells were cultured on coverslips in 6-well dish. Twenty hours post-transfection, HeLa cells were fixed for 5 min in 1.0% paraformaldehyde in PBS pH 7.5 and then permeabilized for 5 min in 0.5% Triton X-100

in PBS. Myc-tagged SMN and SMNΔN27 proteins were labeled using anti-Myc antibody (9E10; 1:400). Cells were then washed with 0.1% Triton X-100 in PBS, followed by PBS and were then incubated with secondary antibody (goat anti-mouse Cy3 from Chemicon) for 30 min. Cells were rinsed with 0.1% Triton X-100 in PBS, in PBS alone and then mounted in 1 mg/ml paraphenylenediamine in PBS/90% glycerol that also contained DAPI at 1 μg/ml. Digital imaging was performed using a SPOT cooled CCD camera (Diagnostic Instruments, Inc.) mounted on a Zeiss Axioplan immunofluorescence microscope.

RESULTS

SMN associates with the human telomerase ribonucleoprotein *in vitro* and *in vivo*. We used functional recombinant human telomerase expressed in *S. cerevisiae* (Bachand and Autexier, 1999) to investigate whether the SMN protein can form a complex with telomerase *in vitro*. First, the GST-hTERT/hTR telomerase complex was immunopurified from yeast extracts using an affinity-purified GST-specific antibody as described in the *Experimental Procedures*. [^{35}S]methionine-labeled luciferase and SMN proteins were generated in rabbit reticulocyte lysates (RRL). The labeled proteins were incubated with the recombinant telomerase RNP previously immobilized on antibody-coated sepharose beads. Following a 2-h incubation, the complexes were washed extensively, eluted, and analyzed by SDS-polyacrylamide gel electrophoresis. Figure 4-1A shows that SMN specifically bound to the GST-hTERT/hTR complex (lane 6), whereas SMN did not bind to GST alone (lane 5). Treatment of the GST-hTERT/hTR complex with a cocktail of RNases before addition of [^{35}S]-labeled SMN did not affect/disrupt the SMN-telomerase interaction (data not shown). Though incomplete RNA digestion cannot be ruled out, the results suggest that the association of *in vitro*-synthesized SMN with recombinant hTERT may be mediated via direct contact with hTERT or a yeast TERT-associated protein.

We also used transient expression of a Flag-tagged hTERT protein in telomerase-positive 293 cells to demonstrate the association between SMN and telomerase. Total cell extracts from 293 cells transiently transfected with a Flag-hTERT construct were subjected to immunoprecipitation using different antibodies and analyzed by immunoblotting with a mouse monoclonal anti-SMN. As previously demonstrated (Liu

et al., 1997; Pellizzoni *et al.*, 1999), an antibody specific to the Sm protein complex efficiently coimmunoprecipitated SMN from 293 cellular extracts (Fig. 4-1B, lane 5). The precipitation of Flag-tagged hTERT using anti-Flag also coimmunoprecipitated SMN from 293 cell extracts (Fig. 4-1B, lane 4). An antibody specific to a telomerase-associated protein (TEP1) (Harrington *et al.*, 1997a), GST antibody, or protein-A-sepharose alone did not precipitate the SMN protein (Fig. 4-1B, lanes 6, 3, and 2, respectively).

Nucleolar-enriched nuclear extracts were prepared from HeLa cells to determine if an endogenous SMN-hTERT complex exists in cells. The SMN protein was found both in the cytosolic and nuclear extracts (Fig. 4-1C, lanes 1 and 2) as expected from its previously determined subcellular localization (Liu and Dreyfuss, 1996). Confirming the efficiency of our nuclear extract preparation, the hTERT protein was primarily nuclear (Fig. 4-1C, lanes 1 and 2) in agreement with the previous immunofluoresence analysis of hTERT (Harrington *et al.*, 1997b) and mTERT (Martin-Rivera *et al.*, 1998). Proteins from the nuclear extracts were subjected to immunoprecipitation using the Sm protein specific antibody (Y12), an affinity-purified hTERT antibody (Moriarty *et al.*, 2002), and a GST-specific antibody as a negative control. After extensive washing of the antibody-coated beads, the immunoprecipitated proteins were analyzed for recovery of SMN and hTERT as determined by Western blotting with antibodies specific for the respective proteins. As demonstrated in Fig. 4-1B, immunoprecipitation performed with the Y12 antibody coprecipitated SMN from HeLa cell nuclear extracts, but not the hTERT protein (Fig. 4-1C, lane 4). hTERT-specific immunoprecipitation also recovered SMN from the HeLa nuclear extracts (lane 5). The coimmunopurification of SMN and hTERT was also

confirmed using a different affinity-purified hTERT antibody (Harrington *et al.*, 1997b). As a control, neither SMN nor hTERT were present in anti-GST immunoprecipitates (lane 3). We conclude that hTERT and SMN can associate *in vitro* and in human cells.

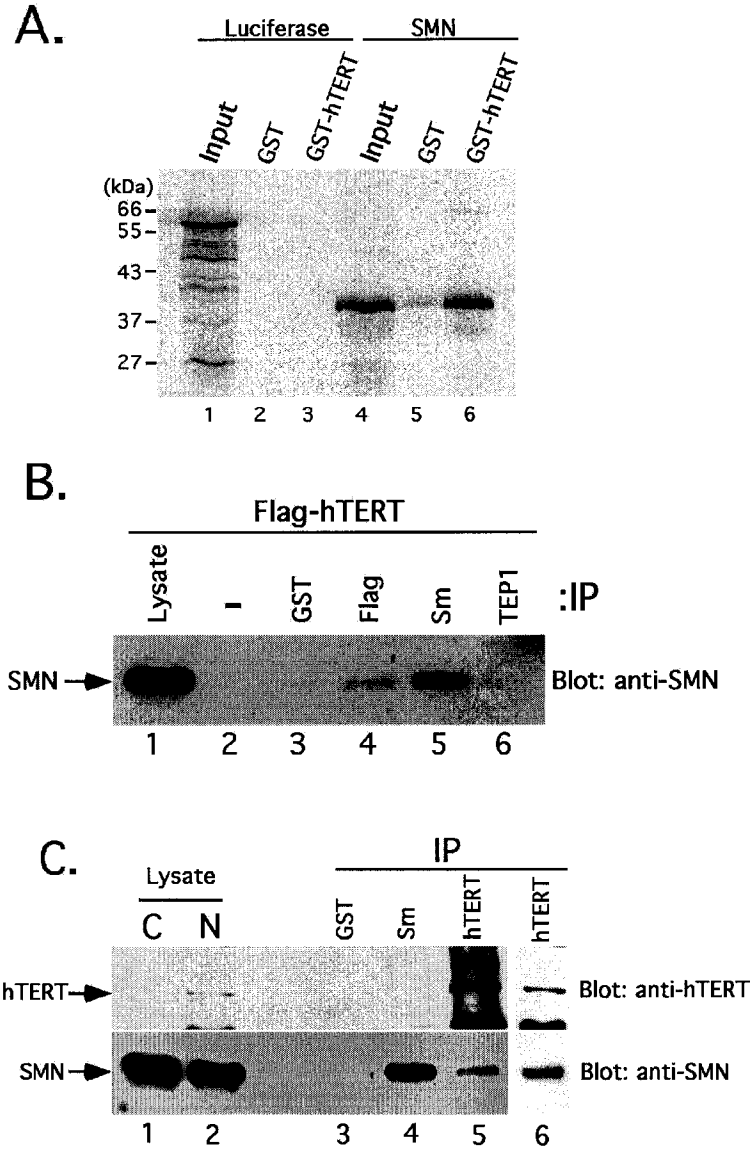

Fig. 4-1. **SMN associates with hTERT *in vitro* and *in vivo*.** (A) Human telomerase was reconstituted by coexpression of GST-hTERT and hTR in *S. cerevisiae* as previously described (Bachand and Autexier, 1999). GST (lanes 2 and 5) and GST-hTERT/hTR (lanes 3 and 6) were affinity-purified from equal volumes of yeast extracts and incubated with *in vitro*-translated [35S]methionine-labeled luciferase (lanes 1-3) and SMN (lanes 4-6). Following extensive washing, bound proteins were analyzed by SDS-PAGE and autoradiography. The input lanes (1 and 4) show 5% of the RRL lysate used in the binding reaction. Molecular mass markers are indicated on the left (in kilodaltons, kDa). (B) 293 cells were transiently transfected with a DNA construct expressing Flag-tagged hTERT. 20h post-transfection, a total cell lysate was prepared and subjected to immunoprecipitation (IP) without antibody or using anti-GST, anti-Flag, anti Sm (Y12), or anti-TEP1. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting for endogenous SMN. The lysate lane corresponds to 5% of the total cell lysate used for the immunoprecipitation. (C) Nucleolar enriched nuclear extracts were prepared from HeLa cells and subjected to immunoprecipitation (IP) using anti-GST, anti-Sm (Y12) and two different affinity-purified hTERT antibodies. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting for endogenous hTERT (top) and SMN (bottom). 5% of the cytosolic (C) and nucleolar-enriched nuclear (N) extracts were also loaded.

SMN is associated with catalytically active human telomerase

We used transient transfection experiments to investigate whether SMN is associated with active human telomerase. A Myc-tagged version of SMN and Flag-hTERT were transiently expressed in human 293 cells and total cell lysates were prepared for immunoprecipitations using either Myc or Flag antibodies. As a control, a lysate from mock-transfected cells was used. Telomeric repeat amplification protocol (TRAP) assays demonstrated that equal levels of telomerase activity were present in the different total cell lysates before immunopurification (Fig. 4-2, lanes 1-3). As previously demonstrated (Harrington *et al.*, 1997a; Mitchell *et al.*, 1999b), Flag antibody resin precipitated the Flag-hTERT protein (data not shown) and human telomerase activity from the Flag-hTERT-containing extracts (Fig. 4-2, lane 8). However, no telomerase activity was present on the Flag-antibody resin incubated with Myc-SMN-containing extracts (lane 7). Telomerase activity (lane 5) was also recovered from Myc-antibody resin prepared from lysates containing the Myc-SMN protein, but not from anti-Myc immunoprecipitates prepared from lysates of mock- and Flag-hTERT-transfected cells (lanes 4 and 6, respectively). Similar results were observed using HeLa cells (data not shown). These results indicate that SMN associates with a fully assembled and catalytically active telomerase RNP.

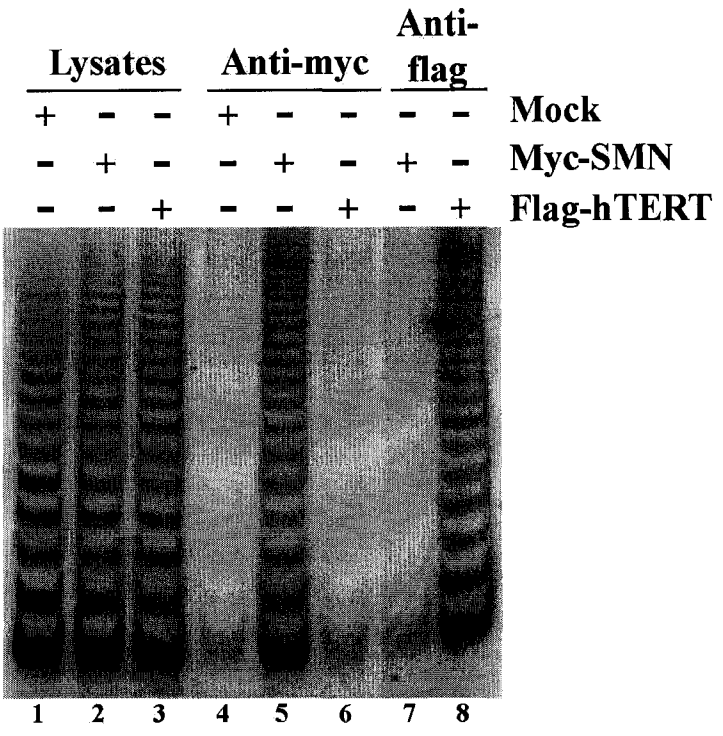

Fig. 4-2. **SMN associates with catalytically active telomerase**. 293 cells were transiently transfected with DNA constructs expressing Myc-tagged SMN (lanes 2, 5, and 7), Flag-tagged hTERT (lanes 3, 6, and 8), or with vector alone (mock-lanes 1 and 4). Total cell lysates were prepared and subjected to immunoprecipitation using either anti Myc (lanes 4-6) or anti-Flag (lanes 7-8). Immunoprecipitates were analyzed for telomerase activity by the TRAP assay. 0.5% of the total cell lysates (lanes 1-3) were also assayed for telomerase activity.

The Sm protein complex is not associated with active human telomerase and hTR

The Sm proteins form an RNA-binding complex that interacts with a specific region of UsnRNAs (Will and Luhrmann, 2001). Direct interactions between SMN and specific members of the Sm proteins are thought to recruit the SMN complex to the Sm-snRNA complex (Liu *et al.*, 1997; Pellizzoni *et al.*, 1999). Interestingly, the *S. cerevisiae* telomerase RNA subunit, TLC1, contains an Sm protein-binding site as determined by the coimmunoprecipitation of the TLC1 RNA and yeast telomerase activity with epitope-tagged versions of the SmD1 and SmD3 proteins (Seto *et al.*, 1999).

Myc-tagged versions of the human SmB, SmD1, and SmD3 proteins were used to examine whether the association of SMN with telomerase could be mediated by the involvement of the Sm protein complex in human telomerase biogenesis. Addition of the Myc epitope to the N-terminus of the SmB, D1, and D3 proteins did not affect their function as determined by their ability to coimmunoprecipitate SMN and by immunofluorescence analysis (data not shown). Constructs expressing the Myc-SmB, Myc-SmD1, and Myc-SmD3 proteins were transfected in human 293 cells and total cell extracts were prepared for immunopurification using the Myc antibody. As was previously shown in Fig. 4-2, Myc-antibody resin incubated with Myc-SMN-containing extracts recovers telomerase activity (Fig. 4-3, lane 9). However, immunoprecipitations performed using the Myc antibody and prepared from either the Myc-SmB, Myc-SmD1, or Myc-SmD3-containing extracts did not recover levels of human telomerase activity (Fig. 4-3, lanes 10-12, respectively) significantly higher than background (lane 8). Western blot analysis of the immunoprecipitated proteins (Fig. 4-3A, bottom panel)

revealed that the myc-tagged Sm proteins were expressed and immunoprecipited to considerably higher levels than myc-tagged SMN, yet only myc-SMN copurified human telomerase activity. As a control, anti-Flag antibody efficiently precipitated telomerase activity from lysates prepared from Flag-hTERT transfected cells (lane 13). These results, in addition to the absence of hTERT in Y12 immunoprecipitates (Fig. 4-1C), indicate that the Sm protein complex is not associated with the human telomerase RNP and suggest that Sm proteins do not mediate the SMN-telomerase association.

To further investigate whether the Sm protein complex is involved in human telomerase biogenesis, we determined if endogenous hTR from HeLa total cell extracts copurifies with the Sm complex using the Y12 antibody. As previously demonstrated (Lerner and Steitz, 1979), endogenous U1 and U6 snRNAs are coprecipitated with Sm proteins using the Y12 antibody. However, the Y12 antibody did not coimmunoprecipitate hTR (Fig. 4-3B, lane 2). hTERT-associated hTR was coprecipitated using an affinity-purified hTERT antibody (Fig. 4-3B, lane 3), while an anti-GST immunoprecipitation did not recover U1, U6, or hTR (Fig. 4-3B, lane 5).

We also determined whether a subpopulation of the human telomerase RNA contains a 2,2,7-trimethylguanosine (TMG) cap structure as was previously shown for the yeast telomerase RNA (Seto *et al.*, 1999). This type of hypermethylated 5'-cap structure is a well-known characteristic of some spliceosomal snRNAs such as U1, U2, U4, and U5 (Will and Luhrmann, 2001). HeLa total cell lysates were subjected to immunoprecipitation using a TMG-specific monoclonal antibody and the copurified RNAs were analyzed by Northern blotting. Both U1 and U2 snRNAs were recovered from the anti-TMG immunoprecipitate (Fig. 4-3B, lane 4 and data not shown), whereas

the presence of the human telomerase RNA in TMG-specific immunoprecipitates was undetectable (Fig. 4-3B, lane 4). Similarly, U6 snRNA was not coimmunoprecipitated by the TMG-specific antibody (lane 4) and was used as a negative control since it does not have a 5'-TMG cap structure (Singh and Reddy, 1989). In conclusion, these results indicate that the Sm proteins, whether endogenous or transiently overexpressed, associate neither with hTR nor with the catalytically active human telomerase RNP. Our results further suggest that hTR does not acquire a 5'-TMG cap.

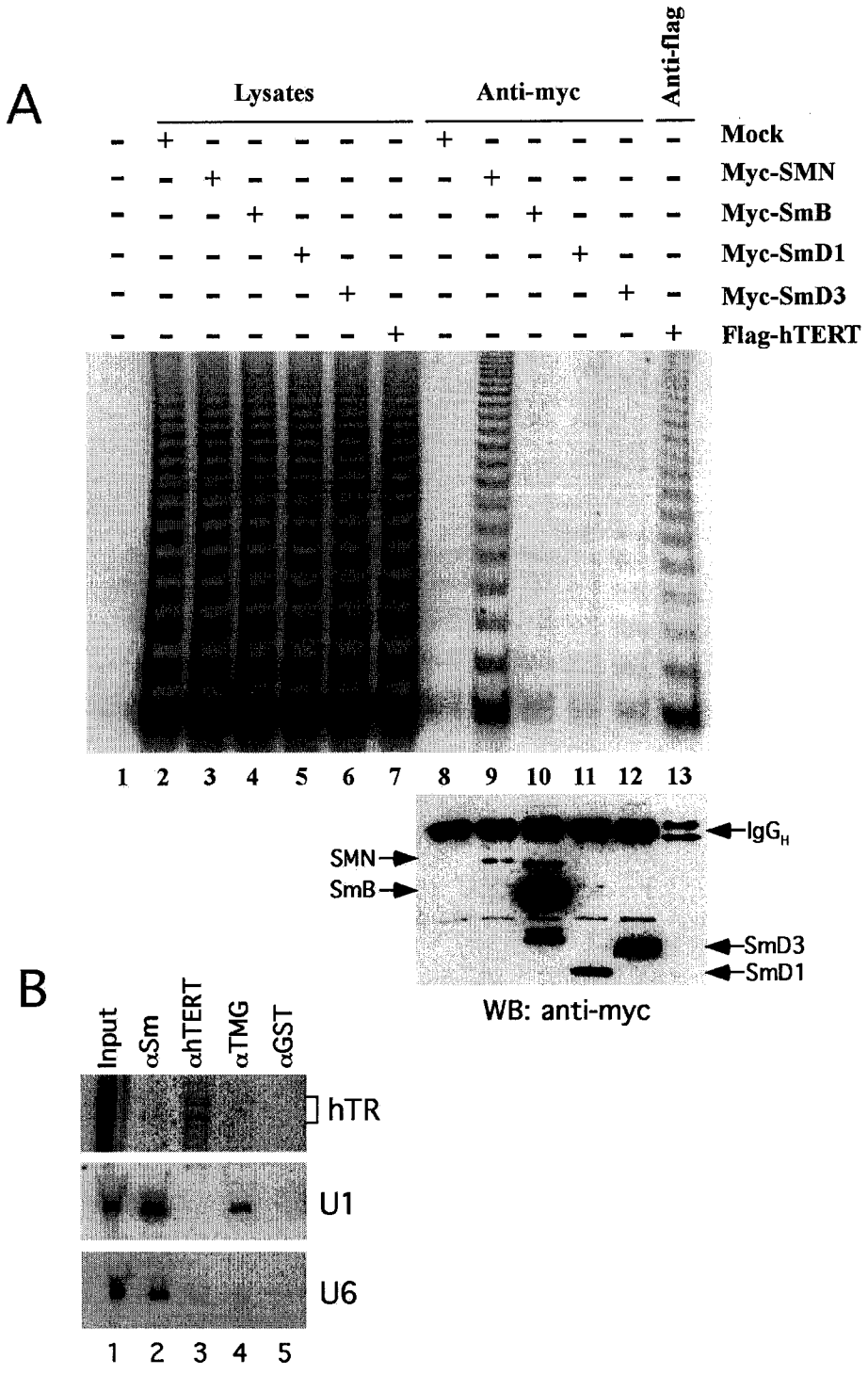

Fig. 4-3. **The human telomerase RNA and telomerase activity are not associated with the Sm protein complex.** (A) 293 cells were transiently transfected with DNA constructs expressing Myc-SMN (lanes 3 and 9), Myc-SmB (lanes 4 and 10), Myc-SmD1 (lanes 5 and 11), Myc-SmD3 (lanes 6 and 12), Flag-hTERT (lanes 7 and 13), or with vector alone (mock-lanes 2 and 8). 20hrs post-transfection, total cell lysates were prepared and subjected tc immunoprecipitation using either anti-Myc (lanes 8-12) or anti-Flag (lane 13). Immunoprecipitates were analyzed for telomerase activity by the TRAP assay (*Top panel*) and for protein content by Western blotting using anti-myc (*Bottom panel*). 1% of the total cell lysates (lanes 2-7) or lysis buffer (lane 1) were analyzed for telomerase activity. (B) A total cell lysate from HeLa cells was prepared and subjected to immunoprecipitation using anti-Sm (Y12-lane 2), affinity-purified anti-hTERT (lane 3), anti-2,2,7-trimethylguanosine (TMG-lane 4), or anti-GST (lane 5) Immunoprecipitates were analyzed by denaturing PAGE and Northern blotting for endogenous human telomerase RNA (hTR, top), U1 snRNA (middle), and U6 snRNA (bottom). The input lane (lane 1) was loaded with 2.5% of the total RNA extracted from the HeLa total cell lysate. For hTR, exposure time was 3 times longer than for U1 and U6.

Expression of a dominant negative version of SMN (SMNΔN27) perturbs the subcellular localization of hTERT

The SMN protein is present in the cytoplasm as well as in the nucleus of cells, where it is known to concentrate in nuclear structures such as gems, Cajal bodies (CB), and nucleoli (Liu and Dreyfuss, 1996; Hebert *et al.*, 2001; Young *et al.*, 2001). A previous study characterizing a dominant-negative mutant of SMN lacking its first N-terminal 27 amino acids (SMNΔN27) revealed that this mutant protein causes the reorganization of snRNPs in the nucleus and in the cytoplasm, and also negatively affects pre-mRNA splicing *in vitro* (Pellizzoni *et al.*, 1998). This SMN mutant protein was also used to demonstrate the functional interaction between SMN and the RNA polymerase II complex (Pellizzoni *et al.*, 2001b) as well as with the snoRNP proteins fibrillarin and hGAR1 (Pellizzoni *et al.*, 2001a).

We generated SMNΔN27 to investigate the effect of this dominant-negative mutant SMN on hTERT cellular localization. Indirect immunofluorescence with the Myc antibody was used to detect the Myc-SMN and Myc-SMNΔN27 proteins in transfected HeLa cells. The Myc-SMN protein localized in the cytoplasm and in the nucleus, where it accumulated in gems/CBs (Fig. 4-4 and 4-5, panel c). When the Myc-SMN construct was cotransfected with a plasmid expressing a yellow fluorescent protein (YFP)-SmB fusion, Myc-SMN and YFP-SmB colocalized in gems/CBs (Fig. 4-4, panels a-d), as previously reported (Liu *et al.*, 1997; Pellizzoni *et al.*, 1998). In cells transfected with Myc-SMNΔN27, the mutant protein accumulated in large cytoplasmic bodies that partially redistributed the YFP-SmB fusion (Fig. 4-4, panels e-h). In constrast, the YFP-SmB fusion protein was barely detectable in the cytoplasm of Myc-SMN transfected cells

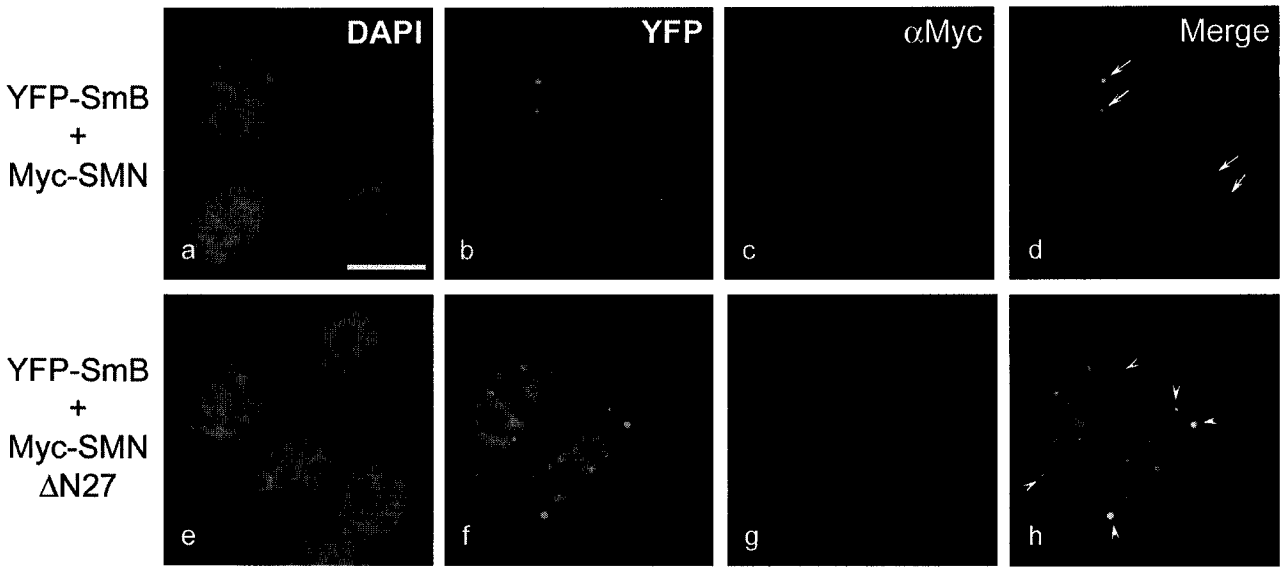

Fig. 4-4. The expression of a dominant-negative mutant of SMN (SMNΔN27) perturbs the localization of Sm proteins. (A) HeLa cells were transiently cotransfected with DNA constructs expressing either Myc-SMN and YFP-SmB (a-d) or Myc-SMNΔN27 and YFP-SmB (e-h). The fixed and permeabilized cells were stained for Myc-SMN (c) and Myc-SMNΔN27 (g) using anti-Myc. DNA stained with DAPI shows the nucleus of each cell (a and e). Images b-c and f-g are merged to form d and h, respectively. The arrows point to the nuclear gems and the arrowheads to the SMNΔN27-induced cytoplasmic accumulations. Bar, 12 mm.

(Fig. 4-4, panel b) and untransfected cells (data not shown), consistent with the localization of endogenous Sm proteins as detected using the Sm-specific Y12 antibody (Liu *et al.*, 1997; Pellizzoni *et al.*, 1998). Similar results were obtained when a YFP-SmD1 fusion was used (data not shown), in agreement with the observations that a mutant of SMN lacking the first 27 amino acids causes a reorganization of Sm snRNPs proteins (Pellizzoni *et al.*, 1998).

We used GFP-tagged hTERT to monitor the steady state subcellular localization of the human telomerase reverse transcriptase. Addition of GFP to the N-terminus of hTERT did not alter its catalytic function, as the GFP-hTERT fusion reconstituted human telomerase activity when expressed in telomerase-negative human fibroblasts (data not shown). The GFP-hTERT fusion protein showed a diffuse nucleoplasmic distribution both in untransfected (data not shown) and in Myc-SMN-transfected HeLa cells (Fig. 4-5, panel b), consistent with previous reports of TERT localization (Harrington *et al.*, 1997b; Martin-Rivera *et al.*, 1998). GFP-tagged hTERT did not specifically colocalize with Myc-SMN in gems/CB (Fig. 4-5, panels a-d), but did frequently localize to the nucleolus of transfected cells (Fig. 4-5 and data not shown). GFP-hTERT expressed in Myc-SMNΔN27-transfected cells prominently accumulated in the cytoplasm in structures that colocalized with the Myc-SMNΔN27 protein (Fig. 4-5, panels e-l), in striking contrast to the restricted and diffuse nucleoplasmic localization of hTERT in Myc-SMN-transfected and untransfected cells. The colocalization of hTERT and Myc-SMNΔN27 was also observed in the nucleus where GFP-hTERT accumulated in gems/CB (Fig. 4-5, panels f-h). The observation that hTERT subcellular localization is affected by expression of

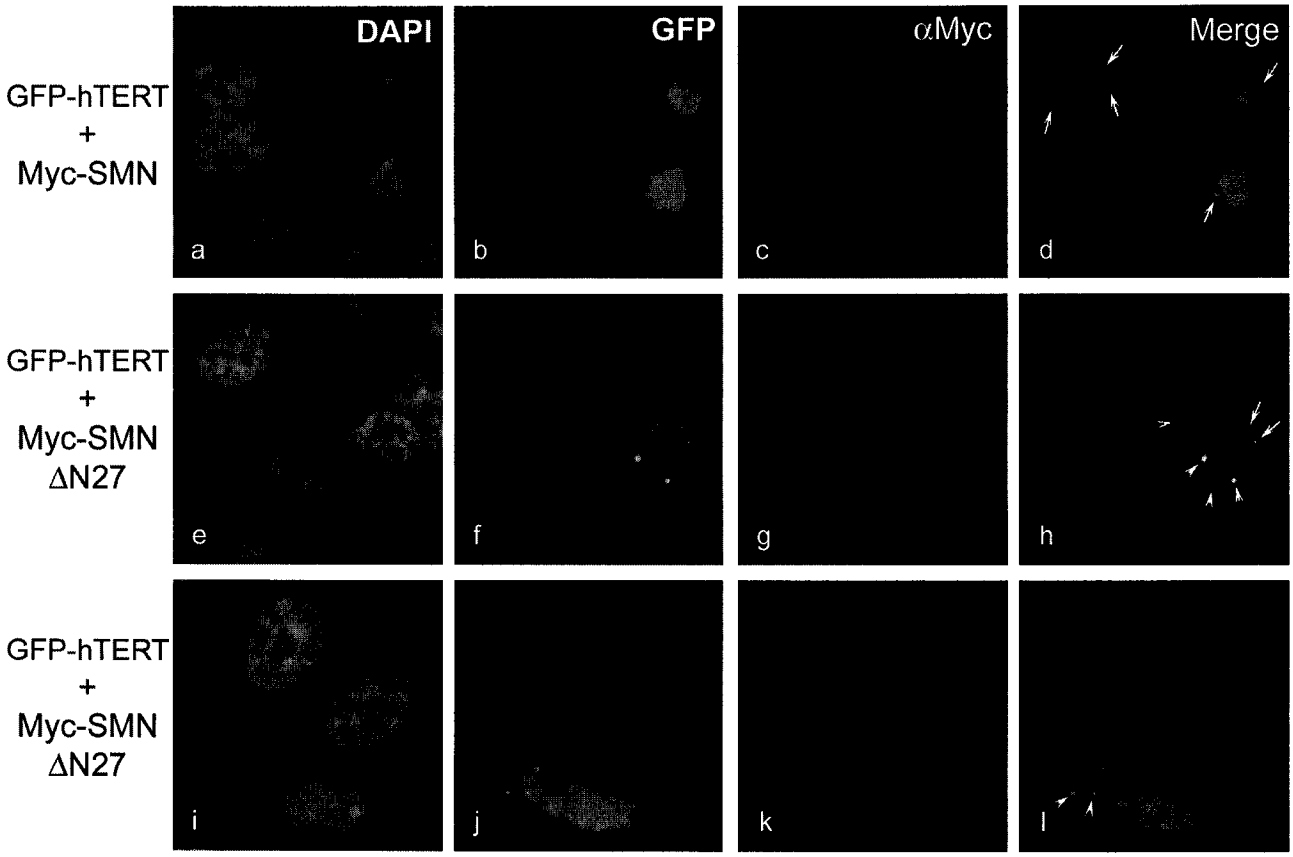

Fig. 4-5. The expression of a dominant-negative mutant of SMN (SMNΔN27) perturbs the nuclear localization of hTERT. HeLa cells were transiently cotransfected with DNA constructs expressing either Myc-SMN and GFP-hTERT (a-d) or Myc-SMNΔN27 and GFP-hTERT (e-l). The fixed and permeabilized cells were stained for Myc-SMN (c) and Myc-SMNΔN27 (g and k) using anti-Myc. DNA stained with DAPI shows the nucleus of each cell (a, e, and i). Images b-c, f-g, and j-k are merged to form d, h, and l, respectively.The arrows point to the nuclear gems and the arrowheads to the SMNΔN27-induced cytoplasmic accumulations.

SMNΔN27 suggests a functional relationship between the SMN complex and human telomerase.

SMNΔN27 affects human telomerase reconstitution *in vitro*

Coexpression of hTERT and hTR reconstitutes human telomerase activity in rabbit reticulocyte lysates (RRL) (Weinrich *et al.*, 1997; Beattie *et al.*, 1998). Studies of human and *Tetrahymena* telomerase suggest that proteins present in reticulocytes extracts may be involved in reconstitution of telomerase assembly and/or activity (Holt *et al.*, 1999; Licht and Collins, 1999). Western blot analysis of crude RRL extracts using a monoclonal SMN antibody revealed the presence of a single 38-kDa protein (data not shown), suggesting the presence of rabbit SMN in reticulocyte lysates. This latter observation and the profound effects of SMNΔN27 on hTERT subcellular localization, led us to examine whether expression of SMNΔN27 in RRL would affect the reconstitution of human telomerase activity.

In vitro-transcribed hTR was added to RRL programmed to express hTERT alone or to coexpress hTERT with SmB, wild-type human SMN, or the SMNΔN27 mutant. Protein synthesis was allowed to proceed for different times, followed by analysis of telomerase activity by TRAP. Human telomerase activity was undetectable after 30 min, whether hTERT was expressed alone or with another protein (Fig. 4-6A, lanes 1, 4, 7, and 10). At 60 min, robust telomerase activity was observed in the control RRL reaction where hTERT was expressed alone (lane 2). Telomerase activity was also detected when SmB or SMN was coexpressed with hTERT (lanes 5 and 8). Lower levels of telomerase activity in RRL reactions coexpressing hTERT and SmB/SMN were observed than in the

control reaction (compare lanes 5 and 8 to lane 2) due to the lower levels of hTERT protein synthesized when additional DNA is present in the RRL reaction (Fig. 4-6B and data not shown). Human telomerase activity reconstituted after 60 min was barely detectable from the reaction that coexpressed hTERT and SMNΔN27 (lane 11). Similarly, after 90 min, the amount of telomerase activity reconstituted in the SMNΔN27-programmed RRL was considerably lower than in SmB- and SMN-containing RRLs (compare lane 12 to lanes 6 and 9). As can be seen in Fig. 4-6B, these differences were not attributed to drastically different levels of hTERT protein (lanes 2-4). Telomerase activity was similar to the results seen in Fig. 4-6A when luciferase was expressed with hTERT rather than SmB (data not shown). The decreased efficiency of human telomerase reconstitution when SMNΔN27 is expressed in RRL was reproduced in three independent experiments. Telomerase activity levels were quantified from the three independent experiments, averaged, and a percentage was calculated relative to the levels of activity reconstituted in the control RRL reaction where hTERT was expressed alone (Fig. 4-6C). These results indicate that a previously characterized dominant-negative SMN mutant, SMNΔN27, significantly decreases the efficiency of human telomerase reconstitution *in vitro*.

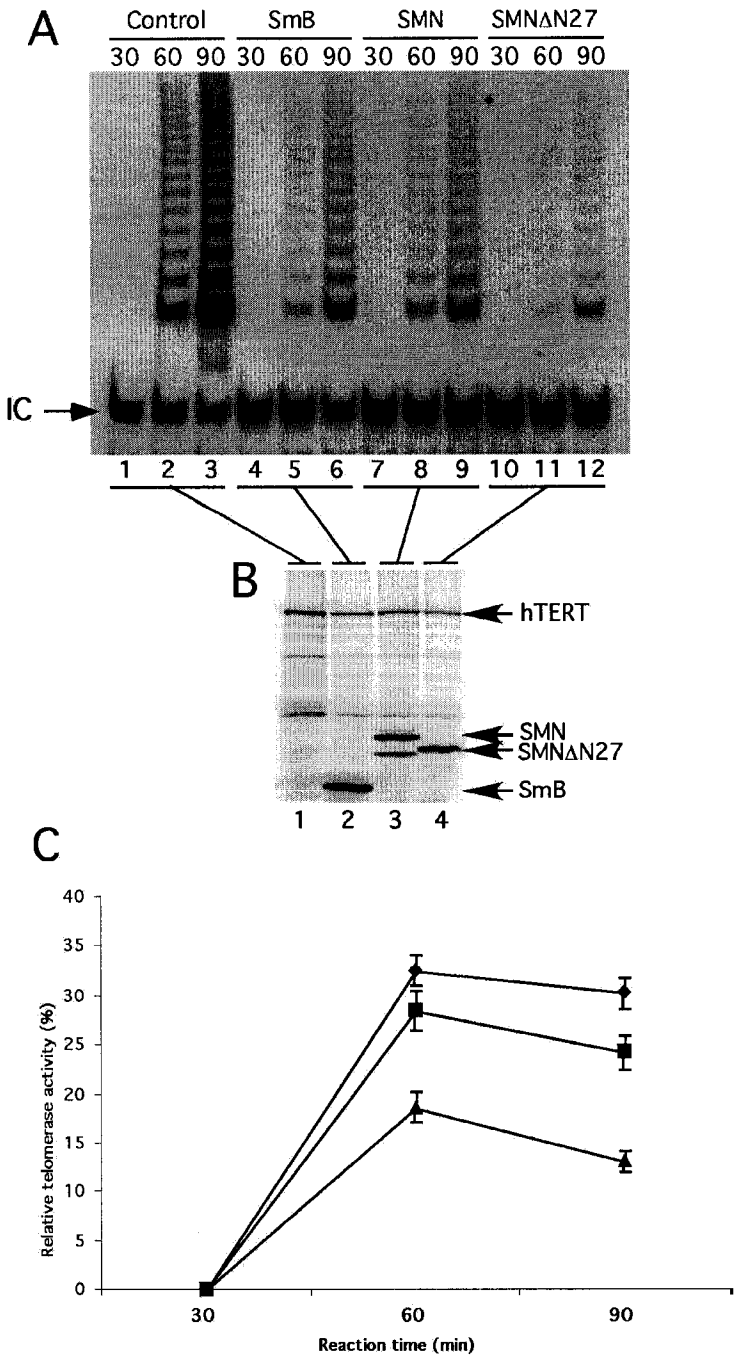

Fig. 4-6. **Expression of SMNΔN27 decreases human telomerase activity reconstitution in rabbit reticulocyte lysates**. (A) hTERT was synthesized in rabbit reticulocyte lysates (RRL) in the presence of hTR, [35S]methionine, and equal amounts of DNA constructs expressing Myc-SmB (lanes 4-6), Myc-SMN (lanes 7-9), Myc-SMNΔN27 (lanes 10-12), or no additional DNA (lanes 1-3). 30, 60, and 90 min after the start of the RRL reactions, telomerase activity was assayed by the TRAP assay. Each TRAP reaction included an internal control (IC) to normalize for variation in PCR efficiency. (B) 90 min after the start of the RRL reactions, equal amounts were analyzed by SDS-PAGE and autoradiography. (C) The telomerase activity was calculated as the ratio between the intensity of the telomerase ladder products and the intensity of the internal PCR control. A ratio of this telomerase activity value to the amount of *in-vitro* translated hTERT measured by the intensity of the S35-labeled hTERT was calculated to generate the relative telomerase activity. The activities from three independent experiments performed in the presence of SmB (diamond), wild-type SMN (square), and SMNΔN27 (triangle) were averaged and compared relative to RRL reactions in which no additional DNA construct was included.

DISCUSSION

Recent results suggest that spliceosomes and transcriptosomes are pre-assembled in a substrate-independent fashion (Gall *et al.*, 1999; Stevens *et al.*, 2002). Similarly, telomerase is likely pre-assembled into a functional RNP before recruitment to its site of action, the telomere. The assembly and maturation of many ribonucleoprotein particles is believed to occur in nuclear structures such as the Cajal bodies (CB) and nucleoli (Matera, 1999; Olson *et al.*, 2000). Vertebrate telomerase RNA is a member of the H/ACA box family of small nucleolar (sno) RNA (Chen *et al.*, 2000) and a fraction of hTR localizes to the nucleolus (Mitchell *et al.*, 1999a; Narayanan *et al.*, 1999), suggesting that vertebrate telomerase assembly and/or maturation transits through the nucleolus. Furthermore, Lukowiak and colleagues recently reported that *in vitro*-transcribed human and *Xenopus* telomerase RNAs microinjected into *Xenopus* oocyte nuclei localize not only to nucleoli, but also to CB (Lukowiak *et al.*, 2001).

Our results support a model in which the human telomerase RNP is assembled and/or matured into a functional enzyme by transit through the nucleoli and/or the CB. The physical association between endogenous hTERT and SMN, a protein involved in RNP assembly and that localizes in both nucleoli and CB, strongly suggests that SMN plays a role in human telomerase biogenesis. Two experimental observations support that the association of SMN and telomerase does not merely reflect the fact they both colocalize to similar nuclear structures; nucleoli and/or CB. First, recombinant telomerase can specifically bind *in vitro*-translated SMN (Fig. 4-1A). Second, hTERT protein and telomerase activity are undetectable in immunoprecipitates performed using antibodies specific to the box C/D snoRNP nucleolar protein fibrillarin (data not shown).

The effect of SMNΔN27 on hTERT subcellular localization and telomerase reconstitution *in vitro* further supports a functional role for SMN in telomerase assembly. The mechanism by which SMNΔN27 disturbs the cellular organization of snRNPs (Pellizzoni *et al.*, 1998) and snoRNPs (Pellizzoni *et al.*, 2001a) has not yet been defined. GFP-hTERT did not specifically colocalize with wild-type SMN in gems/CB, yet it accumulated and colocalized with SMNΔN27 (Fig. 4-5). These results suggest dynamic and transient interactions between the SMN complex and components of the telomerase RNP. Similar results are observed in immunofluorescence analyses of several components of the RNA polymerase II transcription machinery upon expression of SMN and SMNΔN27 (Pellizzoni *et al.*, 2001b). The effect of SMNΔN27 on hTERT cellular localization is consistent with the proposed view that this SMN mutant sequesters associated proteins in cellular bodies by blocking or retarding their release and/or their transport between different nuclear bodies (Pellizzoni *et al.*, 1998; Pellizzoni *et al.*, 2001a; Pellizzoni *et al.*, 2001b; Terns and Terns, 2001). SMNΔN27 expression also resulted in the accumulation and the detection of GFP-hTERT in the cytoplasm of cotransfected cells (Fig. 4-5). The accumulation of GFP-hTERT in the cytoplasm was never observed in cells either cotransfected with wild-type SMN (Fig. 4-5) or transfected with the GFP-hTERT construct alone (data not shown). Sm proteins also colocalize with the SMNΔN27-induced cytoplasmic accumulations (Fig. 4-4; Pellizzoni, 1998), possibly as a result of a perturbed interaction between endogenous SMN and Sm proteins in the cytoplasm (Fischer *et al.*, 1997; Liu *et al.*, 1997). Yet, SMNΔN27 does not elicit the cytoplasmic accumulation of other SMN-associated proteins such as p80coilin (Pellizzoni *et al.*, 1998), components of the RNA pol II complex (Pellizzoni *et al.*, 2001b), and

snoRNP proteins (Pellizzoni *et al.*, 2001a). Our results demonstrating the accumulation of GFP-hTERT in cytoplasmic SMNΔN27-containing aggregates suggest that the interaction between the SMN complex and hTERT could be initiated in the cytosol. The SMN-hTERT complex could then relocalize to the nucleus and encounter a fully processed human telomerase RNA-snoRNP protein complex in subnuclear domains such as the nucleolus (Mitchell *et al.*, 1999a; Narayanan *et al.*, 1999) and/or the CB (Lukowiak *et al.*, 2001).

How might SMN be involved in human telomerase biogenesis? The best characterized function of SMN is its role in snRNP assembly. Experiments in *Xenopus* oocytes and using a cell-free system for *in vitro* reconstitution of UsnRNP assembly suggest that the SMN complex is involved in facilitating the association of distinct snRNAs with Sm proteins (Fischer *et al.*, 1997; Meister *et al.*, 2001). hTERT, hTR, and human telomerase activity are not detected in anti-Sm immunoprecipitates (Figs. 4-1 and 4-3), suggesting that the Sm protein complex is not involved in human telomerase biogenesis. The lack of association between hTR and Sm proteins was previously noted (Le *et al.*, 2000; Lukowiak *et al.*, 2001). Thus, based on our results and recent studies that report interactions between SMN and snoRNP proteins (Jones *et al.*, 2001; Pellizzoni *et al.*, 2001a), we propose that SMN may function in human telomerase assembly through the association of SMN with snoRNP proteins such as hGAR1. The human GAR1 snoRNP protein is an attractive candidate because it associates with the human telomerase RNA (Pogacic *et al.*, 2001). Using antibodies specific for H/ACA snoRNP proteins, we were unable to immunodeplete the SMN-associated telomerase activity (data not shown). However, hGAR1 and dyskerin, two hTR-associated H/ACA snoRNP proteins (Mitchell

et al., 1999b; Pogacic *et al.*, 2001), and SMN are present in partially purified human telomerase fractions generated using anion and size exclusion chromatography followed by differential density ultracentrifugation though a cesium sulfate gradient (data not shown). These observations, coupled with the nucleolar localization of hTR (Mitchell *et al.*, 1999a; Narayanan *et al.*, 1999; Lukowiak *et al.*, 2001), hTERT (Fig. 4-5 and data not shown), SMN (Pellizzoni *et al.*, 2001a; Young *et al.*, 2001), and hTR-associated snoRNP proteins (Pogacic *et al.*, 2001), suggest that SMN may be involved in human telomerase biogenesis as an H/ACA snoRNP.

Our results also establish major differences between *S. cerevisiae* and vertebrate telomerase RNPs. The budding yeast telomerase RNA associates with Sm proteins and gains a 5'-TMG cap structure (Seto *et al.*, 1999); both events are characteristics of snRNP assembly. However, hTR was not recovered from anti-Sm and anti-TMG immunoprecipitates (Fig. 4-3), suggesting that human telomerase is not processed as an snRNP. This conclusion is supported by recent experiments in which *in vitro*-synthesized hTR was microinjected into *Xenopus* oocytes nuclei (Lukowiak *et al.*, 2001). SnoRNAs are generated from two different genomic contexts: pre-mRNA introns or their own independent transcription unit (Weinstein and Steitz, 1999). The U3, U8, and U13 box C/D snoRNAs are transcribed from their own promoters and receive a TMG cap, whereas most intron-generated snoRNAs do not undergo 5' hypermethylation (Yu *et al.*, 1999; Speckmann *et al.*, 2000). The lack of a TMG cap at the 5' end of hTR is thus surprising since it is expressed from its own promoter as an RNA polymerase II transcript (Feng *et al.*, 1995; Hinkley *et al.*, 1998). However, to our knowledge, none of the metazoan H/ACA box snoRNAs have been shown to receive a TMG cap. Further studies will be

necessary to better understand the processing and maturation events required for functional hTR formation.

Previous data supports the view that telomerase reconstitution in rabbit reticulocyte lysates (RRL) is facilitated by the action of proteins present in the extracts (Holt *et al.*, 1999; Licht and Collins, 1999). The negative effect of SMNΔN27 on *in vitro* telomerase reconstitution in RRL (Fig. 4-6) is consistent with this view. However, when SMNΔN27-containing extracts from RRL or 293 cells were added to previously reconstituted human telomerase, the activity of telomerase was not affected (data not shown). Thus, the mutant form of SMN may not affect telomerase by inhibiting its catalytic activity, but rather by affecting assembly *in vitro*. The incomplete inhibition of human telomerase reconstitution by SMNΔN27 *in vitro* could reflect a partial decrease in the efficiency of reconstitution or the inability to obtain concentrations of SMNΔN27 sufficient to completely sequester the endogenous RRL proteins involved in telomerase assembly.

The detailed characterization of the cellular components and progressive steps involved in human telomerase assembly will be critical for the rational design of new telomerase inhibitors. The identification of SMN as a telomerase-associated protein suggests that it will be an important player in the functional assembly and activation of human telomerase. Future studies will focus on understanding the specific role performed by SMN and its associated proteins in human telomerase biogenesis.

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Chapter 5

GENERAL DISCUSSION

5.1 *Saccharomyces cerevisiae*: a genetic and biochemical system to study human telomerase

Several observations suggest that the course of telomerase assembly *in vivo* is an active process that requires energy and the action of different chaperone proteins (Holt *et al.*, 1999; Mitchell *et al.*, 1999b; Wenz *et al.*, 2001). The association between the product of the *survival of motor neuron* (*SMN*) gene and the human telomerase RNP is consistent with the notion of protein-assisted assembly of telomerase. A catalytically active human telomerase RNP is assembled *in vivo* in the yeast *S. cerevisiae* by the coexpression of the human telomerase RNA (hTR) and a GST-hTERT fusion protein (Bachand and Autexier, 1999). These results suggest that the necessary components required to assemble an active human telomerase RNP are present in budding yeast and can functionally interact with the human telomerase protein and RNA subunits. We suspect that these components are the yeast homologs of the proteins involved in the biogenesis of mammalian telomerase. The recent finding that the accumulation of the mature form of hTR in *S. cerevisiae* requires the action of the yeast H/ACA snoRNA-binding proteins supports this model (Dez *et al.*, 2001). Thus, the genetically tractable yeast *S. cerevisiae* will provide an excellent model to characterize different steps and components involved in human telomerase biogenesis.

Similarly to human primary cells lacking telomerase activity, yeast cells that lack telomerase show progressive telomere shortening and a senescence-like phenotype

(Lundblad and Szostak, 1989; Singer and Gottschling, 1994; Lingner *et al.*, 1997b). Consequently, the establishment of a yeast complementation system in which human telomerase maintains *S. cerevisiae* telomeres would be of great value. Our attempts to establish such a complementation system by coexpressing GST-hTERT and hTR in yeast have so far been unsuccessful (A. Amadou, E. Clair, F. Bachand, C. Autexier, and R. Wellinger, unpublished results). Yeast cells can be maintained for several generations with human telomeric sequences (TTAGGG) at their chromosomal ends (Henning *et al.*, 1998), suggesting that the absence of functional complementation is not due to the instability of yeast telomeres containing human telomeric sequences. In fact, we do not detect human telomeric sequences at telomeres of yeast genetically disrupted for the protein and RNA subunits genes of yeast telomerase (A. Amadou, E. Clair, F. Bachand, C. Autexier, and R. Wellinger, unpublished results). Thus, we hypothesize that the catalytically active human telomerase reconstituted in *S. cerevisiae* is not recruited to yeast telomeres. This may reflect the major evolutionary divergence between the telomere-associated proteins of *S. cerevisiae* and mammals (Li *et al.*, 2000). We are currently generating a fusion protein between the DNA-binding domain of the single-stranded telomere-binding protein Cdc13p and hTERT. A $Cdc13_{DBD}$-Est2p fusion has been previously demonstrated to effectively tether the yeast telomerase complex onto telomeres in the absence of other essential recruiting components (Evans and Lundblad, 1999). A complementation system would enable genetic screens to identify functional domains within hTERT and hTR, and other components necessary for human telomerase biogenesis.

5.2 Functional role of the amino- and carboxy-terminal regions of hTERT

We provided evidence that the large and basic amino-terminal region of hTERT was involved in both the polymerase and telomerase RNA-binding functions of this protein (Chapter 3). Furthermore, we showed that these two functions map to separate domains; we could generate an inactive hTERT mutant that binds the telomerase RNA as efficiently as the full-length protein. These results suggested that the TERT amino-terminal region could perform diverse functional roles in the active telomerase complex. Since this discovery (Beattie *et al.*, 2000; Bachand and Autexier, 2001), new studies have identified novel functional domains within the TERT N-terminal region involved in: (i) telomere maintenance independent of telomerase catalytic activity (Armbruster *et al.*, 2001); (ii) hTERT nucleolar localization (Etheridge *et al.*, 2002); (iii) TERT oligomerization (Beattie *et al.*, 2001; Arai *et al.*, 2002). The N-terminal region of hTERT (including the T motif; see Figure 1-6) is approximately 60 kDa (F. Bachand and C. Autexier, unpublished results). Thus, it should be possible to purify the recombinant form of this protein module, either alone or in association with the telomerase RNA, and resolve its 3-dimensional structure. This would provide critical information on how the hTERT protein specifically recognizes the telomerase RNA and the location of the different sites of RNA-protein contact.

TERT subunits do not contain previously identified RNA-binding domains of any pe. Thus, it has been proposed that the telomerase reverse transcriptase is a novel type RNA-binding protein. Results presented in Chapter 3 demonstrate that an hTERT olecule lacking amino acids 280 to 540 within the large N-terminal region was amatically altered in its ability to associate with the telomerase RNA subunit *in vitro*

(Bachand and Autexier, 2001). These results are consistent with other experimental observations indicating that the RNA-binding domain of TERTs from divergent species is located within the N-terminal region of the protein (Friedman and Cech, 1999; Lai *et al.*, 2001; Moriarty *et al.*, 2002). This is intriguing based on the strong divergence in length and nucleotide sequences within the telomerase RNA of different species. Interestingly, we can specifically detect the yeast telomerase RNA in anti-GST immunoprecipitates from extracts of yeast that expressed GST-hTERT (F. Bachand and C. Autexier; unpublished results). However, because these experiments were performed in a strain that expresses the yeast catalytic subunit, we cannot exclude the possibility of hTERT-Est2p interactions. It is possible that the RNA-binding domain(s) of TERTs fold into a similar general structure, albeit with some distinct differences intrinsic to the species-specific telomerase RNA. If the N-terminal region of TERTs (including motif T) is the domain specifying the recognition of the telomerase RNA, then it should be possible to generate a chimeric protein in which the N-terminal domain of human TERT is fused to the RT and C-terminus of *S. cerevisiae* TERT; this chimeric protein should specifically associate with the human telomerase RNA and polymerize human telomeric repeats.

The mechanistic role played by the C-terminal domain of TERTs remains largely ncharacterized. Though it is clear that the TERT C-terminal region is dispensable for :lomerase RNA binding, it is clearly required for the reconstitution of telomerase :tivity *in vitro* (Beattie *et al.*, 2000; Bachand and Autexier, 2001; Lai *et al.*, 2001). ompared to the RT motifs and the N-terminal region, the C-terminal region of TERTs ıows the lowest degree of conservation at the amino acid level. This low degree of

sequence conservation suggests that the C-terminal region might be involved in species-specific telomerase functions.

5.3 Role of the conserved hTR domains

Resolution of the vertebrate telomerase RNA secondary structure reveals five highly conserved regions: (i) a single-stranded template; (ii) a pseudoknot; (iii) the CR4-CR5 domain; (iv) the snoRNA H/ACA box sequence/structure; and (v) the CR7 domain. Several studies have analyzed the sequences and structures within the mammalian telomerase RNA that are important for telomerase activity. The major finding from this collection of studies is the distinction between the sequences/structure required for telomerase activity *in vitro* and *in vivo*. Whereas the H/ACA box and the CR7 domain of the vertebrate telomerase RNA are necessary for its cellular accumulation, these two sequences/structures domains are clearly dispensable for telomerase activity reconstituted *in vitro* in RRL.

The H/ACA snoRNA-binding proteins dyskerin, hNHP2, hNOP10, and hGAR1 associate with the H/ACA box motif of the human telomerase RNA (Mitchell *et al.*, 1999b; Dragon *et al.*, 2000; Pogacic *et al.*, 2001). Substitutions of H/ACA box sequences elements that are predicted to disrupt the association of the telomerase RNA ith H/ACA proteins causes the lack of accumulation of the telomerase RNA in cells /itchell *et al.*, 1999a; Martin-Rivera and Blasco, 2001). This indicates that like the /ACA box of snoRNAs responsible for site-specific modifications of rRNAs, the /ACA domain of hTR recruits a set of proteins that are absolutely required for stability *vivo*. However, do these H/ACA proteins remain associated to the telomerase RNA

once it is bound by hTERT? *In vitro* experiments suggest that the H/ACA box of hTR is not required for the association of hTR with hTERT (Bachand and Autexier, 2001). Consequently, it is likely that the H/ACA sequences/structures remain accessible for H/ACA proteins once hTR is bound by hTERT. Dyskerin is indeed found associated with catalytically active, fully assembled human telomerase (Mitchell *et al.*, 1999b). It is thus possible that H/ACA proteins are an integral part of the human telomerase complex via their association with the H/ACA box motif of hTR, and provide stability to the RNP in cells. As will be discussed in section 5.4, the H/ACA snoRNA-binding proteins could also be involved in human telomerase RNA 3'-end formation.

The H/ACA box domain is not sufficient to provide stability to hTR *in vivo*. Substitutions that disrupt the integrity of the conserved CR7 domain also eliminate the cellular accumulation of the mammalian telomerase RNA (Mitchell and Collins, 2000; Martin-Rivera and Blasco, 2001). The CR7 domain is not a conserved feature of H/ACA snoRNAs and is specific to vertebrate telomerase RNAs. Thus, the CR7 domain is likely to provide a protein-binding site for a telomerase-associated factor.

In addition to the single-stranded region that provides the nucleotide template necessary for telomeric DNA synthesis, the pseudoknot and the CR4-CR5 domains are the conserved RNA elements of the vertebrate telomerase RNA necessary for telomerase activity *in vitro*. What is the role of the telomerase RNA pseudoknot? Although the pseudoknot is critical for telomerase activity (Mitchell and Collins, 2000; Bachand and Autexier, 2001; Martin-Rivera and Blasco, 2001), it is not required for the association of TR with hTERT *in vitro* (Bachand and Autexier, 2001; Bachand *et al.*, 2001). RNAse otprinting analysis of the *T. thermophila* telomerase RNA in the presence and absence

of recombinant TERT also suggest that the pseudoknot is not a binding site of TERT (Sperger and Cech, 2001). Furthermore, recent observations suggest that the pseudoknot domain of telomerase RNAs is subject to conformational changes upon TERT binding (Sperger and Cech, 2001; Antal *et al.*, 2002). As the pseudoknot does not provide a high affinity hTERT-binding site, but is in close physical proximity to the template region (see Figure 1-5), this region of the vertebrate telomerase RNA could be involved directly in a catalytic step necessary for telomeric DNA polymerization.

The CR4-CR5 (see Figure 1-5) domain provides a high affinity-binding site for the protein catalytic subunit (Mitchell and Collins, 2000; Bachand and Autexier, 2001; Chen *et al.*, 2002)). In addition to the CR4-CR5 domain, hTR contains another hTERT-binding site located within the template/pseudoknot half (nt 1-200) of the RNA molecule (Mitchell and Collins, 2000; Bachand and Autexier, 2001). Consequently, an hTR variant substituted or deleted in the CR4-CR5 domain can still bind hTERT, yet this RNA is unable to support telomerase activity (Mitchell and Collins, 2000; Bachand and Autexier, 2001). These results indicate that the hTR CR4-CR5 domain not only provides an hTERT-binding site, but that it is also necessary for telomerase catalytic function. Further characterization will be needed to determine whether these two CR4-CR5-mediated functions (hTERT binding and telomerase activation) are independent. A milar observation was made for the reverse transcriptase (RT) of the Hepatitis B virus. he activity of the viral reverse transcriptase to prime DNA synthesis requires the ɪrmation of a RNP complex where a specific stem-loop structure within the pregenomic NA interacts with the viral RT (Wang *et al.*, 1994). At least two possibilities can be ɪvisaged regarding the mechanistic role of the CR4-CR5 domain in telomerase

activation. First, the binding of the CR4-CR5 domain of hTR with hTERT could induce a conformational change in the reverse transcriptase domain that could contribute to one or more catalytic steps such as: (1) nucleotide binding; (2) DNA primer binding or positioning; (3) template recognition; and (4) processivity. Alternatively, the CR4-CR5 stem-loop structure could be involved directly in a catalytic event of telomerase polymerization. A functional role for an RNA structure in yeast telomerase activity has been reported previously (Tzfati *et al.*, 2000).

5.4 A model for the human telomerase assembly pathway

The molecular machinery responsible for the assembly of a functional telomerase RNP in divergent organisms is likely to differ. This prediction is based on the knowledge that the telomerase RNA (TR) from ciliates, yeast, and mammals are of different classes; the ciliate TR is an RNA polymerase III transcript (Greider and Blackburn, 1989), whereas the yeast and mammalian telomerase RNAs are RNA polymerase II snRNA (Seto *et al.*, 1999) and snoRNA (Mitchell *et al.*, 1999a) transcripts, respectively. Indeed, some of the cellular factors involved in the processing, maturation, and RNP assembly for these different classes of RNAs are known to be different. We identified a novel human telomerase-associated protein that is likely to function in the biogenesis of mammalian lomerase (Chapter 4; (Bachand *et al.*, 2002). This protein, SMN (for survival of motor :uron), is well characterized for its role in the maturation of spliceosomal snRNAs (Will ıd Luhrmann, 2001). More recently, it was also reported to interact with the snoRNA-nding proteins Gar1 and Fibrillarin (Jones *et al.*, 2001; Pellizzoni *et al.*, 2001), ggesting the involvement of SMN in the assembly of snoRNP. There are currently a

growing number of experimental observations suggesting a connection between the nucleolus and vertebrate telomerase biogenesis (Mitchell *et al.*, 1999a; Mitchell *et al.*, 1999b; Lukowiak *et al.*, 2001; Bachand *et al.*, 2002; Etheridge *et al.*, 2002).

Illustrated in Figure 5-1 is a possible model for the pathway driving the assembly of functional mammalian telomerase RNPs. (1) This model starts with the synthesis of an hTR molecule. In contrast to many other snoRNAs that are generated from the intron of a spliced pre-mRNA, hTR is transcribed by RNA polymerase II from its specific gene locus (Hinkley *et al.*, 1998). As for the synthesis of other snoRNAs, the site of transcription of the *hTR* gene is unknown. (2) Experimental data indicate that hTR accumulates in the nucleolus of vertebrate cells (Mitchell *et al.*, 1999a; Narayanan *et al.*, 1999). If hTR is not directly synthesized within the nucleolus, such as ribosomal RNAs, a specific targeting machinery is necessary to transport hTR to the nucleolus. (3) hTR can also accumulate in Cajal bodies (CB) (Lukowiak *et al.*, 2001). (4) However, it is still unclear whether the localization of hTR to CB precedes its accumulation in the nucleolus or mediates a specific function. In the nucleolus, H/ACA snoRNP proteins could participate in hTR 3'-end formation and promote its accumulation. The binding of H/ACA snoRNP proteins to the ACA box of hTR (present 3-nt upstream from the mature 3'-end of vertebrate telomerase RNAs) could sterically block the progression of a 3'-to-5' xonuclease acting on an hTR precursor molecule, yielding a mature hTR snoRNP. (5) he catalytic subunit of human telomerase, hTERT, also localizes to the nucleolus 3achand *et al.*, 2002; Etheridge *et al.*, 2002). An hTERT mutant altered in its ability to ind hTR still localizes to the nucleolus (Etheridge *et al.*, 2002), suggesting that the :cumulation of hTERT to the nucleolus is independent of the telomerase RNA. As

SMN accumulates in the nucleolus, most likely via interactions with snoRNP proteins (such as Gar1 and Fibrillarin; (Pellizzoni *et al.*, 2001)), it is a good candidate for the recruitment of hTERT to nucleoli (Bachand *et al.*, 2002). (6) Alternatively, SMN could also recruit hTERT to Cajal bodies via interactions between p80coilin and SMN (Hebert *et al.*, 2001). However, the presence of hTERT in Cajal bodies has not yet been demonstrated. (7) In the nucleolus, hTERT could associate with a mature and fully processed hTR snoRNP: the nucleolar SMN complex could catalyze and/or stabilize the association of hTERT with hTR in a fashion similar to the stabilizing role of cytoplasmic SMN during the formation of the Sm protein complex onto U snRNAs (Fischer *et al.*, 1997; Meister *et al.*, 2001). Interestingly, the *in vitro* association of the *T. thermophila* TERT with the telomerase RNA induces specific conformational change in the RNA structure (Sperger and Cech, 2001). Similar changes in hTR structure upon association with hTERT could be stabilized via H/ACA snoRNP proteins and/or the SMN complex. Such an assembly pathway would generate a functional human telomerase RNP that is competent for telomere replication upon recruitment to chromosomes ends. Whether some or all of the H/ACA snoRNP proteins or SMN remains associated with the mature telomerase complex throughout the lifespan of the RNP will have to be determined.

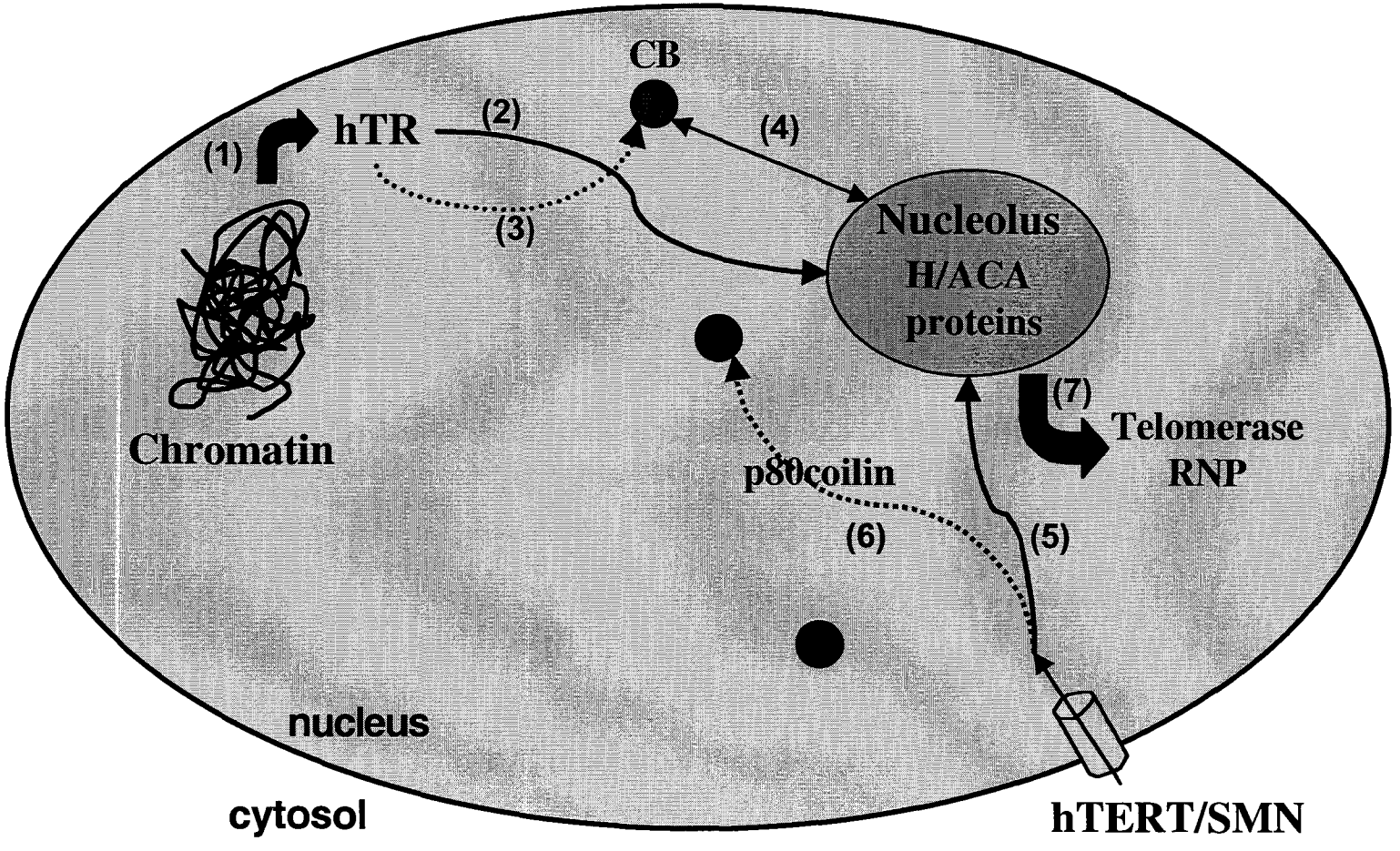

Figure 5-1. A model for the assembly of the human telomerase RNP

5.5 Concluding Remarks

The field of telomerase biology has been rapidly evolving in the last several years. There is a growing body of evidence suggesting that the inhibition of telomerase in human cancer cells could profoundly reduce their indefinite proliferation capacity. In the future, a three-dimensional structure of the human telomerase catalytic site will be required to generate potent and specific inhibitors. Identifying the proteins responsible for the recruitment of telomerase to mammalian telomeres will also represent a major advancement. Finally, a complete understanding of the molecular machinery associated with the assembly and regulation of telomerase RNPs in different species will be essential for the characterization of telomerase function.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The present work has focused on the biochemical and the functional characterization of the human telomerase reverse transcriptase (hTERT) protein, the human telomerase RNA (hTR), as well as the identification of human telomerase-associated proteins. These studies have been published in peer-reviewed journals. The major contributions of this work to original knowledge are summarized below:

1 The establishment of an original heterologous *in vivo* reconstitution system for human telomerase using the yeast *Saccharomyces cerevisiae*.

2 The finding that the reconstitution of human telomerase activity *in vitro* requires regions of hTERT that (i) are distinct from the conserved reverse transcriptase motifs; and (ii) bind nucleotides distal to the hTR template sequence.

3 The identification of two distinct regions of hTR that can independently bind hTERT.

4 The finding that the human telomerase RNA-binding site is located within amino acids 350 to 542 of the human telomerase reverse transcriptase.

5 The identification of a novel telomerase-associated protein: SMN.

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