NOTE TO USERS

Regulation of telomerase-specific catalytic functions by nucleic acid interactions and human telomerase reverse transcriptase N-terminal domains

by

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Your file Votre référence
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To Christine and my parents
And in memory of Mrs. Bayley

ABSTRACT

Telomerase is an unusual reverse transcriptase (RT) that catalyzes the *de novo* addition of telomeric DNA repeats to telomeres. Telomerase activity counteracts the progressive loss of telomeric DNA over successive rounds of DNA replication, and is important for the immortality of most eukaryotic cells. Telomerase is distinct from other RTs in that its catalytic subunit (TERT: telomerase reverse transcriptase) stably associates with a telomerase RNA (TR) component that contains a short template used to direct synthesis of telomeric repeats. Telomerase also exhibits a unique, repeat addition form of processivity that permits the addition of multiple telomeric repeats to a single substrate by repetitive reverse transcription of the template. The TERT proteins consist of a central region of RT-conserved motifs, which is flanked by extensive telomerase-specific N- and C-terminal sequences. The N terminus constitutes nearly half of the TERT protein, and is an excellent candidate site for telomerase-specific catalytic functions. Using a mutagenic approach, we investigated the contributions of human TERT N-terminal sequences to telomerase catalytic function and nucleic acid interactions. We found that the hTERT N terminus contains two RNA interaction domains, RID1 and RID2. RID1 was functionally and physically separable from the remainder of hTERT, and may constitute an hTERT polymerase accessory domain. We investigated the catalytic function of two RID1- and RID2-interacting regions in the human TR, the pseudoknot/template domain and the P6.1 helix. RID1-pseudoknot/template domain interactions were essential for repeat addition processivity, and RID2-P6.1 interactions mediated telomerase assembly and were required for basic polymerase function. Repeat addition processivity is thought to be partly dependent on an anchor site(s) in TERT that stabilizes telomerase-DNA interactions. We found that RID1 mutations also reduced the affinity of human telomerase for its DNA substrate and caused an anchor site-type defect in repeat addition processivity. Human telomerase is a multimer that contains two interacting hTRs and is predicted to contain two hTERTs. We also investigated sequences that were important for physical and functional hTERT-hTERT and hTR-hTR interactions, and determined that multimerization may be required for the repeat addition processivity of human telomerase.

RÉSUMÉ

La télomérase est une transcriptase inverse (RT) inhabituelle qui catalyse l'addition de séquences d'ADN télomériques répétées aux télomères. L'activité de la télomérase prévient la perte progressive des séquences d'ADN télomériques au cours des cycles successifs de réplication de l'ADN; par conséquent, la télomérase est importante pour l'immortalité de la plupart des cellules eucaryotes. La télomérase se différencie d'autres RTs au niveau de sa composante catalytique (TERT: telomerase reverse transcriptase), qui s'associe de manière stable à une composante ARN (TR: telomerase RNA); une courte portion de TR agit comme molécule matrice lors de la synthèse de l'ADN télomérique. La télomérase se caractérise aussi par une unique forme répétitive de la processivité qui permet l'addition de plusieurs répétitions d'ADN télomériques à un substrat unique grâce à la transcription inverse répétée de la molécule matrice. Les protéines TERTs sont constituées d'une région centrale arborant des motifs RT qui sont conservés parmi les membres de la famille des transcriptases inverses (RT motifs), et des larges régions N- et C-terminales qui sont uniques aux membres de la famille TERT. La région N-terminale, qui constitue presque la moitié de TERT, constitue une excellente candidate pour les fonctions catalytiques spécifiques à la télomérase. En utilisant des techniques de mutagénèse, nous avons examiné les contributions des séquences N-terminales de la TERT humaine aux fonctions catalytiques de la télomérase, et l'association de la protéine aux acides nucléiques. Nous avons trouvé que la région N-terminale de hTERT contient deux domaines qui s'associent à l'ARN, RID1 et RID2. RID1 était séparable fonctionnellement et physiquement du reste de hTERT, et peut constituer un domaine accessoire de la polymérase. Nous avons analysé la fonction catalytique des deux régions chez la composante ARN humaine (hTR) qui s'associent à RID1 et RID2, un domaine qui contient le pseudonoeud et la molécule matrice (le domaine pseudonoeud /matrice), et l'hélice P6.1. L'association entre RID1 et le domaine pseudonoeud /matrice était essentielle pour la processivité répétitive, et l'association entre RID2 et l'hélice P6.1 était nécessaire pour la fonction de base de la polymérase et l'assemblage de la télomérase. Il a été proposé que la processivité répétitive soit partiellement régulée par un/des site(s) d'ancrage chez TERT qui stabilise l'association de la télomérase à l'ADN. Nous avons trouvé que les mutations chez RID1 ont réduit aussi l'affinité de la télomérase humaine envers son substrat ADN, et ont causé un défaut de la processivité répétitive qui

ressemblait à un défaut de la fonction de site d'ancrage. La télomérase humaine multimérise, et contient deux hTRs qui agissent l'un sur l'autre, et contient aussi probablement deux hTERTs. Nous avons examiné les séquences qui étaient importantes physiquement et fonctionnellement pour les actions réciproques chez hTERT et chez hTR, et nous avons déterminé que la multimérisation peut être nécessaire pour la processivité répétitive chez la télomérase humaine.

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LIST OF ABBREVIATIONS

Abbreviation	Term
A	adenine
ACD	adrenocortical dysplasia
AD	autosomal dominant
Akt	gene product of AKT8 murine retrovirus
ALT	alternative lengthening of telomeres
APB	ALT-associated PML body
A-T	ataxia telangiectasia
BIR	break-induced replication
BLM	Bloom syndrome helicase
bp	base pair
BRCA	breast cancer-associated
BSA	bovine serum albumin
C	cytosine
C-	carboxy
CAB	Cajal body
c-Abl	cellular orthologue of Abelson murine leukemia virus gene product
C. albicans	*Candida albicans*
CDC	cell division cycle
cDNA	complementary DNA
c-myc	cellular orthologue of myelocytomatosis virus gene product
CP	ciliate-specific
CR	conserved region
CRM1	chromosomal region maintenance protein 1
DAT	dissociates activities of telomerase
DBD	DNA-binding domain
Ddc1p	DNA damage checkpoint protein 1
DKC	dyskeratosis congenita
D-loop	displaced loop
DNA	deoxyribonucleic acid
DNA-PK_{cs}	DNA-dependent protein kinase catalytic subunit
DNA pol	DNA polymerase
Dna2p	DNA replication protein 2
DSB	double-stranded DNA break
dsDNA	double-stranded DNA
E. coli	*Escherichia coli*
ECTR	extra-chromosomal telomeric repeat
EMSA	electrophoretic mobility shift assay
ERCC1	excision repair cross-complementing protein 1
EST	ever-shorter telomeres
Exo1p	exonuclease 1 protein
FISH	fluorescence in situ hybridization
γ-H2AX	phosphorylated histone H2AX
G	guanine
GAPDH	glyceraldehyde phosphate dehydrogenase
GAL4	galactose regulatory element 4
GFP	green fluorescent protein
GAR1	glycine- and arginine-rich protein 1
GST	glutathione-S-transferase

HAT	histone acetyltransferase
HDAC	histone deacetylase
HeT-A	heterochromoatin transposable element A
HIV-1	human immunodeficiency virus-1
hnRNP	heterogeneous nuclear ribonucleoprotein
HP1	heterochromatin protein 1
HR	homologous recombination
HSP	heat shock protein
hTERT	human telomerase reverse transcriptase
hTR	human telomerase RNA
HUS1	hydroxyurea sensitivity protein 1
IC	internal control
IFD	insertion in fingers domain
kb	kilobase
kDa	kilodalton
K. lactis	*Kluyveromyces lactis*
L22	ribosome large subunit protein 22
LTR	long terminal repeat
MDa	megadalton
Mec3p	mitosis entry checkpoint protein 3
MRE11	Meiotic recombination protein 11
mRNA	messenger RNA
MRX	MRE11, RAD50, XRS2
myb	myeloblast-transforming avian leukemia virus gene product
N-	amino
NMR	nuclear magnetic resonance
NBS1	Nijmegen breakage syndrome protein 1
NHEJ	non-homologous end-joining
NHP2	non-histone protein 2
NFκB	nuclear factor κB
NOP10	nucleolar protein 10
nt	nucleotide
OB fold	oligonucleotide/oligosaccharide-binding fold
PAGE	polyacrylamide gel electrophoresis
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	population doubling
PHAX	phosphorylated adaptor for RNA export
PINX1	PIN2-interacting protein X1 (TRF1-interacting protein 1)
PIP1	POT1-interacting protein 1
PKC	protein kinase C
PML	promyelocytic leukemia
Pol	polymerase
POT1	protection of telomeres 1
RAD	radiation-sensitive
RAP	repressor and activator protein
R.A.P.	repeat addition processivity
RCR	rolling circle replication
RecQ	replication protein Q
RID	RNA interaction domain

RNA	ribonucleic acid
RNase	ribonuclease H
RNP	ribonucleoprotein
RPA	replication protein A
RRL	rabbit reticulocyte lysates
rRNA	ribosomal RNA
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
scaRNA	small Cajal body RNA
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
Sgs1p	slow growth suppressor protein 1
SMN	survival motor neuron
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
S. pombe	*Schizosaccharomyces pombe*
Src	sarcoma virus
ssDNA	single-stranded DNA
SV40	simian virus 40
T	thymine
T.A.	telomerase activity
TART	telomere-associated retrotransposon
Taz	telomere-associated in *Schizosaccharomyces pombe*
TEBP	telomere end-binding protein
TEP	telomerase-associated protein
Ten1p	telomere end protection protein 1
TERT	telomerase reverse transcriptase
TGFβ	transforming growth factor β
TIF	telomere-induced DNA damage foci
TIN2	TRF1-interaction nuclear factor 2
TLC1	telomerase RNA component
TMG	trimethylguanosine
TR	telomerase RNA
TRAP	telomeric repeat amplification protocol
TRD	telomere rapid deletion
TRE	template recognition element
TRF	telomere restriction fragment
TRF1	TTAGGG repeat binding factor 1
TRF2	TTAGGG repeat binding factor 2
Ty	transposable element in yeast
VSR motif	vertebrate-specific RNA-binding motif
WRN	Werner syndrome helicase/exonuclease
WS	Werner syndrome
WT	wild-type
X-linked	X chromosome-linked
XRCC3	X-ray repair cross-complementing protein 3
XRS2	X-ray sensitivity protein 2

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PREFACE

This Ph.D. thesis is written in accordance with guidelines for thesis preparation of the Faculty of Graduate and Postdoctoral Studies of McGill University. I have exercised the option of writing a manuscript-based thesis, which includes two previously published original manuscripts (Chapters 2 and 3), and a third original manuscript that has been accepted for publication (Chapter 4). Each of these chapters contains it own abstract, introduction, materials and methods, results, discussion and acknowledgements sections. Prefaces are included at the beginnings of each of Chapters 2 to 4 in order to introduce each manuscript and describe its thematic relationship to preceding material. A comprehensive review of the literature and a summary and general discussion are presented in Chapters 1 and 5, respectively. References for all chapters are consolidated into a single section that is found at the end of this thesis.

Published and accepted manuscripts included in this thesis

Chapter 2: **Moriarty, T. J.**, Huard, S., Dupuis, S., and Autexier, C. (**2002**). "Functional multimerization of human telomerase requires an RNA interaction domain in the N terminus of the catalytic subunit."
- Published in: **Mol Cell Biol** *22*, 1253-1265.
- *Permission to reprint granted by the American Society for Microbiology Journals Department.*

Chapter 3: **Moriarty, T. J.**, Marie-Egyptienne, D. T., and Autexier, C. (**2004**). "Functional organization of repeat addition processivity and DNA synthesis determinants in the human telomerase multimer."
- A shorter version of the manuscript provided in Chapter 3 was published in: **Mol Cell Biol** *24*, 3720-3733.
- *Permission to reprint granted by the American Society for Microbiology Journals Department.*

Chapter 4: **Moriarty, T. J.**, Ward, R. J., Taboski, M. A. S., and Autexier, C. (**2005**). "An anchor site-type defect in human telomerase that disrupts telomere length maintenance and cellular immortalization."
- Accepted for publication in: **Mol Biol Cell**. April 19, 2005. A shorter version of the manuscript provided in Chapter 4 is currently in press.
- *Permission to reprint granted by the American Society for Cell Biology.*

Contributions of authors

The candidate performed the majority of the research presented in this thesis and wrote all of the manuscripts with support from Dr. Autexier. All studies were conducted under the supervision of Dr. Autexier. The contributions of other authors to this work are described as follows. In **Chapter 2**, Sylvain Huard performed approximately 50% of the work associated with cloning of yeast expression plasmids, growth of yeast cultures, preparation of yeast extracts and immunoblotting of yeast extracts (Figure 2.2B). Sophie Dupuis constructed the pet28-GSThTERT D868N RRL expression plasmid used in experiments shown in Figure 2.5B. In **Chapter 3**, Delphine T. Marie-Egyptienne performed half of the hTR dimerization assays shown in Figure 3.5F and G and Figure 3.6B and C. In **Chapter 4**, Ryan J. Ward performed telomere blots and Michael A.S. Taboski performed RT-PCR analysis of hTERT and GAPDH mRNA expression shown in Figure 4.1.

Contributions to original knowledge

The studies presented in this thesis concern the biochemical and functional characterization of the human telomerase catalytic mechanism, and have been published or accepted for publication in peer-reviewed journals. The major, original contributions of these studies to the field of telomerase research include:

1. The development of a quantitative RNA binding assay used for measuring hTERT-hTR interactions (Chapter 2).
2. The identification of distinct hTR interaction domains (RID1 and RID2) in the hTERT N terminus (Chapter 2), and characterization of their catalytic functions and interaction sites in hTR (Chapter 3).
3. The identification of an essential role for RID1 in repeat addition processivity (Chapter 3).
4. Functional mapping of the domain boundaries of hTERT (Chapter 3).
5. The identification of a role for hTR-hTR and hTERT-hTERT interactions in repeat addition processivity (Chapter 3).
6. The identification of RID1 as a mediator of human telomerase anchor site functions (Chapter 4).

Other publications/contributions of the candidate

In addition to the papers included in this thesis, the candidate contributed to the following published or submitted book chapter and original research studies:

2005 **Moriarty, T. J.**, Marie-Egyptienne, D.T. and Autexier, C.
"Regulation of 5' template usage and incorporation of non-cognate nucleotides by human telomerase."
- Original research manuscript submitted for publication to the journal **RNA**, April 25, 2005.

2005 Lue, N.F., Bosoy, D., **Moriarty, T.J.**, Autexier, C., Altman, B. and Leng, S.
"Telomerase can act as a template- and RNA-independent terminal transferase."
- Original research manuscript submitted for publication to the journal **Proc Natl Acad Sci USA**, March 18, 2005.

2003 Huard, S., **Moriarty, T. J.**, and Autexier, C.
"The C terminus of the human telomerase reverse transcriptase is a determinant of enzyme processivity."
- Original research study published in: **Nucleic Acids Res** *31*, 4059-4070.

2003 **Moriarty, T. J.**, and Autexier, C.
"Human, mouse and yeast telomerases."
- Book chapter published in: **Telomeres, telomerase and cancer**., G. Krupp, and R. Parwaresch, eds. (Kluwer Academic/Plenum Publishers).

2002 **Moriarty, T. J.**, Dupuis, S., and Autexier, C.
"Rapid upregulation of human telomerase activity in human leukemia HL-60 Cells treated with clinical doses of the DNA-damaging drug etoposide."
- Original research study published in: **Leukemia** *16*, 1112-1120.

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I thank members of the Autexier laboratory who assisted with my research projects and contributed to my training, especially Ibtissem Triki, Sophie Dupuis, Sylvain Huard, Tamara Marie-Egyptienne, Ryan Ward, Mike Taboski, Annie Dulude and Pooja Jain. Thank you to all past and present Autexier lab members, including Maria Antonietta Cerone and Johans Fakhoury for critical discussion of data and manuscripts and for ensuring that daily life in the lab was stimulating. I thank many research assistants, students, post-doctoral fellows and scientists in my department and at the Lady Davis Institute who have contributed technical and intellectual training and moral support, especially Drs. Andréa LeBlanc, Matthias Götte, Omar Tounekti and Jocelyn Côté, members of my advisory committees, and Dr. Eugenia Wang and her former students Richard Marcotte, Emmanuel Petroulakis and Chantale Lacelle. Thank you also to our chief Canadian competitors, Drs. Lea Harrington and Tara Beattie, for their very non-competitive willingness to share data, ideas and advice.

Salary support during my Ph.D. was provided by a doctoral research scholarship from the Canadian Institutes of Health Research (CIHR), and a McGill Graduate Studies Fellowship. Funding to attend international conferences was provided by the Bloomfield Centre for Research in Aging, Cold Spring Harbor Laboratories, the National Cancer Institute of Canada, the Keystone Symposia Scholarship Fund and CIHR.

Finally, and most importantly, I thank Christine, my mother, father and brothers, and Mrs. Bayley and her family for their support, pride and love through everything . . . and for their unerring ability to find the comical in tragedies big and small. Only they know how close I've come to quitting, and they are, without any doubt, the reason that I didn't. As it's been said before: "Without you, I'm nothing." I love you.

1. CHAPTER 1: Introduction and literature review

1.1. General introduction

The lagging strand DNA synthesis machinery cannot completely replicate both ends of linear DNAs, which results in the progressive shortening of the telomeres, or ends, of linear DNA replicons with each successive round of DNA replication (reviewed in Cech 2004). Telomere shortening eventually results in loss of protective telomeric sequences and subtelomeric coding sequences, and is incompatible with cell survival and proliferation, and replicative immortality. Replicative immortality is common among unicellular organisms such as yeasts and ciliates (reviewed in Counter 1996). In contrast, many of the somatic cells of complex organisms such as humans are not immortal (reviewed in Engelhardt and Martens 1998). However, in complex organisms telomere shortening must be prevented or reduced in germline cells and progenitor cells of highly proliferative tissues, to permit reproductive immortality and tissue self-renewal. All organisms and viruses with linear replicons have developed mechanisms to counteract replicative telomere shortening (reviewed in Kobryn and Chaconas 2001). In most eukaryotes the solution to this problem is the enzyme telomerase.

Telomerase is a unique and evolutionarily ancient reverse transcriptase that counteracts the end replication problem by adding telomeric DNA sequences to the ends of chromosomes in a *de novo* fashion (reviewed in Cech 2004). It is expressed very weakly or not at all in the majority of somatic tissues from adult humans, though it is active in germline cells and highly proliferative tissues such as epithelia and the immune system (reviewed in Engelhardt and Martens 1998). Haploinsufficient telomerase mutations are found in patients with the premature aging syndrome dyskeratosis congenita, who die as a result of bone marrow failure (reviewed in Bessler et al. 2004). Telomerase activity is upregulated in 85-90% of all human cancers, consistent with their enhanced proliferative capacity (reviewed in Shay and Bacchetti 1997). As an important regulator of cellular immortality, telomerase is an attractive target for the design of therapeutic agents intended to limit the proliferation of cancer cells (reviewed in Ulaner 2004). Telomerase is also being used to extend the *in vitro* proliferative life span of various human somatic tissues in the hopes that such tissues might eventually serve as sources for donor-specific transplants (reviewed in Ulaner 2004).

The general subject of this thesis is the biochemical and functional characterization of the human telomerase catalytic mechanism. The specific aim of my doctoral work has been to characterize the roles of telomerase-specific N-terminal sequences of the human telomerase reverse transcriptase (hTERT) in the telomerase catalytic mechanism and in human telomerase RNA (hTR) and DNA substrate interactions. This work may be relevant for the rational design of clinical telomerase inhibitors, and is also important for our understanding of the functional relationship between telomerase and other nucleic acid polymerases. The majority of this thesis discusses the catalytic mechanism of the telomerase enzyme itself; however, telomerase does not act in isolation in the cell. Little is known about how human telomerase functions at telomeres *in vivo.* In the following literature review I present an account of aspects of telomere and telomerase structure and function in diverse eukaryotes that may offer insights into the mechanism of human telomerase action *in vivo* and *in vitro.*

1.2. Telomeres

Telomeres, most simply, are the ends of linear chromosomes or linear DNA replicons. They generally consist of repetitive DNA that contains no coding sequences, and they form specialized structures that distinguish these termini from the ends of broken DNA molecules (reviewed in Pryde et al. 1997; Wellinger and Sen 1997).

1.2.1. Organization

Telomeres can be divided into three regions: 1) sub-telomeric or telomere-associated sequences; 2) repetitive sequences; and 3) protective teminal structures. Sub-telomeric, or telomere-associated DNA sequences are often composed of a mosaic of telomeric and non-telomeric repeat sequences interspersed with and/or adjacent to genes that are frequently involved in recombination events. The repetitive region of telomeric DNA varies in length in different replicons. In eukaryotic chromosomes, the length of this telomeric array is maintained within a fixed range that is specific to each organism. Multicellular eukaryotes tend to have longer telomeres (3-30 kb in humans; >60-100 kb in mice) than unicellular eukaryotes (20-30 bp in *Oxytricha* and *Euplotes*) (Klobutcher et al. 1981; Moyzis et al. 1988; de Lange et al. 1990; Kipling and Cooke 1990) .

1.2.2. DNA sequences

The ends of all linear DNA replicons studied to date contain repetitive, conserved, non-coding DNA sequences that are essential for telomere structure and function. Telomeric DNA sequences in most eukaryotic chromosomes except those of *Dipteran* insects such as *Drosophila* are very similar to one another, suggesting both a common evolutionary origin and mechanism of synthesis (reviewed in Biessmann and Mason 1997; Wellinger and Sen 1997). The 3' strand of most eukaryotic telomeres consists of homogeneous tandem arrays of short, 6 to 8 nucleotide (nt), G/T-rich DNA repeats; in humans and other vertebrates this repeat sequence is TTAGGG (Moyzis et al. 1988; Meyne et al. 1989). The 3' strand is also referred to as the G strand because it rarely contains cytosine residues. The 5' C strand is composed of sequences complementary to the G strand. A few eukaryotes such as the fungal yeasts contain heterogeneous (*Saccharomyces*) or longer, 20-25 nt (*Kluyveromyces, Candida*) telomeric repeats, though these sequences are always T/G-rich (McEachern and Blackburn 1994). *Drosophila* telomeres are composed of the sequences that constitute two retrotransposable elements, *HeT-A* and *TART*; their 3' terminal sequences are also predicted to be T/G-rich (Levis et al. 1993; Pardue and DeBaryshe 2003).

In eukaryotes, the preponderance of T and G residues in the 3' strand of terminal telomeric sequences may reflect similar binding properties among telomere-binding proteins from diverse organisms that evolved from a common eukaryotic ancestor. It has also been proposed that the G-rich sequences of eukaryotic telomeres may play a role in mitotic and meiotic chromatid pairing, since G-rich telomeric sequences exhibit unusual cohesive properties that permit the formation of a range of higher-order DNA structures (reviewed in Wellinger and Sen 1997). Some of the higher-order structures that can form from eukaryotic telomeric sequences *in vitro* include G-G base pairs and four-stranded structures called G-quadruplexes. In theory, such non-canonical base interactions could contribute to a "self-recognition" process that facilitates chromatid pairing; however, such structures have only been detected *in vivo* in *Stylonichia lemnae* macronuclei (Schaffitzel et al. 2001).

1.2.3. Protective terminal structures

Telomeres form structures that protect the ends of the DNA molecule and distinguish them from double-stranded DNA breaks (DSBs); in most eukaryotes, telomeres are packaged into a t-loop structure (Figure 1.1). One exception is in the macronucleus of certain ciliates, where telomeres may instead be capped by a heterodimeric protein complex (section 1.2.4.1.1) (Horvath et al. 1998; Murti and Prescott 1999). The terminal DNA structure of *Drosophila* telomeres is unknown, but is protected or capped by heterochromatin protein 1 (HP1), which exhibits a preferential affinity for binding single-stranded *HeT-A* DNA (Perrini et al. 2004). The ends of the linear replicons of adenoviruses, certain eukaryotic plasmids, RNA viruses and bacteriophages, and *Streptomyces* bacteria are capped by a covalently bound terminal protein (Salas 1991; Casjens 1999). The terminal inverted repeat sequences of some mitochondrial DNAs, and poxvirus, parvovirus, bacteriophage and *Borrelia* replicons form a hairpin structure (Casjens 1999; Kobryn and Chaconas 2001). Interestingly, recent evidence indicates that telomeres of the eukaryote *Saccharomyces cerevisiae* (*S. cerevisiae)* can also form inverted repeat-nucleated terminal palindromes in the absence of telomerase- or recombination-based telomere maintenance mechanisms (section 1.3.1), though it is unknown if these palindromes form terminal hairpins (Maringele and Lydall 2004). Therefore, telomeres can form a variety of protective structures in different linear replicons.

1.2.3.1. 3' overhangs

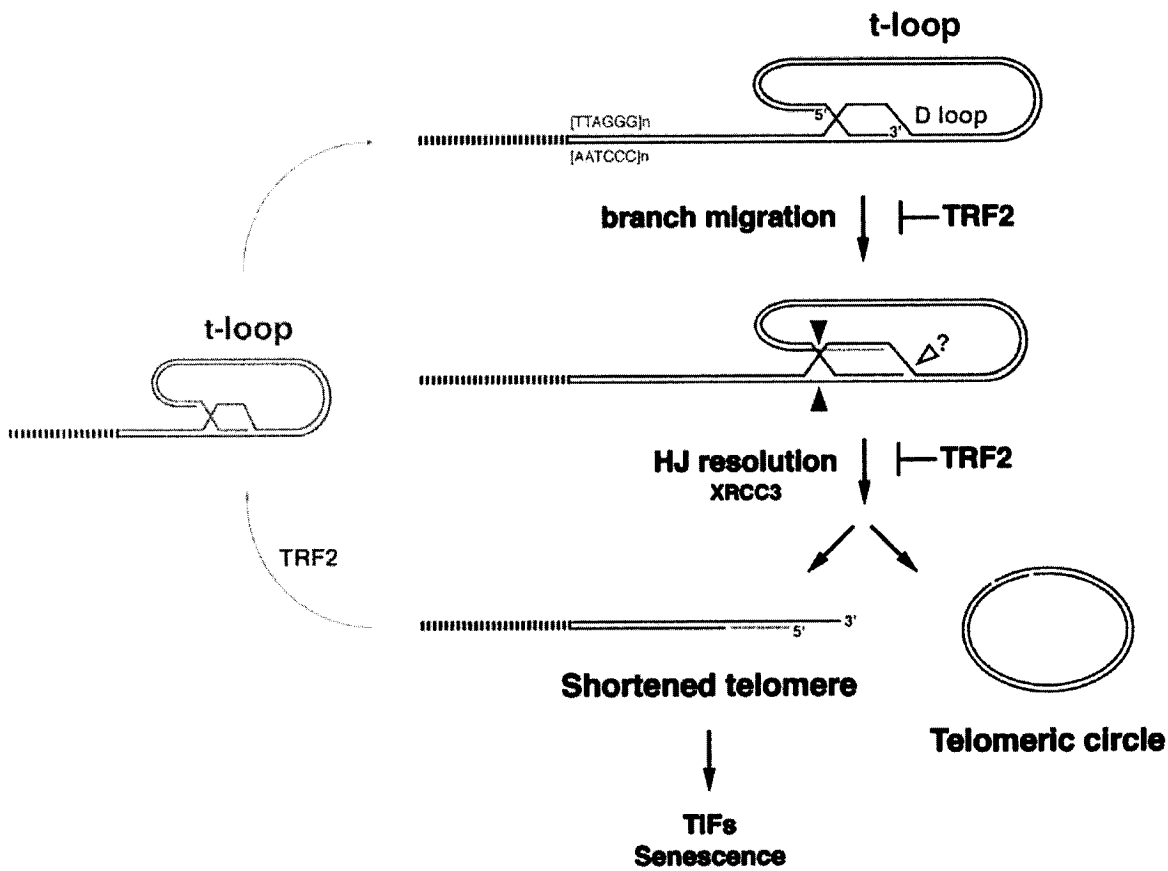
At the extreme end of most eukaryotic chromosomes, the 3' G-rich DNA strand extends beyond the 5' strand to form a 3' single-stranded DNA (ssDNA) overhang (reviewed in Wei and Price 2003). The 3' overhang is thought to be important for the formation of t-loops, and is the substrate for telomerase-mediated telomere elongation (sections 1.2.3.2 and 1.5) (Figure 1.1).

G-rich overhangs are thought to result from nuclease-mediated processing of blunt DNA ends, the inherent end replication problem of lagging strand DNA synthesis, telomerase-mediated extension of the 3' strand, or a combination of these factors. The length of 3' overhangs in slime mold (*Didymium*), ciliated protozoa (*Tetrahymena*, *Euplotes*, *Oxytricha*), yeast (*S. cerevisiae*) and plants (*Silene*, *Arabidopsis*) ranges from 8-

Figure 1.1: Proposed t-loop structure and speculative model of t-loop homologous recombination

(Top) Proposed structure of t-loops. **(Middle)** Model of t-loop homologous recombination (t-loop HR) (section 1.2.3.3). Branch migration at the strand invasion site of the telomere terminus is predicted to result in the formation of a Holliday junction. Two steps are proposed to lead to t-loop deletion in human cells: 1) XRCC3-dependent cleavage of the C strand at two positions by Holliday junction resolvase (green arrowheads); 2) nicking of the D-loop by an unknown nuclease (open arrowhead). **(Bottom)** The products of t-loop deletion are a shortened telomere and a relaxed telomeric circle. The shortened telomere might reform a small t-loop in a TRF2-dependent manner (left) or, if the deletion is too extensive, might activate a DNA damage response, and induce senescence; such damage may be responsible for the formation of telomere-induced foci (TIFs) detected in cells that undergo t-loop HR. In telomerase-negative cells that maintain telomeres by an alternative mechanism (ALT cells), the telomeric circles resulting from t-loop homologous recombination (HR) could function as a template for rolling circle replication and allow telomerase-independent telomere maintenance (section 1.3.2).

This figure is adapted from a an article published by Wang and coauthors in the journal Cell (Wang et al. 2004). *Copyright permission granted.*


t-loop
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5'
3'
D loop
branch migration
TRF2
t-loop
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HJ resolution
XRCC3
TRF2
TRF2
5'
3'
Shortened telomere
Telomeric circle
TIFs
Senescence

30 nt (Klobutcher et al. 1981; Pluta et al. 1982; Henderson and Blackburn 1989; Riha et al. 2000; Wei and Price 2003; Larrivee et al. 2004). This corresponds well to the ssDNA overhang length that results from removal of the most terminal RNA primer during lagging strand DNA synthesis (section 1.3) (reviewed in Wellinger and Sen 1997). However, G-rich overhangs are present at both telomeres in most organisms (reviewed in Wei and Price 2003), suggesting that 3' overhangs are not generated exclusively by the deficits inherent to lagging strand DNA synthesis. In *Euplotes* and *S. cerevisiae*, overhangs of >30 nt appear during *de novo* telomere formation or late stages of DNA replication (Roth and Prescott 1985; Wellinger et al. 1993). At least in yeast, the formation of these long overhangs is not dependent on telomerase activity (Dionne and Wellinger 1996). Furthermore, G-rich overhangs are present in telomerase-negative human and plant cells, implying that telomerase-mediated extension of telomeres is not sufficient for generation of this structure (Wright et al. 1999; Riha et al. 2000). 3' overhangs in mammals are 75-300 nt in length, and are present on both telomeres throughout the cell cycle, though overhang lengths in telomeres generated by leading or lagging strand DNA synthesis may be different (Makarov et al. 1997; McElligott and Wellinger 1997; Wright et al. 1997; Wright et al. 1999).

Collectively, these observations imply that lagging strand synthesis-dependent shortening of 5' strands and telomerase-mediated extension of 3' strands cannot completely account for the presence of G-rich overhangs at both ends of all eukaryotic chromosomes studied to date. There is some evidence supporting a telomeric C stand resection mechanism in telomerase-negative yeasts and *Tetrahymena*; however, identifying C strand-resecting nucleases has proved to be difficult until recently, perhaps due to redundancy or cooperative modes of action among several different nucleases (Dionne and Wellinger 1996; Jacob et al. 2001; Tomita et al. 2004). In *S. cerevisiae*, the MRX nuclease complex (Mre11p, Rad50p, Xrs2p) is required for generation of constitutive 3' overhangs, and other candidates for C-strand resectioning/remodeling include Exo1p, Dna2p and the RAD24 group (Rad24p, Rad17p, Mec3p and Ddc1p) (Maringele and Lydall 2002; Larrivee et al. 2004; Tomita et al. 2004 (reviewed in Lydall 2003). Interestingly, recent evidence from *Tetrahymena* shows that both the telomeric G and C strands are precisely cleaved by unidentified nucleases to reveal specific sequences; the specificity of this cleavage is dependent on the telomerase enzyme, though nuclease

activity itself is telomerase-independent (Jacob et al. 2003). The terminal nucleotides of the human telomeric C strand are also precisely defined, suggesting that a sequence-specific nuclease is responsible for its processing; the identity of the terminal nucleotides in the telomeric G strand is more variable, though the presence of telomerase activity influences terminal base composition (Sfeir et al. 2005). Therefore, though most eukaryotic telomeres have been shown to contain G-rich 3' overhangs, the mechanisms of overhang generation remain unclear, and are likely to be complex.

1.2.3.2. T-loops

Why would eukaryotic cells expend so much energy in the production of ss overhangs at both ends of the chromosome? One hypothesis is that 3' overhangs make an essential contribution to the formation of telomeric t-loop structures (Figure 1.1). T-loops are formed when the telomeric ssDNA overhang invades duplex telomeric DNA with similar sequences, generating a structure that resembles a DNA replication intermediate found in stalled replication forks (Griffith et al. 1999). T-loop formation is dependent on telomere-specific proteins that bind duplex regions of telomeric DNA repeats adjacent to the junction between telomeric ssDNA and double-stranded DNA (dsDNA) (section 1.2.4.2.1) (Griffith et al. 1999; Stansel et al. 2001; Tomaska et al. 2004). However, nucleic acid interactions also play a significant role in t-loop structure. It is probable that 3' overhang-mediated strand invasion contributes to t-loop formation, since *E. coli* single-strand binding protein can bind to a displaced (D)-loop strand at the site where the ssDNA overhang is thought to invade duplex DNA (Griffith et al. 1999). T-loops can also be observed by electron microscopy following psoralen cross-linking and deproteinization of DNA, indicating that DNA base-pairing interactions are important in the t-loop structure (Griffith et al. 1999). Furthermore, t-loops cannot be formed *in vitro* from DNA substrates containing blunt ends, 5' overhangs, or 3' overhangs containing non-telomeric sequences at the junction between dsDNA and ssDNA, whereas t-loops can be generated *in vitro* from DNA molecules composed of natural telomeric sequences and a telomeric 3' overhang (Griffith et al. 1999; Stansel et al. 2001; Tomaska et al. 2004). Therefore, it is clear that at least one function of the ubiquitous 3' telomeric overhang is to contribute to the formation of protective t-loop structures.

T-loop structures have been identified by electron microscopy at the chromosomal telomeres of organisms from diverse eukaryotes: vertebrate (human, mouse, chicken), yeast (*Schizosaccharomyces pombe: S. pombe*), trypanosome (*Trypanosoma brucei*), ciliate (*Oxytricha fallax*), and plant (*Pisum sativum*) (Griffith et al. 1999; Murti and Prescott 1999; Munoz-Jordan et al. 2001; Cesare et al. 2003; Nikitina and Woodcock 2004; Tomaska et al. 2004). The structures of *Drosophila* and *S. cerevisiae* telomeres are unknown. T-loops have been identified at both ends of the same chromosome in trypanosomes, whose chromosomes are short enough for electron microscopic analysis (Munoz-Jordan et al. 2001). This observation indicates that both leading and lagging strand telomeres can form t-loops; however, it is unknown whether t-loops are present on both telomeres of all eukaryotic chromosomes. T-loops and t-loop-like structures have also been found at the ends of linear mitochondrial DNAs from *Tetrahymena* and the yeast *Candida parapsilosis* (Goldbach et al. 1979; Tomaska et al. 2002). Interestingly, t-loops are confined to the long telomeres of the germline DNA of hypotrichous ciliates (micronucleus) (*Oxytricha*), and are absent from the telomeres of the ciliate macronuclear nucleus, which is generated during the vegetative phase of the ciliate life cycle (Murti and Prescott 1999). Instead, the extremely short macronuclear telomeres, which like micronuclear telomeres end in 3' overhangs, are capped by a tightly bound complex of micronuclear telomere-specific proteins (Horvath et al. 1998; Murti and Prescott 1999). These observations suggest that a minimal telomere length may be required for the formation of t-loops. Though the sizes of mammalian and *Oxytricha* t-loops are proportional to telomere length, trypanosome t-loops are much smaller than their telomere lengths, indicating that t-loop formation does not necessarily involve all of the repetitive DNA in telomeres (Griffith et al. 1999; Murti and Prescott 1999; Munoz-Jordan et al. 2001; Cesare and Griffith 2004).

One thing that is not clear is whether t-loops are present at all eukaryotic telomeres at all times. Native t-loops have been isolated from gently lysed mouse, chicken and *Oxytricha* cells without prior cross-linking, confirming that t-loops are a natural structure, and are not an artifact of the psoralen cross-linking methods that have usually been employed for their visualization (Murti and Prescott 1999; Nikitina and Woodcock 2004). However, psoralen cross-linking greatly enhances the yield of t-loops, suggesting that these structures may be labile in cells and/or easily damaged during isolation (Griffith et

al. 1999; Wei and Price 2003). T-loops have been observed at only 15-40% of psoralen cross-linked human telomeres (Griffith et al. 1999). Though this observation might be explained by the fragility of t-loop structures, it is also likely that unwinding of t-loops may be necessary at some point(s) during the cell cycle to facilitate both telomerase-mediated elongation of the 3' strand and replication of telomeric DNA (Griffith et al. 1999; Wei and Price 2003).

1.2.3.3. t-loops and telomere rapid deletions

Recent evidence suggests that the t-loop structure may also provide an explanation for the poorly understood phenomenon of telomere rapid deletion (TRD), which occurs in many eukaryotes and may represent a mechanism for limiting telomere length (reviewed in Lustig 2003). TRDs were first discovered in *S. cerevisiae*, where they are recombination-dependent (Kyrion et al. 1992; Lustig 2003). In the last few years, several authors have noted that if the site of strand invasion in t-loops migrates in a centromeric direction by a branch migration mechanism, the terminus of the telomeric C strand could base-pair with displaced sequences in the D-loop to form a Holliday junction (Figure 1.1) (Griffith et al. 1999; Lustig 2003; Wang et al. 2004). If t-loops can form Holliday junctions, then one prediction is that illegitimate resolution of telomeric Holliday junctions could lead to deletion of all the telomeric sequences in the t-loop, a process that is referred to as t-loop homologous recombination (t-loop HR) (Figure 1.1) (Griffith et al. 1999; Lustig 2003; Wang et al. 2004). A recent study indicates that mutation of TRF2, a protein implicated in mammalian t-loop formation, causes t-loop-sized deletions at human telomeres, and is accompanied by the appearance of t-loop-sized circular DNA molecules containing telomeric sequences (section 1.2.4.2.1.2) (Wang et al. 2004). Human t-loop deletions are dependent on the proteins XRCC3 and NBS1 (a component of the vertebrate MRE11 complex), which are respectively implicated in Holliday junction resolution, and homologous recombination (HR) at both telomeric and non-telomeric sites (Lustig 2003; Wang et al. 2004 and references therein). Interestingly, many of the proteins implicated in TRDs in mammalian and yeast cells may be functionally similar (Lustig 2003; Wang et al. 2004). These observations have led to the suggestion that, like other eukaryotes, *S. cerevisiae* telomeres may also form t-loop structures, though technical limitations related

to the yeast telomeric sequence have made it difficult to test this hypothesis (Lustig 2003; de Lange 2004).

1.2.4. Eukaryotic telomere-binding proteins

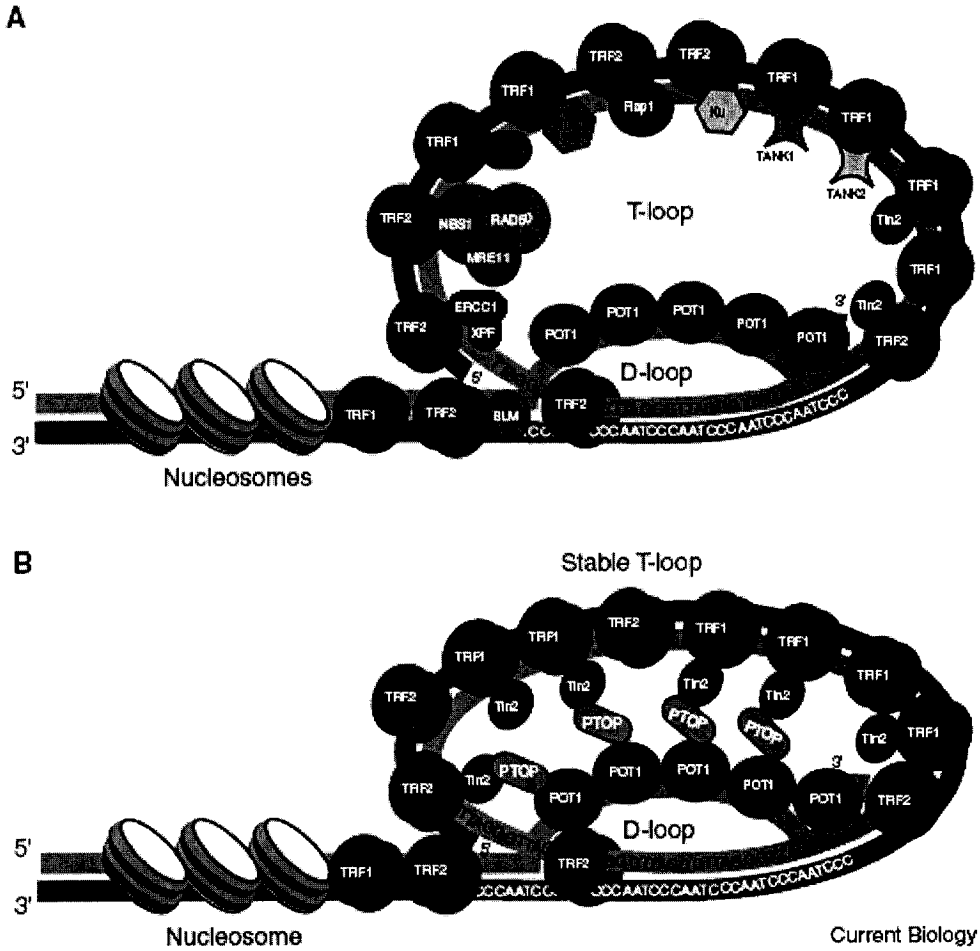
A dizzying number of proteins have been implicated in the regulation of telomere length, integrity and function in various eukaryotes (Figure 1.2). Some of these proteins are key players in the DNA damage response, non-homologous end-joining (NHEJ) and HR; others have telomere-specific roles in regulating telomere structure and length. Readers interested in detailed discussions of these proteins and pathways may consult recent reviews describing different aspects of eukaryotic telomere architecture and function (Lustig 2003; Wei and Price 2003; Colgin and Reddel 2004; d'Adda di Fagagna et al. 2004; Smogorzewska and de Lange 2004). However, in the interests of brevity, this review will necessarily focus primarily on proteins that directly bind the G-rich overhang and dsDNA sequences of vertebrate telomeres.

1.2.4.1. 3' overhang-binding telomeric proteins

Proteins that directly bind the ssDNA overhang of eukaryotic telomeres *in vivo* and/or *in vitro* include members of the oligonucleotide/oligosaccharide-binding fold (OB fold) telomere-binding family (TEBPs, POT1 and Cdc13p), replication protein A (RPA), the telomerase-associated protein EST1, various heterogeneous nuclear ribonucleoproteins (hnRNPs) and the tumor suppressor gene product p53. p53 binding to vertebrate telomeric ssDNA and t-loop junctions has only been observed *in vitro*, though in certain genetic backgrounds p53-negative mouse embryonic fibroblasts exhibit heterogeneous telomere lengths suggestive of rapid telomeric deletions and amplifications that may be related to improper protection/resolution of Holliday junctions at t-loops (Tong et al. 2001; Stansel et al. 2002). Evidently, the telomerase enzyme must also interact with the 3' overhang to permit elongation of this DNA strand, and may additionally interact with the 3' strand in a non-catalytic/protective capacity. The details of telomerase's interaction with its DNA substrate will be discussed in later sections of this thesis.

Figure 1.2: The telomere team

(A) Proteins that bind specifically to human telomeric DNA include TRF1, TRF2 and POT1. In addition, there is an ever growing list of proteins that bind to or modify these core components. *Telomere length-regulating proteins*: TRF1, TRF2, POT1, PINX1, RAP1, Tankyrases 1 and 2, TIN2. *DNA damage response and HR factors associated with TRF2*: NBS1, RAD50, MRE11 (HR pathway) and Ku (NHEJ pathway). For clarity, this figure does not depict all known telomere-associated human proteins. **(B)** PTOP/PIP1/TINT1 is proposed to bridge the gap between TRF1 or TRF2, TIN2 and POT1, possibly creating a stable telomere structure that resists lengthening by telomerase.


A
TRF2
TRF2
TRF1
TRF1
Rap1
KU
TRF1
TRF1
TANK1
TANK2
TRF1
T-loop
TRF2
NBS1
RAD50
Tin2
MRE11
TRF1
3'
ERCC1
Tin2
TRF2
XPF
POT1
POT1
POT1
POT1
TRF2
5'
D-loop
5'
3'
TRF1
TRF2
BLM
TRF2
Nucleosomes
B
Stable T-loop
TRF2
TRF1
TRF1
TRF1
TRF1
TRF1
TRF2
Tin2
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PTOP
PTOP
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TRF1
Tin2
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TRF2
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POT1
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TRF2
D-loop
5'
3'
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TRF2
TRF2
Nucleosome
Current Biology

1.2.4.1.1. OB fold proteins

The major group of 3' telomeric overhang-binding proteins identified to date includes proteins that bind G-rich ssDNA via one or more OB folds. These include the heterodimeric telomere end-binding proteins (TEBPα and TEBPβ) (in *Oxytricha* and *Euplotes*), Cdc13p (in *S. cerevisiae*), and the protection of telomeres (POT1) proteins (in *S. pombe*, humans and other vertebrates, plants and microsporidia) (Gottschling and Zakian 1986; Price 1990; Lin and Zakian 1996; Nugent et al. 1996; Baumann and Cech 2001; Baumann et al. 2002; Pitt et al. 2004; Wei and Price 2004). The POT1 proteins were first discovered based on a weak sequence homology with the OB fold DNA-binding domain (DBD) of the *Oxytricha* TEBPα subunit (Baumann and Cech 2001). Subsequent structural analysis of the *S. pombe* and human POT1 DBDs confirmed that POT1 employs one or more OB folds to interact with telomeric ssDNA (Lei et al. 2003; Lei et al. 2004). The Cdc13p DBD also forms an OB fold, despite very weak sequence similarity between this protein and TEBP or POT1 proteins (Mitton-Fry et al. 2002; Theobald et al. 2003). Human, *S. pombe* and chicken POT1 proteins, ciliate TEBPs and Cdc13p all bind to their respective telomeric ssDNA substrates with extraordinary sequence specificity and high affinity (Lin and Zakian 1996; Nugent et al. 1996; Baumann and Cech 2001; Lei et al. 2004; Wei and Price 2004).

OB folds are common motifs in proteins that recognize single-stranded nucleic acids. Theoretically, any ssDNA-binding protein that binds in a sequence-independent fashion could also interact with 3' telomeric overhangs. Indeed, at least one non-specific OB fold protein, the general replication-, recombination-, transcription- and DNA repair-regulating replication protein A (RPA), has been implicated in telomere maintenance in *S. cerevisiae* (Smith et al. 2000). Recent evidence indicates that *RPA* acts genetically in the telomerase pathway in *S. cerevisiae*, and that RPA is required for telomeric binding of the telomerase recruiting/activating protein Est1p during S-phase, though RPA and Est1p do not interact with each other (Schramke et al. 2004). It has been proposed that proteins such as RPA could compete with telomere-specific OB fold proteins to reveal telomeric 3' ends for replication and telomerase-mediated extension (Smogorzewska and de Lange 2004). However, RPA does not affect the telomeric binding of the telomere-specific OB fold protein Cdc13p or the protein catalytic subunit of telomerase, suggesting that it may

instead modulate the conformation of telomeric ssDNA, or stimulate an unwinding of duplex telomeric DNA that might permit binding of Est1p (Schramke et al. 2004).

1.2.4.1.1.1. POT1 proteins

The minimal *S. pombe* and vertebrate POT1 binding sites contain less than one (*S. pombe*) and two copies (vertebrate) of the core telomeric repeat sequence synthesized by *S. pombe* and vertebrate telomerases (Lei et al. 2002; Lei et al. 2004; Loayza et al. 2004; Wei and Price 2004). POT1 binding to telomeric sequences is stimulated by the presence of an adjacent 3' end, but all POT1 proteins identified to date can bind internal telomeric sequences, suggesting that POT1 may load at the 3' end of the overhang and subsequently coat other regions of telomeric ssDNA, including the D-loop of t-loop structures (Figure 1.2) (Lei et al. 2002; Loayza et al. 2004; Wei and Price 2004). Crystal structure analysis indicates that the 3' end of telomeric DNA protrudes from the *S. pombe* Pot1p DBD (Lei et al. 2003). In contrast, only the 3' hydroxyl group of the terminal nucleotide in the most common permutation of the human telomeric sequence (5'-GGTTAG-3') is solvent-exposed, suggesting that hPOT1 binding may be sufficient to cap or protect the extreme 3' end of human telomeres that terminate with this sequence (Lei et al. 2004; Sfeir et al. 2005). hPOT1 binding to substrates ending with this sequence inhibits telomerase-mediated elongation *in vitro* (Lei et al. 2005). However, the 3' overhang contains many copies of this telomeric repeat, and 60% of the G strands in telomerase-positive cells do not end with the sequence GGTTAG (Sfeir et al. 2005). Recent *in vitro* evidence indicates that hPOT1 binding to internal sites in substrates composed of repeat permutations other than GGTTAG exposes enough 3' overhang sequences for efficient elongation by telomerase (Lei et al. 2005).

POT1 is implicated in both telomere protection and length regulation. Deletion of *S. pombe POT1* causes rapid loss of telomere sequences and chromosome mis-segregation and circularization (Baumann and Cech 2001). Knockdown of hPOT1 expression by RNA interference in telomerase-positive and telomerase-negative human cell lines causes loss of the 3' overhang, end-to-end chromosome fusions, anaphase bridges, cellular senescence and apoptosis (Veldman et al. 2004; Yang et al. 2005). These observations imply that POT1 is important for telomere protection in budding yeast and humans. POT1-dependent

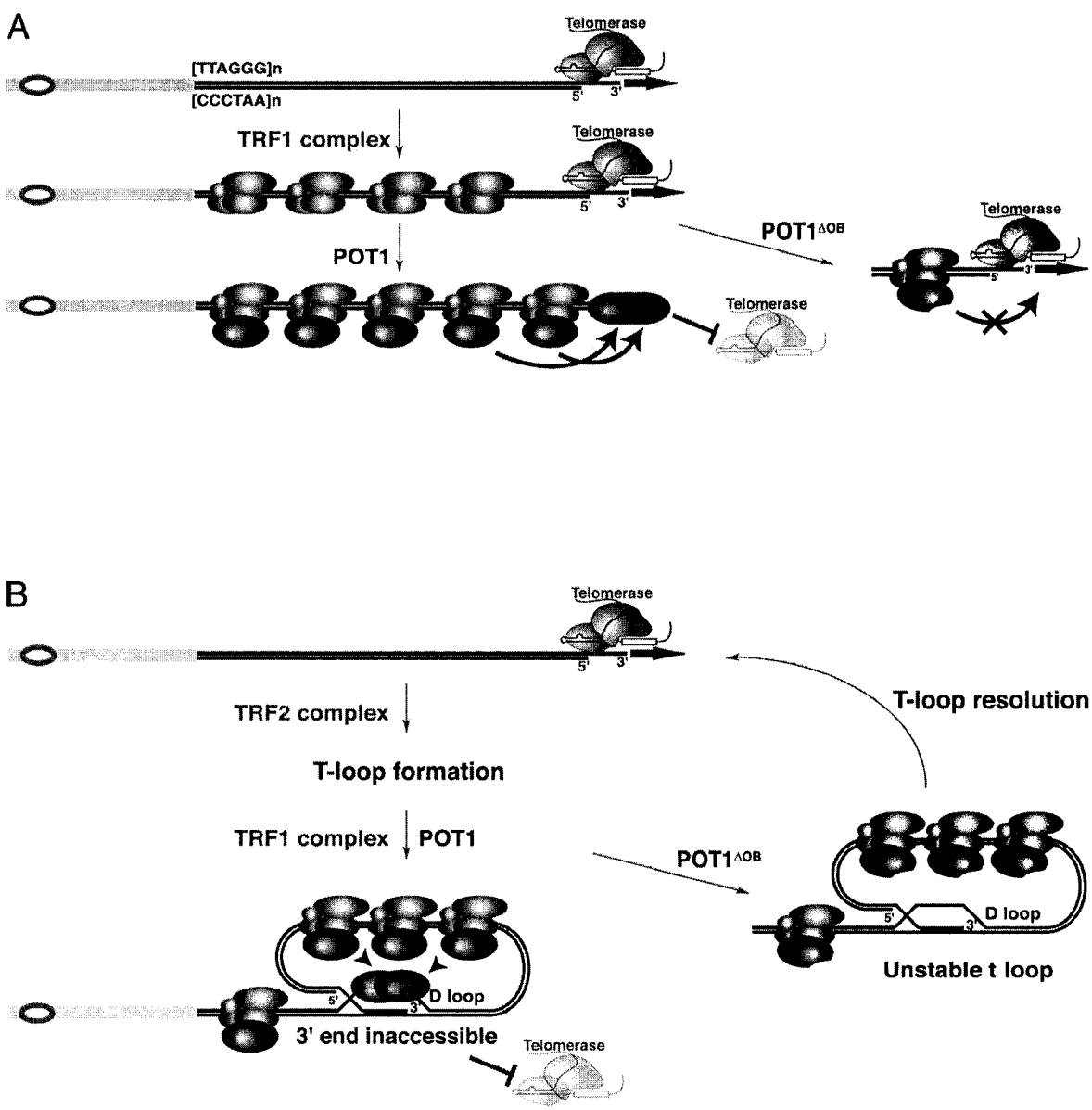
end protection in human cells may be dependent on interactions with the telomere-binding protein TRF2 (section 1.2.4.2.1.2) (Yang et al. 2005).

POT1's role in telomere length regulation is more controversial. In three studies, hPOT1 overexpression was found to cause telomere lengthening in a number of telomerase-positive human cell lines, but not in telomerase-negative cells, suggesting that one of hPOT1's functions is to regulate telomerase-mediated telomere elongation, perhaps by recruiting telomerase (Colgin et al. 2003; Armbruster et al. 2004; Liu et al. 2004a). Recent evidence indicating that hPOT1 can stimulate telomerase-mediated elongation of certain substrates *in vitro*, suggests that it may also regulate the function of the enzyme itself (Lei et al. 2005). In contrast, a fourth group has reported that hPOT1 overexpression does not affect telomere length in at least one telomerase-positive cell line, and reduction of hPOT1 expression in the same cell line by RNA silencing causes rapid telomere elongation (Loayza and de Lange 2003; Ye et al. 2004). It is unknown why overexpression and targeted reduction of hPOT1 expression cause distinct phenotypes in different telomerase-positive cell lines. The observation that reduced POT1 expression can cause telomere lengthening has suggested a model in which hPOT1 negatively regulates telomerase-mediated telomere elongation in certain contexts, perhaps by competing with telomerase for access to its DNA substrate, or by stabilizing the t-loop structure through interactions with its single-stranded D-loop (Figure 1.3) (section 1.2.4.2.1.1) (Smogorzewska and de Lange 2004; Ye et al. 2004). This model may be supported by recent reports that purified hPOT1 inhibits telomerase-mediated elongation of DNA substrates containing consensus hPOT1 binding sites *in vitro*, but does not inhibit telomerase-dependent elongation of substrates that do not contain the minimal hPOT1 binding site (Kelleher et al. 2005; Lei et al. 2005). Curiously, telomere elongation phenotypes in some telomerase-positive cells overexpressing hPOT1, and telomere localization of hPOT1 do not require the hPOT1 minimal DBD (Loayza and de Lange 2003; Liu et al. 2004a). Instead, hPOT1-dependent telomere elongation and hPOT1 localization at telomeres are mediated by a member of the TRF1 telomere length-regulating complex, PIP1 (also known as PTOP and TINT1), which may recruit and/or load hPOT1 onto the telomere (Figure 1.2) (Loayza and de Lange 2003; Houghtaling et al. 2004; Liu et al. 2004a; Ye et al. 2004). One possible explanation for the contrasting telomere length phenotypes of hPOT1-overexpressing cells is therefore that hPOT1's

Figure 1.3: Proposed role for POT1 in vertebrate telomere length regulation by the TRF1 complex

(A) POT1 binding to TRF1 is proposed to act as a molecular counting mechanism that measures telomere length (section 1.2.4.2.1.1). Direct POT1 binding to 3' overhang sequences at the terminus is thought to inhibit access of telomerase to the 3' terminus. **(B)** POT1 is proposed to bind to the single-stranded D-loop in the t-loop structure, and regulated disruption of POT1 binding to the D-loop by unknown cellular proteins, or disruption of binding by expression of a POT1 mutant that cannot bind telomeric DNA ($POT1^{\Delta OB}$) is predicted to result in t-loop resolution and telomerase-mediated telomere elongation.

This figure is adapted from an article published by Smogorzewska and de Lange in the journal Annual Review of Biochemistry (Smogorzewska and de Lange 2004). Reprinted, with permission, from the Annual Review of Biochemistry, Volume 73 © 2004 by Annual Reviews www.annualreviews.org.


A
Telomerase
[TTAGGG]n
[CCCTAA]n
5'
3'
TRF1 complex
Telomerase
5'
3'
POT1
POT1ΔOB
Telomerase
5'
3'
Telomerase
B
Telomerase
5'
3'
TRF2 complex
T-loop formation
T-loop resolution
TRF1 complex
POT1
POT1ΔOB
D loop
5'
3'
Unstable t loop
D loop
5'
3'
3' end inaccessible
Telomerase

function(s) are telomere length- and sequence-dependent, and are regulated by members of the TRF1 complex (Smogorzewska and de Lange 2004; Lei et al. 2005). It has also been proposed that other components of the telomere/telomerase complex such as hEST1 could influence the position of POT1 binding in telomeric sequences, thereby regulating exposure of the telomerase substrate (Lei et al. 2005). Though the current data concerning POT1 function are conflicting, similarly complex observations have been made in the case of the Cdc13p OB fold protein from *S. cerevisiae*, which has been implicated in numerous aspects of telomere length regulation, including end protection, telomerase recruitment and/or activation, telomerase repression, and coordination of G and C strand telomeric DNA synthesis (section 1.2.4.1.1.2).

1.2.4.1.1.2. Cdc13p

The *S. cerevisiae* protein Cdc13p was first discovered in a screen for mutants that disrupt the cell division cycle, and was subsequently identified as a gene whose disruption causes an ever shorter telomere (*est-*) phenotype (Garvik et al. 1995; Lendvay et al. 1996; Nugent et al. 1996). Unlike other OB fold telomere-binding proteins, Cdc13p does not preferentially bind at or near free 3' ends, suggesting that this protein may not directly cap the extreme terminus of the telomere (Lin and Zakian 1996; Nugent et al. 1996). Cdc13p is the most studied member of the OB fold family of 3' overhang proteins, and has been implicated in multiple aspects of telomere maintenance, including end protection, telomerase recruitment/activation, telomerase repression, and coordination of G and C strand telomeric DNA synthesis (reviewed in Wei and Price 2003; Smogorzewska and de Lange 2004). Reminiscent of the conflicting phenotypes observed in hPOT1 overexpression experiments, mutation of different Cdc13p sequences results in gradual or rapid telomere shortening, or telomere lengthening, depending on the mutation site and genetic background. In the case of Cdc13p, the complexity and distinct characteristics of these phenotypes are likely a result of the protein's coordinating role at telomeres. Cdc13p interacts with Stn1p, a protein that is important for recruitment of both the putative end-protecting protein Ten1p and the DNA polα-primase component of the lagging strand DNA synthesis machinery (Grandin et al. 1997; Pennock et al. 2001;Grandin et al. 2001; Grossi et al. 2004). The Stn1p interaction site in Cdc13p overlaps with the site required for interaction with an essential yeast telomerase component, Est1p, which is important for

recruitment and/or activation of telomerase at the telomere (section 1.2.4.1.2) (Chandra et al. 2001). These observations have led to the proposal that Stn1p-mediated recruitment of telomere capping elements and the lagging strand DNA synthesis machinery alternates with Est1p-mediated recruitment/activation of telomerase to regulate both telomere protection (in a non-replicating state) and coordinated synthesis of the telomeric G and C strands (Chandra et al. 2001). If the functional similarities between Cdc13p and the POT1 proteins extend further than their DBDs, then equally complex roles may be anticipated for POT1 proteins in other eukaryotes.

1.2.4.1.2. EST1

EST1 orthologues have been identified in *S. cerevisiae*, *S. pombe*, *Candida albicans* (*C. albicans*) and humans (reviewed in Lundblad 2003). The first member of the EST1 family was discovered in *S. cerevisiae*, where it was found to be essential for telomere maintenance *in vivo*, but not telomerase activity *in vitro* (Lundblad and Szostak 1989; Lingner et al. 1997a). Disruption of *S. cerevisiae EST1* results in an ever-shorter telomere (*est-*) phenotype that is identical to the gradual telomere shortening phenotype observed in cells deficient in either of the minimal components required for telomerase catalytic activity *in vitro* (TERT and TR; in *S. cerevisiae*, Est2p and TLC1, respectively) (section 1.5.1) (Lendvay et al. 1996).

Epistasis analysis indicates that *S. cerevisiae EST1* functions in the same genetic pathway as *CDC13* and the minimal telomerase catalytic components (Lendvay et al. 1996). Budding yeast Est1p interacts with Cdc13p, TLC1 and telomeric DNA (Virta-Pearlman et al. 1996; Qi and Zakian 2000; Zhou et al. 2000). Fusion of Cdc13p and Est1p causes extensive telomere elongation, and fusion of an Est1p mutant with an *est-* phenotype with Cdc13p rescues its telomere shortening phenotype (Evans and Lundblad 1999). Furthermore, fusion of Cdc13p to Est2p bypasses the requirement for Est1p in telomere maintenance, though Cdc13p and Est2p are not known to physically interact with each other in *S. cerevisiae* (Evans and Lundblad 1999). Together, these observations have led to a model in which Cdc13p regulates telomerase access to the telomere by recruiting the telomerase-associated protein Est1p. However, some aspects of this recruitment model are difficult to reconcile with several features of Est1p and telomerase function in budding yeast. First, mutations in the TLC1-associated NHEJ protein Ku disrupt the association of

both Est1p and Est2p with telomeres, suggesting that Ku also regulates Est1p and telomerase localization (Fisher et al. 2004). Second, Est2p is found at telomeres throughout the cell cycle, whereas Est1p association with telomeres does not increase until late S-phase, the period when telomerase-mediated extension of the 3' strand occurs in budding yeast (Taggart et al. 2002). Third, Est1p and Est2p telomere association persists in cells expressing a loss of function Cdc13p mutant that was previously thought to be defective in Est1p-mediated telomerase recruitment, indicating that Est1p telomere-binding likely does not require Cdc13p (Taggart et al. 2002). Therefore, an alternative model has been proposed in which Est1p contributes to telomerase-dependent telomere elongation by activating telomerase complexes already bound to the telomere, perhaps by stimulating telomerase dimerization (section 1.5.1) or conformational changes required for catalytic function; such an activation process could be facilitated by Cdc13p interactions in a recruitment-independent fashion (Taggart et al. 2002).

Though most of the work characterizing EST1 function has been performed in budding yeast, EST1 orthologues have also been identified in fission yeast (*S. pombe*), fungal yeast (*C. albicans*) and humans (Singh et al. 2002; Beernink et al. 2003; Reichenbach et al. 2003; Snow et al. 2003). In contrast to the gradual telomere shortening *est-* phenotype of *S. cerevisiae est1*Δ strains, disruption of *C. albicans EST1* results in rapid telomere loss suggestive of impaired telomere capping (Singh et al. 2002). Furthermore, recent work indicates that *in vitro C. albicans* Est1p stimulates or activates telomerase-mediated elongation of DNA substrates that are not efficiently elongated by the minimal enzyme alone (TERT+TR) (Singh and Lue 2003). Since the effect of *S. cerevisiae* Est1p on telomerase-mediated elongation of similar suboptimal substrates has never been examined, it remains possible that EST1 also contributes to telomerase catalytic function in budding yeast and other organisms (Singh and Lue 2003).

S. pombe EST1-null strains exhibit gradual telomere shortening similar to the *est-* phenotype of strains that do not express TERT; however, deletion of both *EST1* and the gene encoding the t-loop regulating protein Taz1p (section 1.2.4.2.1) is synthetically lethal, suggesting that *S. pombe* Est1p contributes to telomere protection (Beernink et al. 2003). Est1p and TERT are reciprocally coimmunoprecipitated from *S. pombe* cells, and *EST1*-null strains do not appear to exhibit defects in telomerase catalytic activity *in vitro* (Beernink et al. 2003). Curiously, *S. pombe* Est1p does not appear to bind ssDNA or

dsDNA *in vitro*, suggesting that DNA binding may not be conserved in all EST1 orthologues (Beernink et al. 2003).

At least two EST1 orthologues have been identified in human cells, hEST1A and hEST1B; some hEST1A and hEST1B sequences resemble those of certain nucleases and helicases, and it has been proposed that these proteins might play a role in presentation or processing of preferential telomerase substrates (Reichenbach et al. 2003; Snow et al. 2003; Lei et al. 2005). In addition, human EST1 proteins have been implicated in nonsense-mediate mRNA decay (NMD), and contain a 14-3-3-like structure, suggesting that they may confer 14-3-3 function at telomeres (Fukuhara et al. 2005 and references therein). *In vitro*-synthesized hEST1A and hEST1B coimmunoprecipitate with hTERT in an hTR-independent fashion, and hEST1A does not interact with hTR; hEST1A, but not hEST1B, preferentially binds ssDNA (Snow et al. 2003). Overexpression of hEST1B does not affect telomere length or integrity; however, overexpression of hEST1A causes phenotypes that range from telomere shortening to telomere-mediated chromosome fusions assumed to result from telomere uncapping (Reichenbach et al. 2003; Snow et al. 2003). Conflicting data have been reported for the effects of hEST1A overexpression in telomerase-negative and telomerase-positive cells. One group has reported that telomere shortening phenotypes are telomerase-dependent and can be rescued by overexpression of hTERT (Snow et al. 2003); a second study has found that hEST1A-induced catastrophic telomere fusions are independent of telomerase status (Reichenbach et al. 2003). Neither hEST1A nor hEST1B affects the ability of human telomerase to extend certain partially-telomeric substrates *in vitro*, suggesting that these proteins may not functionally resemble the *C. albicans* orthologue in this respect (Chapter 4).

1.2.4.1.3. hnRNPs

The function(s) of the last category of known telomeric G-overhang-binding proteins are poorly understood (reviewed in Ford et al. 2002). A large number of hnRNPs coimmunoprecipitate with telomerase activity in mammalian cells. Most of these proteins bind RNA more specifically than telomeric ssDNA. Several hnRNPs interact primarily with TR and may be involved in telomerase processing and stability *in vivo*. Of the telomeric ssDNA-interacting proteins, the best-characterized is the splicing factor hnRNP A1. Purified hnRNP A1 has been shown to prevent telomeric G strand degradation, though

it inhibits telomerase-mediated extension of telomeric substrates *in vitro* (Dallaire et al. 2000). Mice deficient in hnRNP A1 expression have shortened telomeres (LaBranche et al. 1998). hnRNP A1 may bind simultaneously to both the telomerase RNA and 3' overhang, suggesting that this protein could be involved in regulating telomerase access to the telomere (Fiset and Chabot 2001). However, the telomeric phenotypes caused by disruption or overexpression of certain hnRNPs may also be attributable to the complex roles of many hnRNPs in RNA maturation and turnover.

1.2.4.2. Telomeric duplex-binding proteins

Two major groups of proteins have been identified that interact directly with telomeric dsDNA repeats in eukaryotes. The first are members of the Myb-type/telobox telomeric repeat-binding protein family (reviewed in Kanoh and Ishikawa 2003; Smogorzewska and de Lange 2004). The second are the Ku70 and Ku80/86 subunits of the Ku heterodimer, which have been implicated in telomere function in diverse eukaryotes (reviewed in Downs and Jackson 2004). Eukaryotic telomeres are also known to be packaged into nucleosomes, and native vertebrate telomeres assume a beads-on-a-string conformation with ionic strength-dependent properties suggestive of the presence of histone H1 (Tommerup et al. 1994; Lejnine et al. 1995; Nikitina and Woodcock 2004). Variant forms of histones H2B and H3 have been found, respectively, at a subset of the telomeres of human sperm, and at all telomeres of *Trypanosoma brucei* (Gineitis et al. 2000; Lowell and Cross 2004); however, the contributions of these histones are poorly understood, and will not be discussed in this review.

1.2.4.2.1. Myb-type/telobox proteins

Proteins in this family bind specifically to telomeric dsDNA repeats *in vivo* and *in vitro*, and include TTAGGG repeat binding factors 1 and 2 (TRF1 and TRF2; in mammals) (Figure 1.2), *sc*Rap1p (in *S. cerevisiae*) and Taz1p (in *S. pombe*) (Buchman et al. 1988a; Buchman et al. 1988b; Conrad et al. 1990; Lustig et al. 1990; Chong et al. 1995; Bilaud et al. 1997; Broccoli et al. 1997a; Broccoli et al. 1997b; Cooper et al. 1997). Human cells and fission yeast both express Rap1p-like proteins that are important for telomere length regulation; however, human and *S. pombe* RAP1 proteins do not interact directly with telomeric DNA, but interact indirectly via their associations with TRF2 and

Taz1p, respectively (Li et al. 2000; Chikashige and Hiraoka 2001; Kanoh and Ishikawa 2001; Li and de Lange 2003).

Just as 3' overhang-interacting proteins commonly employ an OB fold structure for binding telomeric ssDNA, TRF1, TRF2, *sc*Rap1p and Taz1p all use a similar Myb-type "telobox" DBD to bind to their telomeric dsDNA targets (Chong et al. 1995; Konig et al. 1996; Bilaud et al. 1997; Cooper et al. 1997). TRF1 and Taz1p contain single Myb DBDs, and bind as dimers (Bianchi et al. 1997; Spink et al. 2000). Based on sequence similarities in the Myb DBDs and dimerization domains of TRF1 and TRF2, it is likely that TRF2 also dimerizes; TRF2 multimers are visible on t-loops *in vitro* (Griffith et al. 1999). *sc*Rap1p contains two Myb DBDs connected by a flexible linker, and binds as a monomer (Konig et al. 1996).

All telobox dsDNA binding proteins have been implicated in telomere length regulation, and TRF2 and Taz1p are important for telomere protection (section 1.2.4.2.1.2). *sc*Rap1p and Taz1p have been implicated in heterochromatin formation and silencing of subtelomeric genes in *S. cerevisiae* and *S. pombe*, respectively; Taz1p is also essential for pre-meiotic telomere-clustering events (reviewed in Kanoh and Ishikawa 2003). Although there is some conflicting evidence for telomere length-dependent silencing of subtelomeric genes in human cells, telomere-binding proteins have not been directly implicated in this process (Baur et al. 2001; Koering et al. 2002; Ning et al. 2003). TRF1 and TRF2 are found at mammalian telomeres during meiosis, but their potential role in meiotic processes has not been directly examined (Scherthan et al. 2000).

1.2.4.2.1.1. Negative regulation of telomere length

Negative regulation of telomere length is likely to be important for species-specific maintenance of telomere length within a characteristic range (reviewed in Smogorzewska and de Lange 2004). Telomerase-mediated extension of budding yeast telomeres is progressively inhibited as telomeres elongate (Marcand et al. 1999). Furthermore, telomerase appears to preferentially elongate the shortest telomeres in mice and budding yeast (Hemann et al. 2001; Liu et al. 2002; Teixeira et al. 2004). In yeast, preferential elongation of the shortest telomeres by telomerase is not caused by more efficient telomerase activity on these substrates, but is likely dependent on enhanced accessibility of these substrates to telomerase (Teixeira et al. 2004). Though the mechanism inhibiting

excessive telomerase-dependent telomere elongation is not yet known, the telobox DNA binding proteins likely play an important role in this process.

Overexpression of any of TRF1, TRF2 or *sc*Rap1p in their respective cellular contexts causes telomere shortening, suggesting that these proteins are involved in negative telomere length regulation (Conrad et al. 1990; van Steensel and de Lange 1997; Smogorzewska et al. 2000) The essential status of these genes has precluded analysis of their null phenotypes. However, TRF1 and *sc*Rap1p mutations that abrogate interactions with other telomere length-regulating proteins cause substantial telomere elongation, indicating that TRF1 and *sc*Rap1p likely play central roles in negative telomere length regulation in mammals and budding yeast, respectively (reviewed in Smogorzewska and de Lange 2004). Taz1p has also been implicated in negative regulation of telomere length, since deletion of *TAZ1*, which is non-essential under certain conditions, causes substantial telomere lengthening (Cooper et al. 1997).

The current model of TRF1- and *sc*Rap1p-dependent telomere length regulation is that binding of these proteins acts as a counting mechanism (Figure 1.3) (reviewed in (Smogorzewska and de Lange 2004). This model is based partially on the observation that the number of *sc*Rap1p and TRF1 molecules bound to *S. cerevisiae* and human telomeres, respectively, is directly proportional to telomere length (Smogorzewska and de Lange 2004). The most direct evidence for the counting model comes from experiments in *S. cerevisiae*, where artificial tethering of a *sc*Rap1p-GAL4 fusion protein to GAL4-binding sites in subtelomeric DNA creates a new, shorter setpoint length at the proximal telomere that is proportional to the number of subtelomeric GAL4-binding sites (Marcand et al. 1997). Evidence for the counting model in human cells is less direct, and is based on the observations that TRF1 overexpression or partial inhibition of endogenous TRF1 (via expression of a dominant-negative protein) respectively reduces or increases the average telomere length in telomerase-positive human cancer cells (van Steensel and de Lange 1997; Smogorzewska et al. 2000). Interestingly, telomere shortening does not occur in telomerase-negative human cells overexpressing TRF1, suggesting that TRF1-dependent telomere length regulation is at least partly effected through restriction of telomerase activity at the extreme terminus of the telomere (Karlseder et al. 2002). This hypothesis is consistent with the observation that TRF1 overexpression-induced telomere shortening in

telomerase-positive cells is gradual, as is seen in telomerase-negative and telomerase-inhibited cells (van Steensel and de Lange 1997; Smogorzewska et al. 2000).

TRF1-mediated regulation of telomere length in mammals is not yet understood, but involves a highly complex network of interacting factors (Figures 1-2 and 1-3) (reviewed in Colgin and Reddel 2004; Smogorzewska and de Lange 2004). At one level of regulation, tankyrases 1 and 2 stimulate telomere elongation by the addition of poly(ADP-ribose) to TRF1, thus removing TRF1 from telomeric DNA. Inhibition of tankyrase 1 promotes telomere shortening and cell death in cancer cells treated with a telomerase inhibitor, indicating that tankyrase 1 is a critical regulator of telomere length (Seimiya et al. 2005). A second TRF1-interacting protein, TIN2, protects TRF1 from tankyrase-mediated modification, thus suppressing telomere elongation. TRF1 also interacts indirectly with POT1, through interactions between TIN2 and a newly identified telomere length regulation factor, PTOP/PIP1/TINT1 (Figure 1.2) (reviewed in Colgin and Reddel 2004). Defective splicing of the transcripts from the PTOP mouse orthologue *ACD* is implicated in the disease adrenocortical dysplasia, though the role of telomeres in this disease has not yet been investigated (Keegan et al. 2005). The levels of telomere-associated POT1 under various experimental conditions correspond closely to the levels of telomere-associated TRF1, and to telomere length, suggesting that TRF1 recruits POT1 to telomeres, and/or that TRF1-dependent telomere length regulating signals may be 'transduced' to the extreme terminus of the telomere via indirect interactions with POT1 (Figure 1.3) (Loayza and de Lange 2003). TIN2 and PTOP/PIP1/TINT1 also appear to regulate the association of TRF1 with TRF2, a major telomere-protecting protein in mammalian cells (section 1.2.4.2.1.2) (reviewed in Colgin and Reddel 2004). Finally, one TRF1-interacting factor that may not be acting at the level of telomere structure, *per se*, is the *in vitro* telomerase inhibitor and telomerase-interacting factor PINX1 (Zhou and Lu 2001). Overexpression of PINX1 inhibits *in vitro* telomerase activity and causes telomere shortening *in vivo*, whereas depletion of endogenous PINX1 enhances telomerase activity and causes telomere elongation (Zhou and Lu 2001). However, though PINX1 may function to disrupt telomerase ribonucleoprotein assembly/stability in both yeast and human cells (section 1.5.3.4), little is known about its ability to regulate telomerase at the telomere, or how such regulation is related to PINX1's association with TRF1 (Banik and Counter 2004; Lin and Blackburn 2004). Thus, TRF1 is found at the centre of a network of

proteins that connect the extensive telomeric dsDNA tracts to the ssDNA overhang elongated by telomerase, but the mechanism(s) by which these proteins regulate telomerase-mediated telomere extension are still not understood.

1.2.4.2.1.2. Telomere protection

In addition to regulating telomere length (by unknown mechanisms) telobox telomere-binding proteins TRF2 and Taz1p also play a role in telomere protection. In fact, TRF2 overexpression causes telomere shortening even in telomerase-negative cells, suggesting that TRF2 may be associated with a telomere degradation pathway, and not with negative regulation of telomerase-mediated telomere elongation (Smogorzewska et al. 2000; Ancelin et al. 2002). In *S. pombe* subjected to nitrogen starvation or grown at cold temperatures, deletion of *TAZ1* results in lethal telomere-mediated chromosome fusions and missegregation, DSBs and cell cycle arrest (Ferreira and Cooper 2001; Miller and Cooper 2003). In nitrogen-starved cells arrested at the G1 phase of the cell cycle, telomere fusions that arise in *taz1-* cells are caused by an inability to suppress Ku-dependent NHEJ (Ferreira and Cooper 2001). Similarly, in mammalian cells, transient expression of a dominant-negative TRF2 mutant that removes endogenous TRF2 from telomeres causes immediate loss of the 3' overhang, replicative senescence, p53- and ATM (ataxia telangiectasia mutated) kinase-dependent apoptosis, and inter-telomeric covalent chromosome fusions that are dependent on the NHEJ factor DNA ligase IV (van Steensel et al. 1998; Karlseder et al. 1999; Smogorzewska et al. 2002). Recent evidence indicates that TRF2 can interact with hPOT1, and that the telomere-destabilizing function of the dominant-negative TRF2 mutant is caused by its ability to remove POT1 from telomeres (Yang et al. 2005). TRF2 also interacts with at least one member of the NHEJ machinery, Ku70, and the TRF2-associated protein hRAP1 may also recruit Ku86 to the TRF2 complex (Song et al. 2000; O'Connor et al. 2004). Interestingly, recent data indicates that TRF2 rapidly localizes to genomic DSBs before activation of the ATM-dependent DSB response, suggesting that it may serve an additional role in protecting or processing of non-telomeric DNA ends (Bradshaw et al. 2005).

Both Taz1p and TRF2 stimulate the formation of t-loops *in vitro*, and associate in oligomeric form with the junction between dsDNA and the ssDNA overhang in t-loops (Figure 1.2) (Griffith et al. 1999; Tomaska et al. 2004). Taz1p- and TRF2-dependent DNA

binding and t-loop formation require the presence of a 3' overhang containing *S. pombe* or vertebrate telomeric sequences, respectively, suggesting that both proteins may be loaded onto telomeric DNA via the 3' tail (Stansel et al. 2001; Tomaska et al. 2004). The Taz1p DBD is required for t-loop formation *in vitro* (Tomaska et al. 2004). Interestingly, a TRF2 mutant that retains the TRF2 DBD and the ability to localize to telomeres and stimulate t-loop formation, but lacks other, TRF2-specific sequences, prevents inter-telomeric NHEJ in mammalian cells, suggesting that illegitimate NHEJ between telomeres is blocked by the t-loop structure (Wang et al. 2004). However, this mutant fails to suppress HR-mediated deletion of t-loops (section 1.2.3.3), implying that TRF2 also plays a role in suppressing HR at telomeres (Wang et al. 2004). HR-dependent telomere maintenance in *S. pombe* requires deletion of *TAZ1*, suggesting that telobox TRF2 orthologues may have a common function in suppressing HR (Nakamura et al. 1998).TRF2 interacts with the HR MRE11 complex (RAD50, MRE11, NBS1), which is implicated in t-loop deletion, though the function of this interaction is unknown (Zhu et al. 2000; Wang et al. 2004). TRF2 also interacts with three components of the base excision repair pathway, DNA polymerase β, poly(ADP-ribose) polymerase-2 (PARP-2) and ERCC1/XPF (Zhu et al. 2003; Dantzer et al. 2004; Fotiadou 2004). hRAP1 may also recruit the DNA damage response enzyme PARP-1 to the TRF2 complex (O'Connor et al. 2004). Furthermore, TRF2 interacts with and stimulates the activities of the RecQ-like Bloom syndrome helicase (BLM) and Werner syndrome helicase/exonuclease (WRN), and TRF2 interactions may also suppress the ability of WRN to dissociate the t-loop structure *in vitro* (Opresko et al. 2002; Stavropoulos et al. 2002; Lillard-Wetherell et al. 2004; Machwe et al. 2004; Opresko et al. 2004). Though a complex set of DNA recombination, repair and modification factors are associated with the TRF2 complex at telomeres, it is still not clear how TRF2, or TRF2 orthologues such as Taz1p, mediate telomere protection *in vivo*.

1.2.4.2.2. Ku

Chromatin immunoprecipitation and immunolocalization studies have demonstrated that the Ku heterodimer physically associates with telomeres in *S. cerevisiae*, *S. pombe* and mammals (Gravel et al. 1998; Laroche et al. 1998; Hsu et al. 1999; d'Adda di Fagagna et al. 2001; Nakamura et al. 2002). It is unknown if this interaction is direct, but Ku should, in principle, be able to directly bind the double-

stranded ends of telomeres. Human Ku can also interact with TRF1, TRF2, the catalytic subunit of telomerase, hTERT, and the telomerase RNA (Hsu et al. 2000; Song et al. 2000; Chai et al. 2002; Ting et al. 2005). Yeast and human Ku specifically associate with a catalytically inessential stem-loop of the TR (Peterson et al. 2001; Ting et al. 2005).

Ku functions as a heterodimer containing two subunits, Ku70 and Ku80/Ku86 (reviewed in Downs and Jackson 2004). Ku binds in a sequence-independent fashion to DNA structures with blunt ends and 3' or 5' overhangs. When bound to DNA, the Ku heterodimer from higher eukaryotes also associates with the catalytic subunit of the DNA-protein kinase (DNA-PK_{cs}). Ku70, Ku80 and (in mammals) DNA-PK_{cs} have been implicated in telomere length regulation and protection in diverse eukaryotes, including *S. cerevisiae*, *S. pombe*, *Arabidopsis*, mice, trypanosomes and humans; Ku also contributes to localization of telomeres to the nuclear periphery and transcriptional silencing of subtelomeric genes in yeast (reviewed in (d'Adda di Fagagna et al. 2004; Downs and Jackson 2004). Interestingly, in *S. cerevisiae*, Ku and Ku-interacting proteins have been shown to dissociate from telomeres and relocalize to DSB sites following DNA damage, suggesting that telomeres may act as a storage reservoir for DNA repair proteins (Haber 1999; Martin et al. 1999).

Ku and the NHEJ factor DNA ligase IV have also been implicated in the chromosome fusions that are found in mammalian cells with critically short and/or dysfunctional telomeres (Espejel et al. 2002; Smogorzewska et al. 2002). These observations indicate that Ku-mediated NHEJ can impair telomere function. What prevents Ku from causing fusions between normal telomeres? Several recent reports of mutations in the *S. cerevisiae Ku80* subunit indicate that the telomeric functions of Ku are separable from its role in NHEJ (Bertuch and Lundblad 2003; Stellwagen et al. 2003; Roy et al. 2004). One of these mutations abolishes the ability of Ku80 to interact with TR, implying that Ku's ability to interact with telomerase, and perhaps to recruit it to telomeres, is one of the properties that distinguishes between its different functions at telomeres and DSBs (Stellwagen et al. 2003). Indeed, Ku plays an important role in regulating the association of telomerase and Est1 with telomeres during different phases of the budding yeast cell cycle (Fisher et al. 2004).

1.3. The end replication problem

DNA replication in all living organisms is accomplished by DNA polymerases that catalyze the addition of nucleotides to preexisting DNA or RNA primers in a 5'-3' direction, using parental DNA as a template (reviewed in Lingner et al. 1995). The two strands of DNA have opposite orientations, with one strand (the leading strand) running 5'-3' toward the origin of replication, whereas the lagging strand runs 5'-3' away from the replication origin (Figure 1.4). The parental DNA leading strand is copied starting at the 5' origin of replication in an uninterrupted fashion. The lagging strand is replicated by lagging strand DNA synthesis, which proceeds along the parental strand in a direction toward the origin of replication, and is primed by short RNA oligonucleotides laid down periodically on the parental strand; the DNA added to each RNA primer is referred to as an Okazaki fragment. After synthesis of Okazaki fragments is complete, the RNA primers are removed and the intervening gaps between Okazaki fragments are filled in by specialized DNA polymerase and ligase activities of the lagging strand synthesis machinery. However, as early as the 1970s it was realized that, in theory, lagging strand synthesis cannot completely replicate the extreme 3' ends of DNA molecules, since removal of the most terminal RNA primer would leave an unreplicated region of DNA (Figure 1.4) (Watson 1972; Olovnikov 1973). More recently, this prediction has been formally proven by examining the synthesis of linear viral DNA molecules in a reconstituted replication system (Ohki et al. 2001). The inherent inability of lagging strand DNA synthesis to completely replicate the 3' ends of DNA is referred to as the end replication problem. The end replication problem causes the loss of telomeric sequences, and possibly coding sequences, and is incompatible with the longterm survival of organisms with linear genomes.

1.3.1. Solutions to the end replication problem in various linear genomes

All organisms with linear genomes have evolved mechanisms to counteract the end replication problem. The most common solution to this problem in eukaryotes is the telomerase enzyme, which extends the 3' ends of telomeres to permit more complete replication of this strand (section 1.5). Some viruses such as adenoviruses and certain bacteriophages use a protein-primed mechanism that initiates replication at DNA ends (Salas 1991). DNA replication in prokaryotic *Streptomyces* species is also protein-primed,

Figure 1.4: The end replication problem

The end-replication-problem (section 1.3). Double stranded DNA (top) is replicated by the movement of replication forks throughout the entire chromosome (middle). RNA primers are required for initiation of DNA synthesis by DNA polymerase. RNA primers are replaced by gap-filling DNA synthesis following replication everywhere except for the extreme 5′-end (bottom). Thus, the newly synthesized DNA is slightly shorter, with a loss of about 50–200 bp per cell division (as observed in telomerase negative human fibroblasts *in vitro*).

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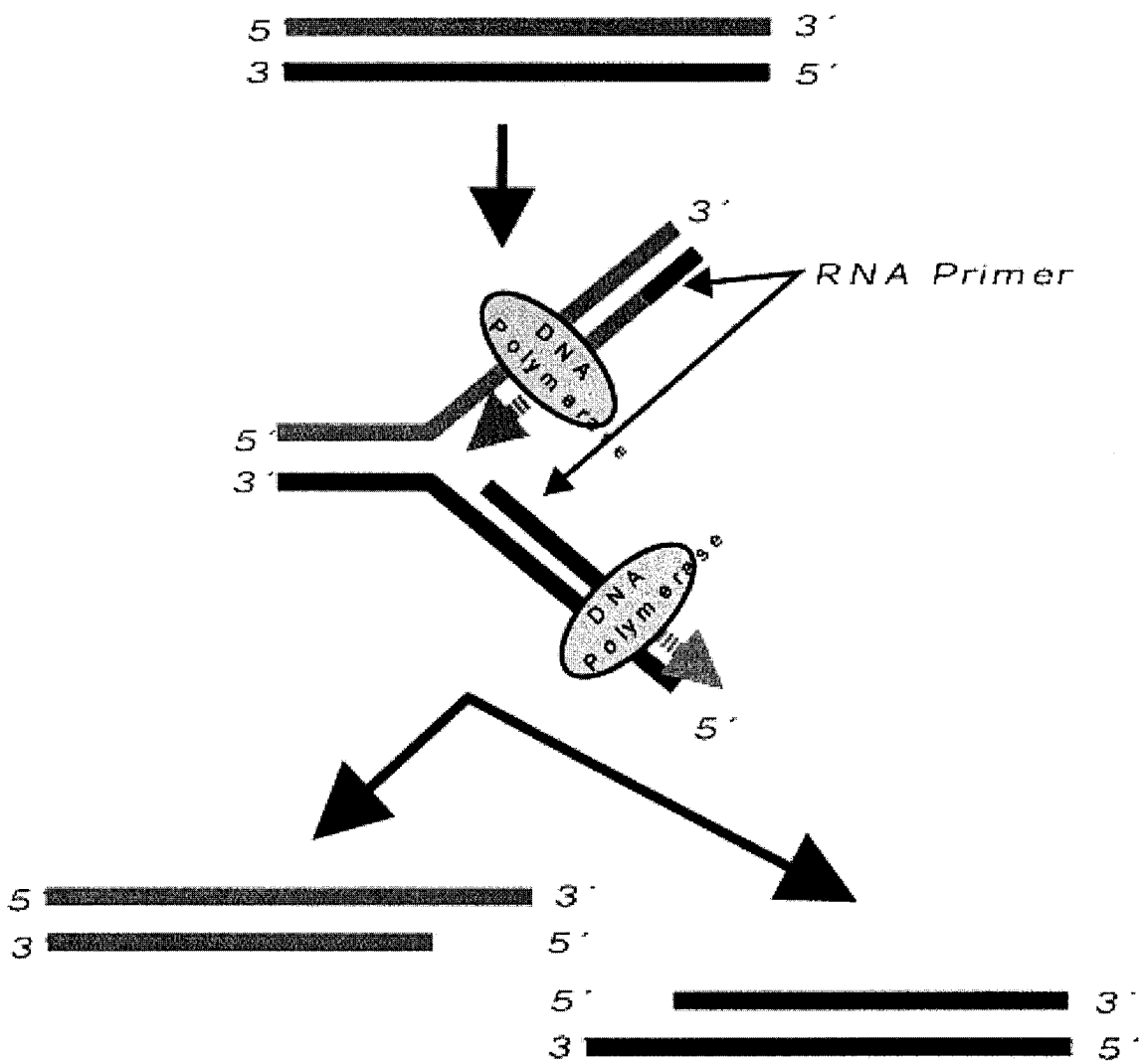
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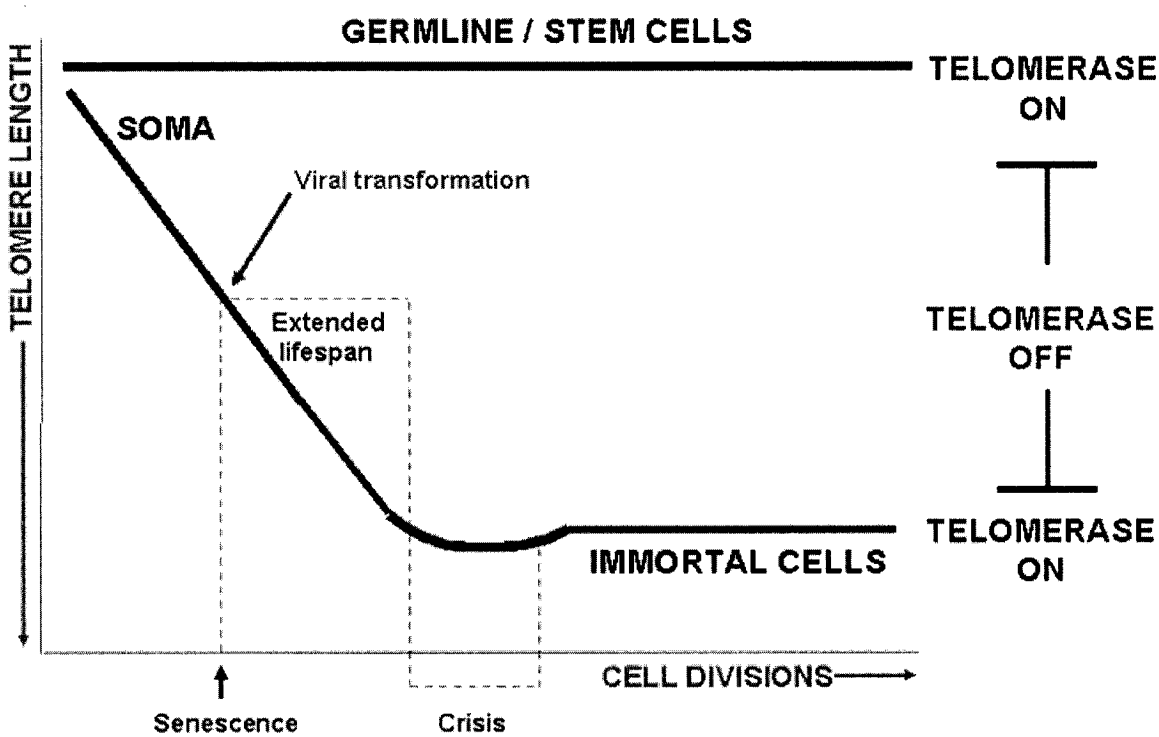
GERMLINE / STEM CELLS
TELOMERASE ON
SOMA
Viral transformation
TELOMERE LENGTH
Extended lifespan
TELOMERASE OFF
IMMORTAL CELLS
TELOMERASE ON
CELL DIVISIONS
Senescence
Crisis

but DNA replication is initiated at an internal origin and results in recessed 5' ends that may be filled in by a telomere-associated DNA polymerase or topoisomerase with reverse transcriptase activity (Qin and Cohen 1998; Bao and Cohen 2004). *Drosophila* use retrotransposition of telomere-embedded non-long terminal repeat (LTR) retroelements to counteract telomere loss, and *Chironomus* species (biting midges) may use a retrotransposition-independent reverse transcriptase-mediated mechanism to maintain their telomeres (reviewed in Pardue and DeBaryshe 2003). Furthermore, an LTR retrotransposon, Ty1, is activated by telomere erosion in *S. cerevisiae*, and may play a role in telomere maintenance in the absence of telomerase (Scholes et al. 2003; Maxwell et al. 2004). Other genomes, including those of poxviruses and *Borrelia* species, have covalently closed hairpin ends formed from inverted repeats. Replication of these hairpins results in covalently linked circular (*Borrelia*) or concatameric head-to-head or tail-to-tail dimers (poxvirus) containing duplicated telomere sequences at the dimer junctions (reviewed in Kobryn and Chaconas 2001). These dimeric intermediates are resolved by sequence-specific cleavage of hairpin junctions and formation of new hairpin ends, a process that is catalyzed in *Borrelia* by a novel telomere-specific resolvase enzyme that exhibits properties similar to both site-specific recombinases and topoisomerases (Bankhead and Chaconas 2004 and references therein). Intriguingly, recent evidence suggests that inverted repeats play an important role in palindrome-mediated immortalization in *S. cerevisiae* when telomerase- and recombination-based mechanisms of telomere maintenance are disrupted (Maringele and Lydall 2004). Maringele and Lydall have proposed that in these yeast, inverted repeats could form a stable or transient hairpin structure that permits break-induced replication (BIR)-mediated duplication of centromere-proximal DNA sequences to form palindromes that flank the inverted repeat sequence; it is unknown if telomeric or any other form of chromosome resolution is required for palindrome-dependent telomere maintenance. Finally, many eukaryotes employ recombination-based mechanisms of telomere maintenance when the telomerase pathway is disrupted or absent; these mechanisms may include rolling circle replication of extragenomic circular DNA molecules containing telomeric repeats, intra-telomeric BIR within the t-loop structure, and inter-telomeric recombination. Though it is evident that the end replication problem can be circumvented by multiple strategies, sometimes even in the same organism (*S. cerevisiae*), the remainder of this review will focus on telomerase- and

recombination-based mechanisms of telomere maintenance, as these are the best-understood forms of telomere replication in eukaryotes.

1.3.2. Recombination-dependent telomere length maintenance

Telomerase-independent mechanisms of telomere maintenance in eukaryotes were first observed in *S. cerevisiae* strains deficient in the telomerase-associated protein Est1p, but have since been identified in all *S. cerevisiae* strains null for one of the five genetic components of the telomerase pathway (*TLC1*, *EST1*, *EST2*, *EST3*, *CDC13*) (reviewed in Lundblad 2002) (Lundblad and Blackburn 1993; Singer and Gottschling 1994; Lendvay et al. 1996; Teng and Zakian 1999). Telomerase-independent mechanisms of telomere maintenance have also been observed in *K. lactis* (*Kluyveromyces lactis*) and mammalian cells, and in a subset of *S. pombe* cells that do not form circular chromosomes in response to telomerase deficiency (McEachern and Blackburn 1996; Bryan et al. 1997; Nakamura et al. 1998).

Telomere maintenance in telomerase-defective budding yeast, which are called "survivors" because of their ability to escape telomere shortening-induced cell death and replicative senescence, is dependent on the *RAD52* gene product, which is essential for most forms of homologous recombination in yeast (Lundblad and Blackburn 1993). Though all survivors exhibit unstable growth patterns over time, and dynamic, rapid changes in the length and sequence of individual telomeres, two types of *RAD52*-dependent survivors have been identified. Type I survivors have short telomeres and exhibit extensive exchange and amplification of subtelomeric DNA sequences, whereas the telomeres of Type II survivors are very long and heterogeneous in length, and contain multiple copies of the entire G-rich telomeric DNA tract (Lundblad and Blackburn 1993; Teng and Zakian 1999). Though Type I survivors are more common, they frequently convert to the more stable Type II pattern of telomeric amplification (Teng and Zakian 1999). *RAD52*-dependent survivors also arise in telomerase-deficient strains of the budding yeast *K. lactis*, but are exclusively Type II (McEachern and Blackburn 1996). The G-rich telomeric DNA tracts of Type II survivors in *S. cerevisiae* and *K. lactis* are frequently longer than the telomeric DNA of telomerase-positive cells, and appear to undergo both rapid amplification and rapid deletion (McEachern and Blackburn 1996; Teng and Zakian 1999; Teng et al. 2000). The telomeres of Type II survivors also

resemble the telomeres of immortalized telomerase-negative human cells (referred to as ALT cells: alternative lengthening of telomeres), which are long and heterogeneous in length (section 1.3.2.2) (Bryan et al. 1997; Teng and Zakian 1999). Since the Type I telomere phenotype has not been identified in telomerase-negative cells from any organism except *S. cerevisiae*, it is possible that Type I telomere maintenance is not universal. Interestingly, Ty1 retrotransposon activity substantially increases the mobility of subtelomeric DNA sequences in Type I but not in Type II *S. cerevisiae* survivors or telomerase-positive cells, suggesting that retrotransposition might contribute to the *S. cerevisiae*-specific Type I survivor phenotype (Scholes et al. 2003; Maxwell et al. 2004). This hypothesis may be supported by the observation that Ty1 elements present in the *K. lactis* genome are predicted to be catalytically non-functional (Neuveglise et al. 2002). However, disruption of *RAD52* usually enhances activation of Ty1 retroelements in *S. cerevisiae*, though *RAD52* is required for integration of Ty1 cDNA sequences under certain conditions, implying that Ty1 activity cannot completely account for the subtelomeric amplifications seen in Type I survivors (Sharon et al. 1994; Rattray et al. 2000).

1.3.2.1. Break-induced replication and recombination-dependent telomere length maintenance

Break-induced replication (BIR) has been proposed as a major mechanism of recombination-dependent telomere length maintenance (Figure 1.5) (reviewed in Biessmann and Mason 2003). BIR is a one-ended recombination event that occurs when ssDNA with a free 3' hydroxyl group (normally present in the 3' overhang of telomeres, or generated by processing of DSBs) invades and hybridizes with homologous sequences in an intact donor DNA molecule, generating a D-loop (reviewed in Kraus et al. 2001); this BIR intermediate is very similar to the structure that has been proposed for t-loops (Griffith et al. 1999). The free 3' end is then extended by the DNA replication machinery, using the non-displaced donor strand as a template; synthesis of DNA complementary to the newly extended invading 3' strand results in the incorporation into the invading molecule of dsDNA sequences identical to those of the donor molecule. BIR often results in extensive elongation of the invading DNA molecule and could therefore permit copying ("capture") of all of the telomeric DNA sequences from a donor chromosome (Figure 1.5)

Figure 1.5: Break-induced replication (BIR)-dependent telomere replication using different templates.

Possible choices of different templates for BIR (section 1.3.2.1). (*A*) intermolecular homologous telomere, (*B*) intramolecular invasion forming a D-loop on the same chromosome, (*C*) extrachromosomal circular and (*D*) small linear DNA. The arrows indicate direction of 3' extension of the invading strand.

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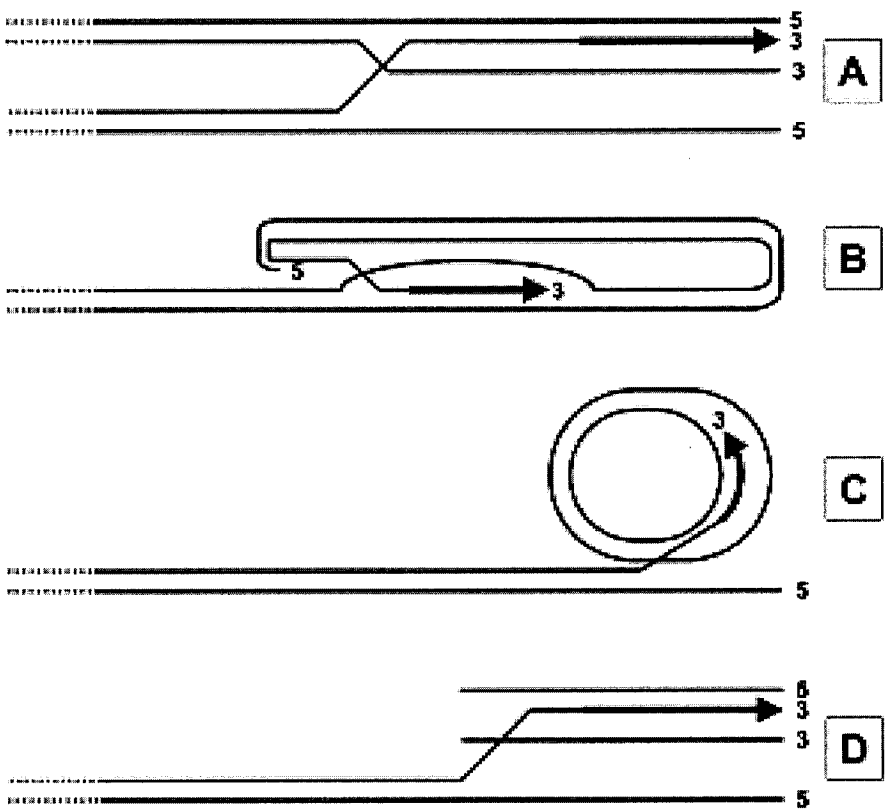

5
3
3
5
A
5
3
B
3
C
5
5
3
3
D
5

(Bosco and Haber 1998). This kind of event could explain the rapid telomere elongation phenotype of Type II survivors in *S. cerevisiae* and *K. lactis*, whose telomeres often contain multiple copies of the entire telomeric DNA sequence (McEachern and Blackburn 1996; Teng and Zakian 1999; Teng et al. 2000; Hackett et al. 2001). BIR could also be used to copy extrachromosomal DNA molecules containing telomeric or subtelomeric sequences (extrachromosomal telomere repeat-ECTR DNA) (Figure 1.5). In Type I survivors, such donors could include autonomously replicating circular DNAs or Ty1-mobilized linear DNAs containing subtelomeric DNA sequences (Lundblad 2002; Maxwell et al. 2004). Transfection of Type II *K. lactis* survivors with very small circular ECTR DNA molecules results in extensive elongation of telomeric sequences by a rolling circle replication (RCR) mechanism; telomerase-negative *S. cerevisiae* Type II survivors can also use circular ECTR DNA molecules for recombination-dependent telomere length maintenance (Natarajan and McEachern 2002; Lin et al. 2005). Recent evidence indicates that ECTR-containing DNA circles (t-circles) are far more abundant in human ALT cells than in telomerase-positive cells, and that these circles may be derived from rapid, HR-dependent deletion of t-loops (Cesare and Griffith 2004; Wang et al. 2004). These observations suggest that BIR-mediated elongation of telomeres using RCR of t-circle templates might facilitate the extensive telomere elongation present in mammalian ALT cells (Figure 1.5). Another possibility, that would be relevant for the majority of eukaryotic cells, is that the t-loop itself could be used as a template for BIR/RCR (Figure 1.5) (Griffith et al. 1999; Lundblad 2002). This hypothesis is supported by experiments in *S. pombe*, where deletion of *TAZ1*, which has been implicated in t-loop formation *in vitro*, promotes the emergence of telomerase-negative survivors that maintain their telomeres by alternative mechanisms (Nakamura et al. 1998; Tomaska et al. 2004). The association of the RAD50/MRE11/NBS1 complex with the t-loop-regulating protein TRF2 at mammalian telomeres might facilitate such a process (Zhu et al. 2000).

1.3.2.2. Alternative lengthening of telomeres in mammalian cells

Approximately 10% of immortal human tumor cells are telomerase-negative and maintain their telomeres by alternative mechanisms (ALT) (reviewed in Henson et al. 2002). The mean telomere length in ALT cells is at least twice the mean telomere length of telomerase-positive immortal human cells, suggesting that they resemble yeast Type II

survivors (Henson et al. 2002). Furthermore, telomeres in the cells of a single cell line, and even within individual cells, exhibit a very broad length distribution, ranging from 3-50 kb (Henson et al. 2002). Fluorescence *in situ* hybridization (FISH) analysis performed using telomeric probes in ALT cells reveals that telomeric signals are frequently undetectable on individual chromosomes (Yeager et al. 1999; Perrem et al. 2001). ALT cells also resemble Type II survivors in that individual telomeres undergo rapid changes in length, including rapid amplification and rapid deletions (Murnane et al. 1994; Perrem et al. 2001). ALT cells undergo frequent telomeric exchanges, and tagged telomere sequences are copied from one telomere to another in ALT cells, but not in telomerase-negative mortal cells or telomerase-positive immortal cells, indicating that the ALT mechanism is recombination-based (Murnane et al. 1994; Dunham et al. 2000; Varley et al. 2002; Bailey et al. 2004; Londono-Vallejo et al. 2004). Similar to Type II survivors, telomeric recombination occurs in the G-rich telomeric sequences and not in the sub-telomeric regions of ALT cells (Dunham et al. 2000; Henson et al. 2002). ALT cells do not exhibit enhanced rates of recombination in genomic regions outside the telomeres, implying a telomere-specific recombination mechanism (Bechter et al. 2003). Defects in mismatch repair, which inhibits recombination between non-identical sequences, promote the emergence of telomerase-independent survivors/ALT cells in *S. cerevisiae*, *K. lactis* and human cells (Rizki and Lundblad 2001; Bechter et al. 2004). The tumor suppressor p53 inhibits recombination in a transactivation-independent fashion, and mutation or disrupted expression of p53 is strongly associated with the emergence of ALT in human cells (Zaineb et al. 2004 and references therein). The mechanisms of recombination-based telomere length maintenance in ALT cells are unknown, and may include unequal sister chromatid or inter-chromosomal telomere exchange, BIR-mediated recombination using intratelomeric, intertelomeric or ECTR DNA as a template, RCR of ECTR circular DNAs or within t-loops, or ligation of ECTR DNA to chromosomal telomeres (reviewed in Henson et al. 2002); (Bailey et al. 2004; Cesare and Griffith 2004; Londono-Vallejo et al. 2004; Wang et al. 2004).

ECTR DNA in ALT cells is frequently found in foci referred to as ALT-associated promyelocytic leukemia bodies (APBs) (Yeager et al. 1999). APBs contain the telomere-binding proteins TRF1 and TRF2 and telomere-associated proteins hRAP1 and RPA, as well as promyelocytic leukemia (PML) protein, members of the MRE11 complex

(RAD50, MRE11, NBS1), p53, RAD51, RAD52, BLM, WRN, BRCA1, phosphorylated histone H2AX (γ-H2AX) and the DNA damage-associated clamp complex hRAD9/hHUS1/hRAD1; many of these proteins are implicated in DNA damage signaling, DNA repair and recombination (Nabetani et al. 2004 and references therein). APBs are also found at telomeres in ALT cells (Nabetani et al. 2004). APBs appear in late S to G_2 phases of the cell cycle, and are sites of DNA synthesis (Wu et al. 2000; Nabetani et al. 2004). Together, these observations suggest that ALT-mediated telomere elongation may occur at APBs, though they could also constitute telomere-specific DNA damage foci.

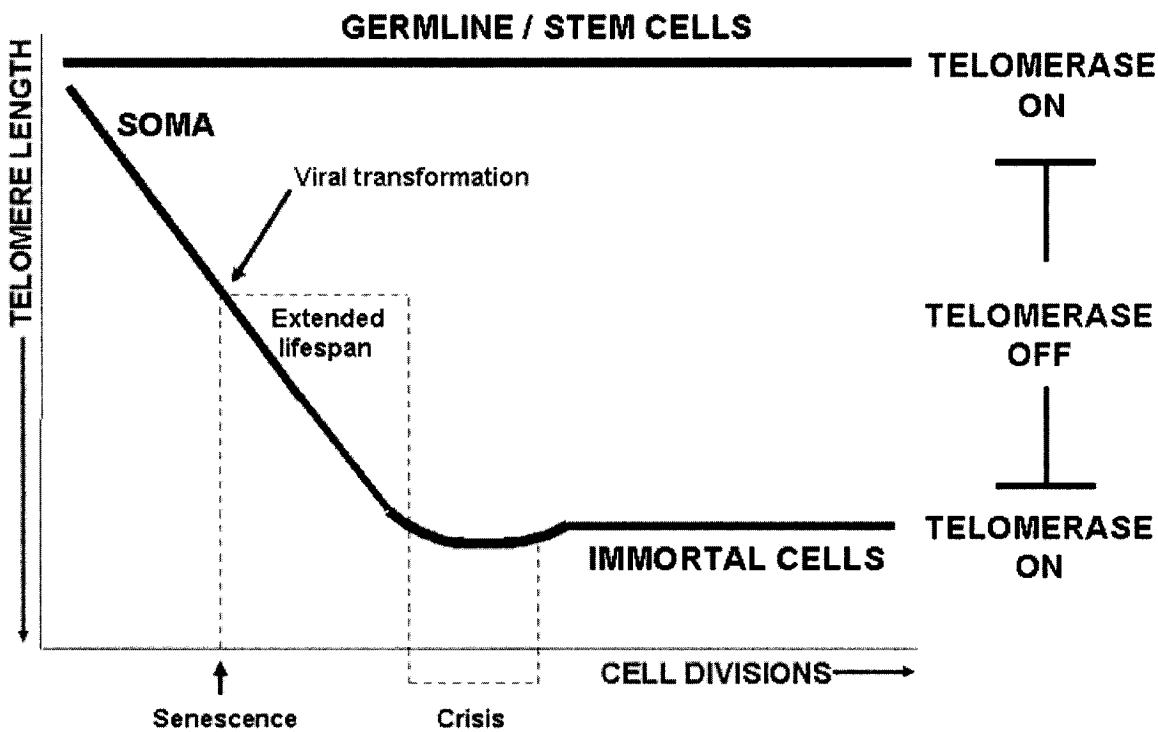
1.4. Consequences of telomere shortening and dysfunction

In the late 1930s, Barbara McClintock and Herman Muller observed that natural chromosome ends are distinct from those of broken chromosomes in that they are protected from fusion with other DNA ends (Muller 1938; McClintock 1939). These conclusions have subsequently been confirmed by hundreds of studies in diverse organisms demonstrating that telomere dysfunction leads to chromosome fusions and breakage, genome damage and rearrangements, and ultimately, permanent cell cycle arrest, cell death and (in multicellular eukaryotes) cellular transformation and tumorigenesis (reviewed in Artandi and DePinho 2000; Harrington and Robinson 2002; Godinho Ferreira et al. 2004). Telomere dysfunction can be directly induced in numerous ways, including mutation of the telomeric DNA sequences synthesized by telomerase, and inhibition, mutation or genetic ablation of telomerase components or telomere-associated proteins (reviewed in Harrington and Robinson 2002; Godinho Ferreira et al. 2004).

Some multicellular organisms such as humans do not express robust telomerase activity in the majority of their somatic tissues after development (reviewed in Aviv et al. 2003; Bekaert et al. 2004). Fibroblasts and many other human somatic cells with little or no telomerase activity have a limited lifespan *in vitro*, and stop dividing after a fixed number of cell divisions (replicative senescence) (Figure 1.6). This replicative arrest is referred to as the Hayflick limit, and is partially attributable to the progressive shortening of telomeres with each round of DNA replication (Hayflick 1965; Harley 1990; Allsopp et al. 1992). The hypothesis that telomere shortening limits the *in vitro* proliferative lifespan of at least some human cell types has been directly demonstrated by the immortalization of telomerase-negative fibroblasts through ectopic expression of telomerase

Figure 1.6: Telomere hypothesis of cellular aging and immortalization

Telomerase is inactive in most human somatic cells, and telomere shortening over successive rounds of DNA replication eventually results in replicative senescence *in vitro* (Hayflick limit) (Section 1.4). Viral transformation can sometimes extend the proliferative lifespan of telomerase-negative cells by inactivating tumor suppressor pathways and DNA damage checkpoints; however, even transformed cells eventually undergo a period of massive cell death referred to as crisis when their telomeres become critically short. In almost all cases, the few cells that escape crisis reactivate telomerase, which is essential for the replicative immortality of most human cells.


GERMLINE / STEM CELLS
TELOMERASE ON
TELOMERE LENGTH
SOMA
Viral transformation
Extended lifespan
TELOMERASE OFF
IMMORTAL CELLS
TELOMERASE ON
CELL DIVISIONS
Senescence
Crisis

(Bodnar et al. 1998). Furthermore, telomere length in blood cells is predictive of age at mortality in human donors 60 years or older, implying that telomere length is also a determinant of human lifespan *in vivo* (Cawthon et al. 2003). This conclusion is supported by the observation that autosomal dominant dyskeratosis congenita (DKC), which is a fatal progeroid syndrome caused by hypomorphic mutations in the human telomerase RNA component (section 1.4.1.3), is characterized by progressive telomere shortening and anticipation of disease severity and age of onset in successive generations of genetic carriers (Vuillamy et al. 2004). Therefore, telomere length in humans contributes to the regulation of *in vitro* and *in vivo* lifespan.

Telomere shortening in mouse models of telomerase deficiency also results in many of the phenotypes predicted by *in vitro* models of telomere disruption, including telomeric chromosome fusions, impaired cellular proliferation, apoptosis, tumorigenesis and aging-related pathologies (reviewed in Blasco 2003) (Blasco et al. 1997; Lee et al. 1998; Rudolph et al. 1999). In telomerase-negative mice null for one allele of the tumor suppressor gene p53, telomere shortening and telomeric chromosome fusions result in complex nonreciprocal translocations that are the hallmark of human epithelial cancers; p53 deficiency strongly enhances tumorigenesis in telomerase-null mice, suggesting that telomere shortening is a tumor-suppressing mechanism in a wild-type p53 background (Artandi et al. 2000). Indeed, though telomere shortening-induced genomic instability may promote cellular transformation, continuous loss of telomeric sequences is ultimately incompatible with cellular immortality, and must be rescued by the upregulation of telomere maintenance mechanisms such as telomerase or ALT. This hypothesis is supported by the observations that telomerase activity is upregulated in approximately 90% of all human cancers and immortal laboratory cell lines, whereas the remaining 10% of cancers and cell lines maintain their telomeres by ALT mechanisms (reviewed in Shay and Bacchetti 1997; Henson et al. 2002). Telomere shortening in mouse models also leads to phenotypes that are associated with human aging, particularly in highly proliferative tissues. These phenotypes include impaired immune and stress responses, reduced wound healing and fertility, and dysfunction in tissues such as the spleen, bone marrow and intestines (Lee et al. 1998; Rudolph et al. 1999). The hypothesis that telomere shortening is central to the processes of carcinogenesis and aging is confirmed by the observation that

reintroduction of telomerase in telomerase knockout mice with short telomeres rescues chromosomal instability and premature aging (Samper et al. 2001).

1.4.1. Telomeres and human disease

In addition to its contributions to tumorigenesis and the phenotypes associated with "normal" human aging, telomere dysfunction is also thought to play a role in at least three human diseases, Werner syndrome (WS), ataxia-telangiectasia (A-T) and dyskeratosis congenita (DKC). All of these diseases are characterized by elevated incidence of cancer and aging-associated pathologies.

1.4.1.1. Werner syndrome

WS is an autosomal recessive progeroid syndrome caused by mutations in the Werner syndrome helicase (WRN), a member of the RecQ family of helicases (reviewed in Kipling et al. 2004). Individuals with WS are prone to the development of sarcomas, and exhibit early onset, severe manifestations of many pathologies associated with human aging, including arteriosclerosis, Type II diabetes, osteoporosis, cataracts, thymic atrophy and infertility; however, the immune response and central nervous system function are normal in WS patients (Kipling et al. 2004). Interestingly, unaffected tissues in WS patients are those that normally express telomerase or are post-mitotic (Kipling et al. 2004). WS fibroblasts have a very limited replicative lifespan *in vitro*, but can be immortalized by ectopic expression of telomerase, implicating telomere shortening and/or dysfunction in the WS phenotype (Wyllie et al. 2000). Furthermore, *WRN* knockout mice do not display signs of premature aging, whereas telomere shortening and aging phenotypes associated with telomerase ablation are accelerated in mice that express neither WRN nor TR; mice doubly null for *WRN* and *TR* exhibit pathologies such as loss of bone mass that are not observed in telomerase knockout mice, and their phenotype recapitulates the pathologies found in WS patients (Lombard et al. 2000; Chang et al. 2004; Du et al. 2004). Similar synergistic phenotypes have also been observed in telomerase knockout mice that express a hypomorphic allele of the Bloom syndrome RecQ helicase (BLM) (Du et al. 2004). Both WRN and BLM interact with TRF2 *in vitro* and *in vivo*, though colocalization of WRN and TRF2 may be restricted to the telomeres and APBs of telomerase-negative ALT cells (Opresko et al. 2002; Opresko et al. 2004). Disruption of

the *S. cerevisiae* RecQ helicase Sgs1p also accelerates replicative senescence and prevents the emergence of survivors in telomerase-negative yeast, suggesting that the RecQ helicase family is involved in telomere maintenance in the absence of telomerase (Huang et al. 2001; Johnson et al. 2001). The RecQ helicases contribute to genome integrity, and have been implicated in DNA replication, suppression of illegitimate recombination, and recombination-dependent repair of collapsed replication forks (a process which may resemble BIR) (reviewed in Khakhar et al. 2003). *In vitro*, they catalyze unwinding of G-quadruplexes and Holliday junction-like structures, and can resolve telomeric D-loops (Orren et al. 2002; Khakhar et al. 2003; Opresko et al. 2004). WRN colocalizes with TRF2 at the APBs and telomeres of ALT cells during S-phase, suggesting that in these cells WRN may be directly involved in telomeric DNA replication, especially as S-phase DNA synthesis has previously been observed at the telomeres and APBs of ALT cells (Wu et al. 2000; Nabetani et al. 2004; Opresko et al. 2004). Recent evidence indicates that telomeres replicated by lagging strand DNA synthesis are deleted in human cells lacking WRN, implying that this helicase may be important for the replication of telomeric DNA or suppression of illegitimate recombination (Crabbe et al. 2004)..

1.4.1.2. Ataxia telangiectasia

A-T is an autosomal recessive disease whose phenotypes include premature aging, infertility, immunodeficiency, progressive neurological degeneration and extreme sensitivity to ionizing radiation (reviewed in Pandita 2002). The product of the *ataxia-telangiectasia mutated* (*ATM*) gene, ATM kinase, is an evolutionarily-conserved phosphoprotein that plays central roles in cell cycle progression, maintenance of genome integrity and the cellular response to DSBs. ATM is activated in response to telomere uncapping and fusion in human cells, resulting in p53-dependent apoptosis (Karlseder et al. 1999). The loss of ATM or ATM orthologue function in diverse eukaryotes, from humans to *Drosophila*, causes loss of telomeric DNA sequences and/or extensive telomere-mediated chromosome fusions, suggesting that ATM is important for telomere protection (Pandita et al. 1995; Metcalfe et al. 1996; Naito et al. 1998; Ritchie et al. 1999; Bi et al. 2004; Silva et al. 2004). A-T cells have a reduced replicative lifespan *in vitro*, which can be extended by ectopic telomerase expression (Wood et al. 2001). Furthermore, TRF1 inhibition-induced telomere lengthening in A-T cells rescues their radiosensitive

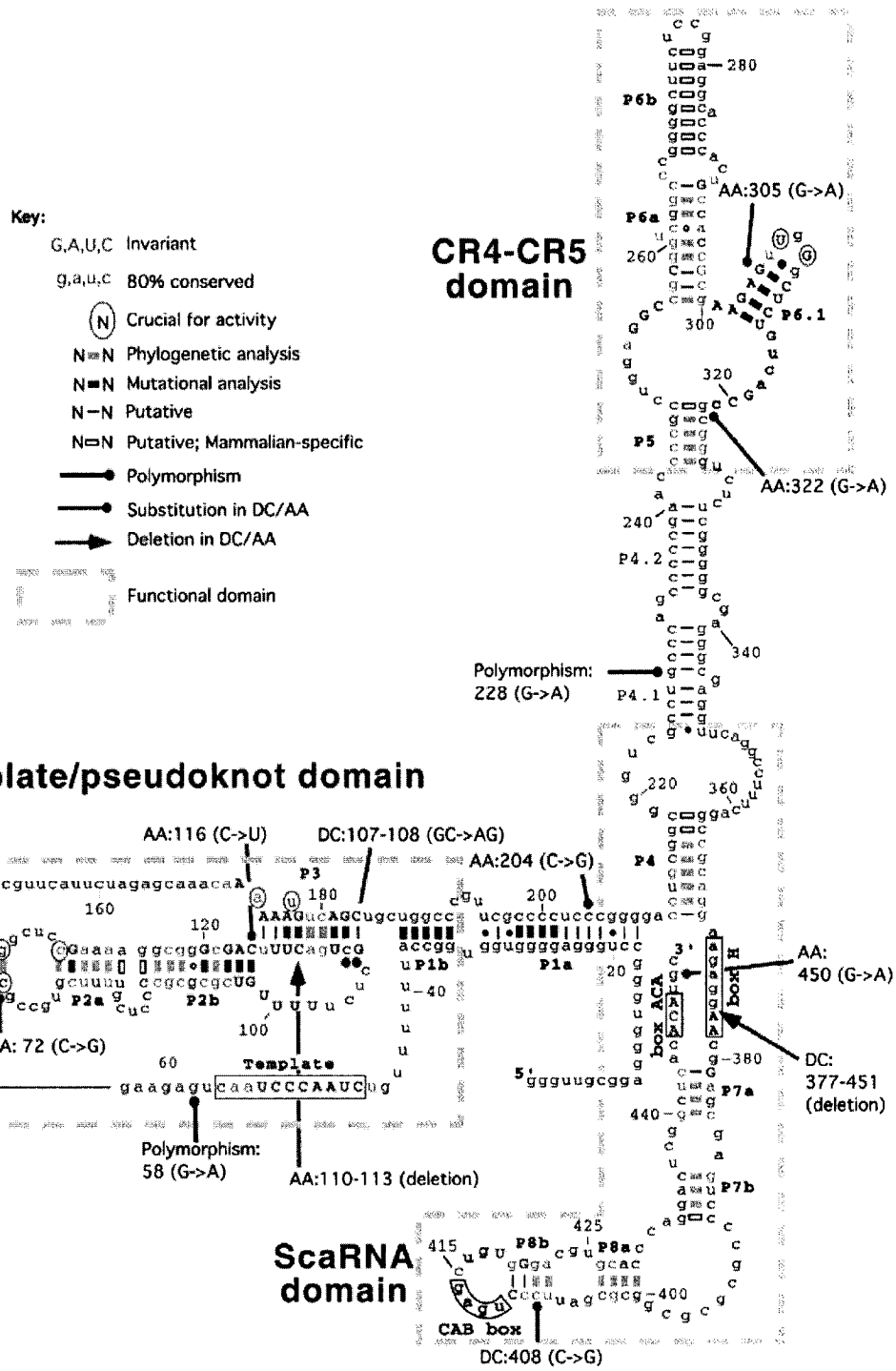
phenotype (Kishi and Lu 2002). ATM deficiency in telomerase-negative mice with long telomeres does not cause telomere-mediated chromosome fusions, whereas telomerase-negative ATM-deficient mice with short telomeres exhibit a pronounced telomere fusion phenotype and several of the pathologies associated with A-T (Qi et al. 2003; Wong et al. 2003). ATM may play multiple roles in HR-dependent repair of DSBs, and is known to directly or indirectly regulate many of the proteins found at normal telomeres and in the APBs of ALT cells, including TRF1, NBS1, BLM and BRCA1, γH2AX and RAD51 (reviewed in Valerie and Povirk 2003). ECTR-containing circular DNAs are also observed in murine cells with defective ATM function, as well as in A-T patients, though A-T mutations do not affect HR-dependent t-loop deletions (Hande et al. 2001; Wang et al. 2004). These observations suggest that, like the RecQ helicases, ATM might promote telomerase-independent telomere replication in cells with short telomeres. However, studies performed in yeast indicate that ATM may instead protect telomeres from NHEJ (Chan and Blackburn 2003). Though the mechanism(s) by which ATM prevents telomere shortening-induced telomere fusions in mammalian cells are not yet known, telomere shortening likely plays an important role in the expression of A-T phenotypes.

1.4.1.3. Dyskeratosis congenita

At clinical diagnosis, the DKC disease phenotype consists of a combination of abnormal skin pigmentation, nail dystrophy and mucosal leukoplakia, although other pathologies are also found in most highly proliferative tissues; the major cause of death in DKC patients is bone marrow failure (reviewed in Mason 2003). Two forms of DKC have been identified in humans, X-linked recessive, which is characterized by mutations in the gene encoding dyskerin (*DKC1*), and autosomal dominant (AD), which is associated with mutations in the genes encoding hTR; telomeres are abnormally short in the cells of patients with all forms of DKC (Mitchell et al. 1999b; Vulliamy et al. 2001; Ly et al. 2004 and references therein). Mutations in the gene encoding hTERT are also found in patients with aplastic anemia, a disease that is often associated with DKC (Yamaguchi et al. 2005). Dyskerin is a protein that interacts with H/ACA box small nucleolar RNAs (snoRNAs) in diverse eukaryotes, and in yeast and bacteria is involved in pseudouridylation of ribosomal RNAs (reviewed in Mason 2003). Dyskerin also interacts with an H/ACA box domain in hTR that is essential for hTR accumulation, and dyskerin mutations that cause DKC

Figure 1.7: Secondary-structure model of human telomerase RNA indicating sites of mutations found in patients with dyskeratosis congenita and aplastic anemia.

hTR contains three vertebrate-conserved structural and functional domains, the template/pseudoknot, CR4-CR5 and small Cajal-body RNAs (scaRNA) domains, which are indicated by broken boxes. The template/pseudoknot and CR4-CR5 domains are important for telomerase catalytic activity and hTERT interactions, and the scaRNA domain is implicated in processing, stability and subnuclear localization of hTR *in vivo*, but not catalytic activity *in vitro*. The template/pseudoknot domain consists of the template region, the pseudoknot structure and the P1 helix that defines the template boundary. The CR4-CR5 domain consists of two conserved regions (CR), CR4 and CR5, and includes the P6.1 helix. The scaRNA domain consists of three conserved motifs, box H, box ACA and CAB box. Base pairings supported by phylogenetic co-variation and mutational analysis are shown in green and blue, respectively. Putative base pairings that might exist in mammals and are not contradicted by phylogenetic data are shown as open dashes. Invariant residues are shown as capital letters in red, whereas residues that are 80% conserved are shown as lower-case letters in red. Individual residues crucial for telomerase activity are indicated by red ovals. Conserved sequence motifs, template, box H, box ACA and CAB box are indicated in black boxes. Mutations identified in patients with autosomal dominant dyskeratosis congenita (DC) and aplastic anemia (AA) are indicated by orange lines. Polymorphisms found in both healthy individuals and some aplastic anemia patients are indicated by blue lines.


Key:
G,A,U,C Invariant
g,a,u,c 80% conserved
N Crucial for activity
N=N Phylogenetic analysis
N=N Mutational analysis
N–N Putative
N⊐N Putative; Mammalian-specific
Polymorphism
Substitution in DC/AA
Deletion in DC/AA
Functional domain
CR4-CR5 domain
AA:305 (G->A)
AA:322 (G->A)
Polymorphism: 228 (G->A)
Template/pseudoknot domain
AA:116 (C->U)
DC:107-108 (GC->AG)
AA:204 (C->G)
AA: 72 (C->G)
Polymorphism: 58 (G->A)
AA:110-113 (deletion)
AA: 450 (G->A)
DC: 377-451 (deletion)
ScaRNA domain
CAB box
DC:408 (C->G)

impair the stable accumulation of hTR in human cells (Mitchell et al. 1999a; Mitchell et al. 1999b; Mitchell and Collins 2000). DKC dyskerin mutations have no effect on modification or accumulation of other cellular RNAs, suggesting that the DKC phenotype is telomerase-specific (Mitchell et al. 1999b). This conclusion is supported by the observation that hTR H/ACA box mutations are found in AD forms of DKC (Figure 1.7) (Vulliamy et al. 2001). Many other mutations in hTR sequences outside the H/ACA box have been implicated in AD DKC, as well as in diseases that occur alone, or in late stages of DKC; these diseases include aplastic anemia, myelodysplastic syndrome and essential thrombocytopenia (Figure 1.7) (Ly et al. 2004 and references therein). These mutations are found in hTR regions such as the H/ACA box that are required for *in vivo* accumulation of hTR but not *in vitro* telomerase catalytic activity, as well as in hTR sequences that are important for *in vitro* catalytic activity (Figure 1.7); however, most hTR mutations implicated in DKC and associated diseases result in reduced telomerase activity when reconstituted *in vivo*, either because of impaired hTR accumulation or innate catalytic defects (reviewed in Ly et al. 2004; Marrone et al. 2004). Human telomerase contains two functionally- and physically-interacting hTR molecules, and *hTR* mutations found in AD DKC and other hematopoietic diseases could in theory act in a dominant-negative fashion in the telomerase complex (Wenz et al. 2001; Ly et al. 2003b). Several groups have investigated the potential dominant-negative properties of DKC and other mutant hTRs by transiently or stably expressing equal amounts of wild-type (WT) and mutant hTRs in cells that express the telomerase catalytic subunit (hTERT), but not endogenous hTR (Fu and Collins 2003; Ly et al. 2004; Marrone et al. 2004; Cerone et al. 2005). In these *in vivo* reconstitution systems, none of the disease-associated hTR mutants identified to date inhibit WT telomerase catalytic activity, suggesting that disease phenotypes may be caused by haploinsufficiency (Fu and Collins 2003; Ly et al. 2004; Marrone et al. 2004; Cerone et al. 2005). Haploinsufficiency is also observed in mice heterozygous for *mTR* and a null allele, suggesting that WT levels of TR expression and activity are required for normal telomere length maintenance in mammals (Hathcock et al. 2002).

The human telomerase complex is also thought to contain two hTERTs, and haploinsufficiency is found in mice heterozygous for *mTERT* (Beattie et al. 2001; Wenz et al. 2001; Erdmann et al. 2004). Interestingly, deletion of a single hTERT allele is found in all patients with the severe neurological and developmental syndrome Cri du chat, which

is caused by deletion of terminal sequences of chromosome 5p (Zhang et al. 2003). Cells from affected children exhibit shortened telomeres, telomeric chromosome fusions, and reduced proliferative lifespan *in vitro*, phenotypes that are rescued by ectopic expression of hTERT. Though many of the symptoms of Cri du chat may be caused by loss of other genes in the terminal deletion, the observation that *hTERT* heterozygosity is insufficient for telomere length maintenance suggests that *hTERT* haploinsufficiency may also contribute to human disease (Zhang et al. 2003). This conclusion is supported by recent observations that telomeres are abnormally short in aplastic anemia patients bearing mutations in the gene encoding hTERT (Yamaguchi et al. 2005).

1.5. Telomerase: structure, function and regulation

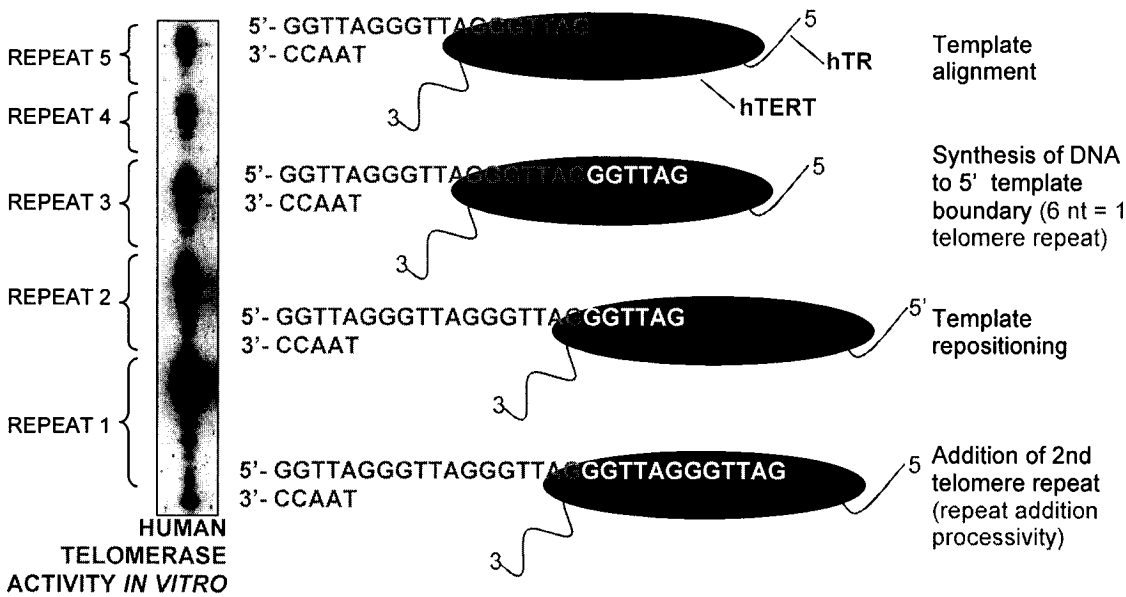
The first indication that eukaryotic telomeric DNA might be synthesized by an unconventional mechanism came from studies performed in Elizabeth Blackburn's lab in which linear yeast plasmids capped with *Tetrahymena* telomere sequences were transfected into yeast. Amazingly, the similar but distinct sequences of yeast telomeres were added to these plasmids, implying that telomeric DNA sequences were added in a *de novo* fashion by a yeast-dependent activity that did not use plasmid DNA sequences as a template (Szostak and Blackburn 1982; Shampay et al. 1984). Carol Greider in the Blackburn lab partially purified and characterized the agent responsible for the corresponding terminal transferase activity in *Tetrahymena*, a protein- and RNA-dependent enzyme that is now called telomerase (Greider and Blackburn 1985; Greider and Blackburn 1987). Telomerase has since been identified as the enzyme responsible for *de novo* addition of telomeric DNA sequences to telomeres in all eukaryotic organisms studied to date, with the exception of some insects (section 1.3.1).

1.5.1. Telomerase minimal components

Tetrahymena and all other telomerases synthesize telomeric DNA by reverse transcription of a short template region in their integral RNA molecule, the telomerase RNA (TR; also referred to as TERC, TER, and TLC1) (Figure 1.8) (Greider and Blackburn 1989; Yu et al. 1990). More than ten years after the discovery of telomerase, its essential protein component, telomerase reverse transcriptase (TERT; also referred to as TRT, hEST2, TP2 and Est2p) was finally identified (Lingner et al. 1997b). TERT and TR

Figure 1.8: Telomerase catalytic cycle and repeat addition processivity.

Telomerase synthesizes a single telomeric DNA repeat (GGTTAG) by reverse transcription of a short template in the telomerase RNA. Translocation at the 5' template boundary permits telomerase to add a second telomeric repeat to the same DNA molecule. Multiple rounds of this catalytic cycle result in the characteristic 6-bp ladder of telomerase activity shown at left.


REPEAT 5
REPEAT 4
REPEAT 3
REPEAT 2
REPEAT 1
HUMAN TELOMERASE ACTIVITY *IN VITRO*
5'- GGTTAGGGTTAGGGTTAG
3'- CCAAT
5
hTR
hTERT
3
Template alignment
5'- GGTTAGGGTTAGGGTTAGGGTTAG
3'- CCAAT
5
3
Synthesis of DNA to 5' template boundary (6 nt = 1 telomere repeat)
5'- GGTTAGGGTTAGGGTTAGGGTTAG
3'- CCAAT
5'
3
Template repositioning
5'- GGTTAGGGTTAGGGTTAGGGTTAGGGTTAG
3'- CCAAT
5
3
Addition of 2nd telomere repeat (repeat addition processivity)

are the only components required for reconstitution of human, mouse, *Xenopus* and *Tetrahymena* telomerase activities in expression systems such as rabbit reticulocyte lysates (RRL), yeast and insect cells, implying that these are the minimal subunits of the telomerase enzyme (Weinrich et al. 1997; Beattie et al. 1998; Collins and Gandhi 1998; Greenberg et al. 1998; Bachand and Autexier 1999; Masutomi et al. 2000; Kuramoto et al. 2001; Wenz et al. 2001). However, native telomerase complexes are large (1 MDa and larger in mammals and yeast), and likely contain additional proteins that are required for *in vivo* function and/or regulation (sections 1.5.3.4 and 1.5.4.4) (reviewed in Collins 1999). Reconstitution of yeast telomerases in heterologous expression systems has not been reported, and it is unknown if this reflects a requirement for catalytic components in addition to TR and TERT.

Though catalytically active *Tetrahymena* telomerase purified from RRL and native extracts under stringent conditions is a monomer containing one TR and one TERT, several lines of evidence suggest that human, yeast and *Euplotes* telomerases may be dimers (Bryan et al. 2003). First, the minimal active human telomerase complex purified from recombinant and endogenous sources is 600 kDa in size, contains two hTRs, and is predicted by molecular weight calculations to contain two hTERTs (Wenz et al. 2001). Similarly, the minimal endogenous *Euplotes* telomerase complex is predicted to be a dimer containing two TRs and two TERTs (Wang et al. 2002). Second, TRs containing different, "marked" templates have been shown to function cooperatively in human and *S. cerevisiae* telomerases; yeast and *Euplotes* telomerases can also cooperatively elongate distinct DNA substrates (Prescott and Blackburn 1997a; Wenz et al. 2001; Wang et al. 2002). Third, functional complementation between distinct, inactive hTERT or hTR variants can reconstitute human telomerase activity (Chapters 2 and 3) (Tesmer et al. 1999; Beattie et al. 2001). Finally, hTR dimerizes *in vitro*, and reciprocal coimmunoprecipitation has also been observed for TERTs from human and *Euplotes*, but not *Tetrahymena* (Arai et al. 2002; Wang et al. 2002; Bryan et al. 2003; Ly et al. 2003b). Together these observations suggest the possibility that some telomerases may contain two active sites, whereas *Tetrahymena* telomerase contains only one. It has been proposed that the presence of two active sites could permit the coordinated elongation of two sister chromatid telomeres *in vivo*, or that alternating use of two templates (template-switching) might facilitate the addition of multiple telomeric repeats to a single DNA substrate (repeat addition

processivity) (section 1.5.2.1) (Prescott and Blackburn 1997a; Wenz et al. 2001). Recent evidence suggests that human telomerase may not use both of its templates to synthesize multiple telomeric repeats, at least after the first ten or more repeats have been added; however, it is not clear if template-switching is obligatory during initial rounds of DNA synthesis (Rivera and Blackburn 2004). The number of active sites in various telomerases is still unknown, and dimerization in certain organisms' telomerases could also be attributable to substantial differences in the architecture of their TRs, a more cooperative mode of catalysis in these organisms' enzymes, or differences in the context and requirements of telomerase catalytic function *in vivo*. A number of RTs such as HIV-1 (human immunodeficiency virus-1) use template-switching between dimeric templates during genome replication *in vivo* but do not require a dimeric template to function catalytically *in vitro*; the HIV-1 RT dimer also contains a single active site cooperatively formed by interactions between two asymmetrically-arranged RT monomers (reviewed in Götte et al. 1999). This example implies that multimerization of some telomerases must not be interpreted simplistically as an indicator of the presence of more than one active site, and that the role of telomerase dimerization may be complex. These issues will be discussed further in Chapters 2 and 3.

1.5.2. Telomerase catalytic properties

Like all other DNA polymerases, telomerase catalyzes the addition of nucleotides to substrates with a free 3' OH group (Greider and Blackburn 1985). Telomerase extends ssDNA primers and duplex DNA with 3' overhangs, but cannot elongate blunt dsDNA molecules, dsDNA with 5' overhangs or DNA substrates that form G-quadruplex structures (Greider and Blackburn 1985; Greider and Blackburn 1987; Zahler et al. 1991; Lingner and Cech 1996). The 3' overhang length requirement varies for different organisms' telomerases; in humans the minimal length required for catalytic function is 6 nt (Rivera and Blackburn 2004). Human telomerase can elongate hPOT1-bound DNA *in vitro* if 8 nt of the substrate 3' end are exposed, but hPOT1 binding to more terminal 3' sequences inhibits activity, consistent with the minimal substrate length requirements of human telomerase (Kelleher et al. 2005; Lei et al. 2005). Many telomerases, including human, can elongate primers that do not contain their cognate telomeric DNA sequences (non-telomeric primers), a property which may be important for healing broken

chromosomes *in vivo* (section 1.5.2.4) (Greider and Blackburn 1987; Harrington and Greider 1991; Morin 1991). Though telomerase can elongate non-telomeric primers, it exhibits a preference for G-rich content in these sequences, especially at the substrate 5' end, and the minimal primer length required for elongation is longer than for telomeric sequences; the specificity of these substrate preferences is thought to reflect the presence of a template-independent anchor site in telomerase that regulates interactions with the 5' sequences of substrates (section 1.5.2.2) (Greider and Blackburn 1987; Morin 1991; Wang and Blackburn 1997). Telomerase is a highly specialized and unusual reverse transcriptase. The mechanism of telomerase reverse transcription will be discussed in the following sections, as will a number of telomerase catalytic properties that are not shared with other reverse transcriptases.

1.5.2.1. Telomerase catalytic cycle and repeat addition processivity

Telomerase-mediated elongation of telomeric DNA substrates is thought to be initiated by a recognition step in which the 3' end of the primer aligns and base-pairs with complementary sequences at the 3' end of the TR template, forming a short RNA-DNA hybrid (Figure 1.8) (reviewed in Harrington 2003). After the initial alignment/recognition step, telomerase reverse transcribes the TR template one nucleotide at a time until it reaches the 5' boundary of the template, where it pauses and remains tightly associated with its DNA product (yeast telomerases), or translocates so that the 3' end of the template is aligned with the newly synthesized DNA 3' end (ciliate and human telomerases) (Figure 1.8). This translocation enables another round of reverse transcription of the template, and repetitions of this cycle generate a periodic pattern of repeated DNA sequences that is characteristic of the catalytic activity of many telomerases (Figure 1.8). Telomerase's ability to add multiple complements of the TR template sequence to a single DNA molecule by translocating to the beginning of its template is referred to as repeat addition processivity (also called Type II or reiterative processivity), which is distinct from the conventional form of processivity used to translocate along the TR template during synthesis (Type I or nucleotide addition processivity). Yeast telomerases exhibit very weak repeat addition processivity, whereas human and ciliate telomerases can add hundreds of telomeric repeats to DNA substrates. Type I and/or Type II translocations during telomerase-mediated DNA synthesis presumably require the unwinding of

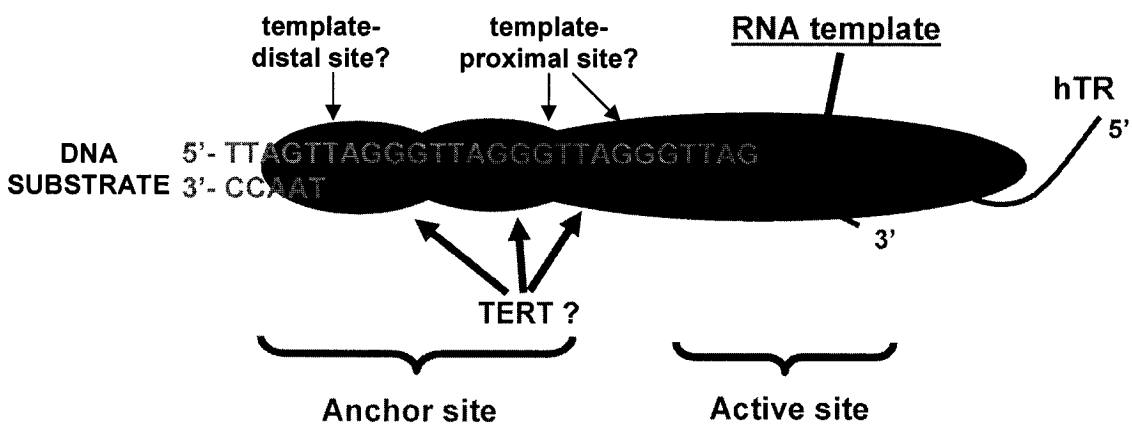
RNA/DNA hybrids formed during the alignment and elongation phases. The repeat addition processivity of ciliate, vertebrate and yeast telomerases is stimulated by dGTP, and the nucleotide addition processivity of yeast telomerase is enhanced by ATP, but does not require ATP hydrolysis (Lue and Wang 1995; Hammond and Cech 1997; Maine et al. 1999; Sun et al. 1999; Hardy et al. 2001; Bosoy and Lue 2004b). Though binding of these nucleotides might provide energy for translocation, it has also been proposed that conformational rearrangements in TR structures such as the pseudoknot could also drive unwinding of the RNA/DNA duplex (Collins 1999).

1.5.2.2. Anchor site

The 5' end of the DNA substrate also interacts with a template-independent site in telomerase (anchor site) that has been implicated in repeat addition processivity, in default placement of the DNA 3' end in the catalytic active site during elongation of completely non-telomeric DNA substrates, and in removal of non-telomeric 3' sequences from partially telomeric primers; the anchor site may also help to stabilize telomerase interactions with short DNA primers that cannot anneal extensively to 3' template sequences (Figure 1.9) (Harrington and Greider 1991; Morin 1991; Melek et al. 1996; reviewed in Collins 1999; Harrington 2003). Anchor site-facilitated telomerase activity does not require the presence of telomeric sequences in the substrate, but is enhanced by G-rich sequences in the 5' region of the primer (Harrington and Greider 1991; Morin 1991). Anchor site interactions might facilitate repeat addition processivity by preventing enzyme-substrate dissociation during Type II translocation, or by regulating the alignment or positioning of the DNA substrate in the active site (Greider 1991; Morin 1991; Lee and Blackburn 1993; Melek et al. 1994). The anchor site may be protein-dependent, and cross-linking studies in *Euplotes* indicate that this protein is likely TERT; our data suggest that a unique domain of the telomerase reverse transcriptase (RID1: section 1.5.4.3) may be part of the human telomerase anchor site (Chapter 4; Hammond et al. 1997). Catalytic evidence from ciliate, yeast and human telomerases suggests that the anchor site is bipartite; putative anchor site interactions with template-proximal and template-distal regions of the DNA substrate may contribute to different catalytic functions, though both anchor sites contribute to telomerase's affinity for DNA (Chapter 4; Collins and Greider 1993; Lee and Blackburn 1993; Hammond et al. 1997; Wang and Blackburn 1997; Lue and Peng 1998;

Figure 1.9: The putative telomerase anchor site.

Telomerase interactions with DNA substrates in the active site are mediated by base-pairing interactions between the TR template and the 3' end of the DNA substrate. A telomerase anchor site(s) regulates template base-pairing-independent interactions with the 5' sequences of substrates. The anchor site may be divided into two regions that interact with template-proximal and template-distal DNA sequences, and is thought to be TERT-dependent.


template-
distal site?
template-
proximal site?
RNA template
hTR
5'
DNA
SUBSTRATE
5'- TTAGTTAGGGTTAGGGTTAGGGTTAG
3'- CCAAT
3'
TERT ?
Anchor site
Active site

reviewed in Collins 1999). The bipartite anchor site and its catalytic functions will be discussed in greater detail in Chapter 4.

1.5.2.3. Nuclease activity

Most telomerases, including human can remove non-telomeric sequences from the 3' end of DNA primers (Collins and Greider 1993; Huard and Autexier 2004; Oulton and Harrington 2004; reviewed in Collins 1999). Telomerase-associated nuclease activity may be dependent on the minimal components of telomerase, since it is retained following stringent purification, and is present in recombinant *Tetrahymena* and human telomerases reconstituted in RRL (Collins and Gandhi 1998; Greene et al. 1998; Niu et al. 2000; Huard and Autexier 2004; Oulton and Harrington 2004). Interestingly, functional swapping of the TRs from two ciliates causes aberrant telomerase-mediated cleavage, suggesting that nuclease activity may be dependent on the TR, or on TR-interacting proteins (Bhattacharyya and Blackburn 1997). Telomerase-dependent nuclease activity requires the presence of telomeric sequences immediately 5' of the cleavage site and is template-directed under most conditions. In *Tetrahymena* and human telomerases, cleavage may occur at the 5' end of the template, but *Euplotes* and yeast telomerases can direct cleavage from a number of internal template positions; *Euplotes* and yeast telomerases may cleave by an endonucleolytic mechanism, whereas it is unclear if human telomerase-mediated cleavage is endo- or exonucleolytic (Collins and Greider 1993; Melek et al. 1996; Greene et al. 1998; Niu et al. 2000; Huard and Autexier 2004; Oulton and Harrington 2004). Telomerase nuclease function can remove internal mismatched DNA sequences that are not complementary to the TR template, suggesting that it may provide a proofreading function (Collins and Greider 1993; Melek et al. 1996; Huard and Autexier 2004). Human and other telomerases can also cleave entirely telomeric primers in a sequence-specific fashion that may reveal preferred substrates for elongation (Huard and Autexier 2004). Telomerase-dependent nuclease activity could also contribute to reinitiation of stalled replication complexes, as is observed for the nuclease activity of RNA polymerases; such a function might facilitate translocation at the 5' template boundary (Melek et al. 1996). It has also been proposed that telomerase-dependent cleavage might be important for elongation of partially-telomeric, G-rich substrates during chromosome healing/*de novo* telomere synthesis, and that this nuclease function could be dependent on the telomerase

anchor site (Harrington and Greider 1991). Recent reports of human telomerase nuclease function present conflicting data concerning the effects of primer length and 5' DNA sequences on nucleolytic cleavage; a potential anchor site influence on nuclease function has not been examined in other telomerases (Huard and Autexier 2004; Oulton and Harrington 2004). Nuclease activity is a property of many different nucleic acid polymerases, and is usually conferred by polymerase accessory domains (section 1.5.4.1); however, sites mediating nuclease function have not been identified in either of the telomerase minimal components.

1.5.2.4. *De novo* telomere synthesis

The first observation that telomerase can "heal" broken chromosomes by *de novo* addition of telomeric DNA sequences to DNA ends was made in *Tetrahymena* (Yu and Blackburn 1991). The life cycle of *Tetrahymena* and other ciliates consists of two phases, a vegetative phase, and a mating phase. During the mating phase (conjugation), the germline micronucleus is amplified into polytene chromosomes, from which gene-sized DNA molecules are excised, then stably capped by long telomerase-synthesized telomeric DNA sequences. Macronuclear telomeres are eventually trimmed to generate the short telomeres of the mature macronucleus found in vegetative cells (reviewed in Vermeesch and Price 1994). Since the initial observation in *Tetrahymena*, chromosome healing, identified as the presence of telomeric repeats at chromosome breakpoints, has been observed in other ciliates, mammals (rodents and humans), plants and yeast (Praznovsky et al. 1991; Kramer and Haber 1993; Lamb et al. 1993; Vermeesch and Price 1994; Tsujimoto et al. 1999). *De novo* telomere addition is thought to be facilitated by the presence of telomeric or G-rich seed sequences near the chromosome breakpoint, suggesting that the telomerase anchor site could be important in this process (Harrington and Greider 1991; Morin 1991; Wang and Blackburn 1997; Bottius et al. 1998; Klobutcher et al. 1998; Diede and Gottschling 1999). In yeast, chromosome breaksites healed by telomere addition frequently contain DNA sequences that can anneal with the TR template, suggesting that this mode of recognition is also important (Putnam et al. 2004). Telomerase-mediated addition of telomeric repeats to completely non-telomeric substrates during macronuclear development in *Euplotes* appears to be dependent on a dissociable member of the telomerase complex, implying that in this organism chromosome healing is

not entirely telomerase-dependent (Bednenko et al. 1997). Interestingly, the *Euplotes* genome contains three different *TERT* genes, one of which (*EcTERT-2*) is expressed specifically during the *de novo* telomere addition phase of macronuclear development (Karamysheva et al. 2003). EcTERT-2 differs most substantially from EcTERT-1 and EcTERT-3 in a telomerase-specific insertion between RT motifs A and B', which has been implicated in repeat addition processivity in *S. cerevisiae* (Karamysheva et al. 2003; Lue et al. 2003). The *Candida albicans* genome also contains two *TERT* genes, which encode proteins with sequence variations in RID1, RID2 and the C terminus (sections 1.5.4.2 and 1.5.4.3), though it is unknown if the expression patterns or functions of these variants are distinct (Metz et al. 2001). The telomerase-associated protein Est1p promotes the ability of *Candida* telomerase to elongate short primers or DNA substrates with non-telomeric 5' sequences *in vitro*, suggesting that this protein may stimulate the anchor site function of telomerase and/or chromosome healing (Singh and Lue 2003). In *S. cerevisiae*, *de novo* telomere formation requires components of the lagging strand DNA synthesis machinery that have been implicated in normal telomere length maintenance, members of the MRX complex (Mre11p, Rad50p/Xrs2p), the TR-interacting function of Ku, and telomerase pathway members Cdc13p and Est1p (Diede and Gottschling 1999; Diede and Gottschling 2001; Stellwagen et al. 2003; Bianchi et al. 2004). Together, these observations imply that *de novo* telomere addition may be dependent on many of the same factors that are important for the replication and processing of normal telomeres. It is still unknown how cells prevent telomerase-mediated chromosome healing from occurring at all DSBs.

1.5.3. Telomerase RNA

Telomerase RNAs are widely divergent in size and sequence among different eukaryotes, but share certain conserved features, including the presence of a C/A-rich template that directs synthesis of G/T-rich telomeric DNA sequences specific to each organism, a pseudoknot structure, and various family-specific stem-loop structures that mediate protein interactions (Figure 1.10) (reviewed in Chen and Greider 2004b).

Figure 1.10: Secondary structures of ciliate, vertebrate, and yeast (*Saccharomyces*) telomerase RNAs.

Structural elements in green represent conserved regions that bind to TERT. The structures that are present in some but not all species within a group are shown by dashed lines. The structures that define template region (red) and the template boundary (blue) are indicated. The RNA structural elements bound by protein components are indicated. In the *Saccharomyces* RNA, the putative stem-1 is indicated by red brackets.

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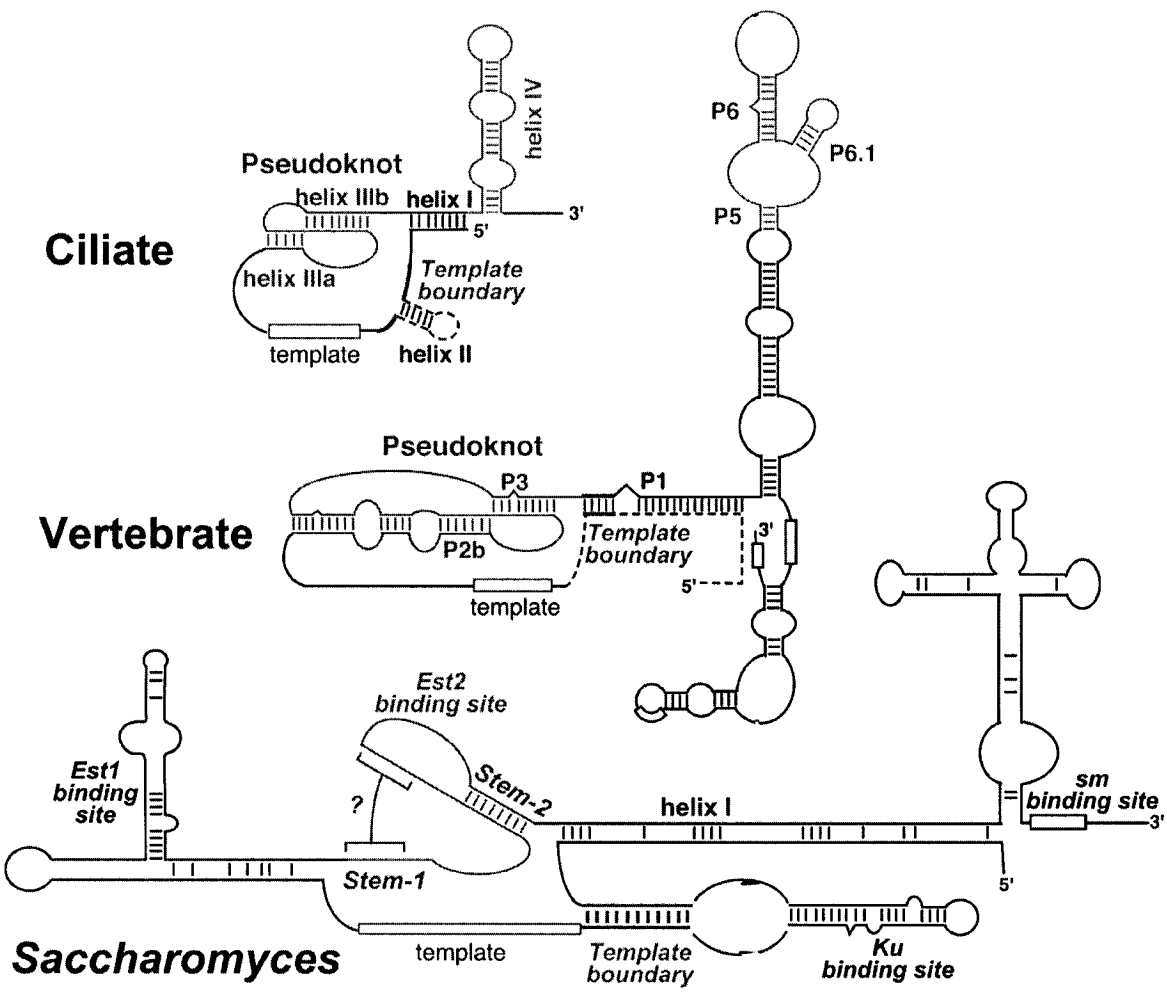

Ciliate
Pseudoknot
helix IIIb
helix I
helix IV
3'
5'
helix IIIa
Template boundary
template
helix II
P6
P6.1
P5
Vertebrate
Pseudoknot
P3
P1
P2b
Template boundary
3'
5'
template
Est2 binding site
Est1 binding site
?
Stem-2
helix I
sm binding site
3'
5'
Stem-1
Saccharomyces
template
Template boundary
Ku binding site

1.5.3.1. Template definition and use

The templates of TRs have been identified by RNase H cleavage of DNA/RNA hybrids produced by annealing complementary oligonucleotides containing telomeric DNA sequences to TR, and by testing the ability of these oligonucleotides to inhibit telomerase-mediated elongation of telomeric DNA substrates (Greider and Blackburn 1989). Template identification has been confirmed by analyzing the synthesis products of telomerases in which TR template residues are substituted with non-telomeric DNA sequences (Yu et al. 1990). Such analyses indicate that the template region of most TRs contains a permuted telomeric sequence that is longer than the length of the DNA repeat synthesized by telomerase, and typically encodes ~1.5 telomeric repeats (reviewed in Lue 2004). The hTR template is 11 nt in length (3'-CAAUCCCAAUC-5') and potentially encodes nearly two telomeric repeats, but directs synthesis of a single, 6 nt telomeric repeat sequence (5'-GGTTAG-3') (Figure 1.7) (Feng et al. 1995).

Since all TRs are very large with respect to their templates (451 vs 11 nt in hTR) (Figures 1.7 and 1.10), telomerase must also define the 3' and 5' boundaries of the TR sequence that is used as a template (Feng et al. 1995; reviewed in Collins 1999). Definition of the 3' boundary is important for directing the proper alignment of DNA substrates with the template during initiation, and is also critical for realignment of the 3' end of newly elongated DNA after translocation. Definition of the 5' boundary is essential to prevent synthesis of non-telomeric DNA sequences, since the presence of mutated sequences in telomeres is known to have serious consequences for telomere function and viability (Yu et al. 1990; McEachern and Blackburn 1995; Marusic et al. 1997; Guiducci et al. 2001).

Site-directed mutagenesis studies of hTR indicate that the two nucleotides 3' of the template coding sequences (3'-CAAUCCCAAUC-5') are likely essential for base-pairing-mediated alignment of the 3' end of the DNA substrate with the hTR template (substrate recognition); these alignment nucleotides are conserved in all vertebrate TRs (Gavory et al. 2002; Chen and Greider 2003a). In hTR, an additional three nucleotides 3' of the alignment sequences (3'-CAAUCCCAAUC-5') stimulate repeat addition processivity and overall activity levels by increasing the number of base-pairs that can form between the template and substrate; these processivity-enhancing sequences in hTR are degenerate in the mouse TR (mTR), and account at least partly for the greatly reduced repeat addition processivity of mouse telomerase compared to human enzyme (Gavory et al. 2002; Chen

and Greider 2003a). As in vertebrates, ciliate telomerases employ telomeric repeat-complementary sequences at the 3' end of their template coding sequences for base-pairing- dependent substrate alignment and stimulation of processivity (reviewed in Collins 1999). Many ciliate telomerases can also use much of the template region (up to 13 nt) for coding, though ciliate telomeric repeats are 6-8 nt in length (reviewed in Collins 1999). Template usage in *Tetrahymena* telomerase may be dependent on dGTP concentrations, and also depends on non-telomeric sequences 3' of the template (TRE: template recognition element) and on a ciliate-specific CP2 motif in the TERT N terminus that may stabilize or promote base-pairing-mediated alignment of the template and substrate (section 1.5.4.3) (Hammond and Cech 1997; Miller and Collins 2002). It is unknown if human telomerase, like *Tetrahymena*, can use its 3' alignment region for coding, or if it employs a 3' TRE or TERT-dependent 3' template alignment mechanism. Substitutions in alignment sequences affect the number of repeats synthesized by mouse and human telomerases, but do not appear to alter the pausing patterns or number of nucleotides in each repeat product, suggesting that the alignment region may not have a coding function (Chen and Greider 2003a). DNA substrates align in multiple registers with the template of the predominantly non-processive *S. cerevisiae* telomerase, resulting in the synthesis of the degenerate telomeric DNA repeats characteristic of *S. cerevisiae* telomeres (Forstemann and Lingner 2001).

The 5' boundary of the TR template is defined by template-proximal TR sequences and stem structures in all telomerases studied to date (Figure 1.10). A template-adjacent helix (Helix II) and flanking sequences define the 5' template boundary in *Tetrahymena* telomerase (Figure 1.10) (Autexier and Greider 1995; Autexier and Greider 1998; Lai et al. 2002). Helix II is not conserved in other ciliates, and even in some *Tetrahymena* species; however, template-adjacent sequences that are important for template boundary definition in *Tetrahymena* are highly conserved in all ciliates (Lingner et al. 1994; Autexier and Greider 1995; McCormick-Graham and Romero 1995). The template boundary definition mechanism in other ciliates has not been characterized. Template-proximal stem structures formed by long-range base-pairing interactions confer 5' template boundary definition in human, *K. lactis* and *S. cerevisiae* telomerases (Figure 1.10) (Prescott and Blackburn 1997b; Tzfati et al. 2000; Chen and Greider 2003b; Seto et al. 2003). In hTR, the 5' boundary-regulating stem structure is the P1b helix, which is

separated from the 5' template boundary by an 8 nt uridine-rich tract the length of which is important for boundary definition (Figures 1.7 and 1.10) (Chen and Greider 2003b). Interestingly, the high affinity TR interaction domain of *Tetrahymena* TERT that interacts with Helix II also contributes to 5' template boundary definition, and the 5' boundary-regulating element of *S. cerevisiae* TR is implicated in TERT interactions (Figure 1.10) (Miller et al. 2000; Lai et al. 2002; Seto et al. 2003). These observations suggest that TR-TERT interactions may be important for 5' template boundary regulation in all telomerases; however, 5' template boundary-regulating sequences have not yet been reported in non-ciliate TERTs. Recently, we have found that mutation of the hTERT RID1 domain and C-terminal hTERT sequences results in the incorporation of noncognate nucleotides in a manner that may be consistent with read-through past the 5' template boundary; our data also suggest that RID1 interacts with the hTR P1b helix and regulates 5' template usage and in a P1b-dependent fashion (Moriarty et al. 2005). hnRNPs C1 and C2 also interact with the single-stranded uridine-rich tract between the 5' boundary and the P1b helix; however, it is unknown if these hnRNPs contribute to 5' template boundary definition in human telomerase (Ford et al. 2000).

1.5.3.2. TR pseudoknot

All TRs are predicted to contain a central pseudoknot structure located near the template (Figure 1.10) (reviewed in Chen and Greider 2004a). Enzymatic probing, chemical modification and nuclear magnetic resonance (NMR) studies of naked ciliate and human TRs *in vitro* suggest that the pseudoknot is not stable, and may exist in equilibrium with a hairpin structure; it has been proposed that such conformational changes could be important for the telomerase catalytic mechanism, perhaps by functioning as a molecular switch (Bhattacharyya and Blackburn 1994; Lingner et al. 1994; Zaug and Cech 1995; Antal et al. 2002; Theimer et al. 2003; Theimer et al. 2005; Yingling and Shapiro 2005). Pseudoknots have been implicated in protein recognition and binding, and pseudoknot mutations abolish telomerase activity and disrupt the ability of the *Tetrahymena* TR to assemble with TERT *in vivo*, but not *in vitro*, indicating that the pseudoknot structure is essential for the formation of active, stable *Tetrahymena* telomerase complexes *in vivo* (Figure 1.10) (Autexier and Greider 1998; Gilley and Blackburn 1999). Pseudoknot mutations in the yeast TR also disrupt TERT interactions (Figure 1.10) (Lin et al. 2004). In

contrast, the human TR pseudoknot is catalytically important *in vitro*, but has not been implicated in high affinity *in vitro* interactions with hTERT; our data indicate that disruption of several hTR pseudoknot sequences abolishes repeat addition processivity (Chapter 3; Autexier et al. 1996; Bachand and Autexier 2001; Ly et al. 2003a; Theimer et al. 2005). Conserved regions 2 and 3 (CR2 and CR3) of hTR, which form the P3 helix of the pseudoknot structure, are also involved in intermolecular base-pairing interactions that mediate hTR dimerization *in vitro*; reconstitution of CR2-CR3 base-pairing *in trans* restores catalytic activity to telomerase complexes assembled *in vitro* and *in vivo* (discussed in greater detail in Chapter 3; Ly et al. 2003b). Recent structural evidence indicates that CR2-CR3 interactions *in cis* are also essential for the stable conformation of the pseudoknot and make important contributions to telomerase activity that are independent of dimerization (Theimer et al. 2005). A telomerase activity-inhibiting hTR pseudoknot mutation found in patients with autosomal dominant DKC enhances hTR dimerization and destabilizes the pseudoknot structure in favor of a hairpin structure *in vitro*, suggesting that hTR dimerization may play a role in a (putative) pseudoknot conformational switch mechanism (Chapter 3; Vulliamy et al. 2001; Theimer et al. 2003; Yingling and Shapiro 2005). However, this DKC hTR mutant does not impair telomerase activity by a dominant-negative mechanism, implying that it does not affect assembly of higher-order telomerase complexes, or that interactions between hTR monomers in the pseudoknot region may be transient (Fu and Collins 2003). Finally, complementary oligonucleotides that can hybridize to a single-stranded region of the hTR pseudoknot may stabilize the interactions of naked hTR with telomeric DNA, suggesting a possible role for the pseudoknot in regulating human telomerase's association with its substrate; the pseudoknot region of the *S. cerevisiae* TR may also affect DNA interactions, possibly by regulating TR conformation or folding (Lue 1999; Yeo et al. 2005). It is unknown if the distinct catalytic and TERT interaction phenotypes of *Tetrahymena* and human TR mutants are dependent on differences in TR structure, function and dimerization potential, or on differences in reconstitution efficiency and/or requirements *in vivo* and *in vitro*. The putative pseudoknot structures of yeast TRs have not yet been investigated by mutagenic or other structural probing methods, and their function is unknown.

1.5.3.3. TERT interactions

Vertebrate, ciliate and yeast telomerases interact with their respective TERT components in the pseudoknot/template region, and via stem structures that are sometimes, but not always part of the pseudoknot/template domain (Figure 1.10) (reviewed in Chen and Greider 2004a). Two catalytically-essential sites in the vertebrate TR interact independently with TERT in coimmunoprecipitations: the CR4/CR5 domain and the pseudoknot/template domain (Figure 1.10) (Beattie et al. 2000; Mitchell and Collins 2000; Bachand and Autexier 2001; Chen et al. 2002). The P6.1 helix is essential for CR4/CR5 interactions with mouse and human TERTs, and additional CR4/CR5 sequences have also been implicated in hTR association with hTERT; P6.1 mutations that disrupt TERT interactions abolish telomerase catalytic function (Figure 1.10) (Chapter 3; Mitchell and Collins 2000; Chen et al. 2002). The hTERT-interacting CR4/CR5 domain sequences identified by coimmunoprecipitation assays correspond closely to hTR residues that are protected from chemical and enzymatic modification in the presence of hTERT and other cellular proteins *in vivo*, supporting the conclusion that CR4/CR5 interacts with TERT (Mitchell and Collins 2000; Antal et al. 2002). The hTR CR4/CR5 domain associates with hTERT RNA interaction domain 2 (RID2) in a P6.1-dependent fashion, and is likely the major site mediating telomerase RNP assembly (section 1.5.4.3; Chapter 3; Lai et al. 2001). Interestingly, though two-dimensional schematics of the vertebrate TR secondary structure depict the P6.1 helix as physically distant from the pseudoknot/template domain (Figure 1.10), recent evidence indicates that the isolated P6.1 helix can physically interact with the isolated hTR template *in vitro*, suggesting that this region of hTR may be closer to the pseudoknot/template domain than previously assumed (Ueda and Roberts 2004). Furthermore, recent evidence indicates that the CR4/CR5 region also regulates interaction of naked hTR molecules with DNA substrates (Yeo et al. 2005). It will be important to determine if such hTR-hTR interactions occur in the context of the full-length hTR molecule and in the presence of hTERT.

Site-directed mutagenesis studies indicate that most structural and sequence elements in the hTR pseudoknot/template domain (nt 1-209) are important for human telomerase activity in the context of the full-length hTR molecule (Autexier et al. 1996; Bachand and Autexier 2001; Ly et al. 2003a; Ly et al. 2003b). The smallest hTR pseudoknot/template domain fragment that can interact with hTERT extends from hTR nt

10-91, a region which includes residues that are part of the P1a, P1b, P2a and P2a.1 helices, as well as the template and template-adjacent sequences; this region does not include elements that contribute to pseudoknot formation (Figure 1.7) (Beattie et al. 2000; Chen et al. 2000). In the presence of hTERT *in vivo*, helices P1a, P1b and P2a.1 are all protected from chemical and enzymatic modification, suggesting that these elements could interact with hTERT or other cellular proteins (Antal et al. 2002). This hypothesis may be supported by our unpublished observation that disruption of the P1b helix impairs hTR interactions with an hTERT variant with a mutation in hTERT RNA interaction domain 1 (RID1) (Moriarty et al. 2005), which interacts independently with the hTR pseudoknot/template domain (Chapter 3;). In the absence of the pseudoknot structure, the first 40 nt of hTR, which include helices P1a and P1b, are important for hTERT interactions (Figure 1.7) (Beattie et al. 2000). However, the mouse TR does not contain the first 43 nt found in hTR, and hTR fragments that contain pseudoknot-forming sequences associate with hTERT in the absence of nt 1-43, suggesting that pseudoknot residues might also mediate hTERT interactions under certain conditions (Chen and Greider 2003a). This hypothesis is supported by *in vivo* protection data that implicate the pseudoknot region in interactions with hTERT and/or other cellular proteins (Antal et al. 2002). Furthermore, oligonucleotides that can hybridize to sequences from the P1b helix and the pseudoknot P3 helix impair hTR pseudoknot/template domain association with hTERT by 50% when telomerase is assembled *in vitro*, implicating both P1b and the pseudoknot in hTERT interactions (Keppler and Jarstfer 2004). Though hTR pseudoknot/template domain interactions with hTERT are specific (Chapter 3), mutagenic approaches have failed to identify a single specific hTR structure or sequence essential for this association, implying that multiple sites in the pseudoknot/template domain may be involved in cooperative interactions with hTERT (Chapter 3; Bachand and Autexier 2001; Ly et al. 2003a).

1.5.3.4. TR processing, stability, subnuclear localization and telomerase RNP assembly *in vivo*

The TRs from different organisms are transcribed, processed and regulated by different pathways. Yeast and vertebrate TRs are transcribed by RNA polymerase II, whereas the ciliate TR is an RNA polymerase III transcript (Yu et al. 1990; Chapon et al.

1997; Hinkley et al. 1998; Chen et al. 2000). Yeast TR resembles RNAs from the small nuclear (sn)RNA family because its 5' end is modified by a trimethylguanosine (TMG) cap and associates with the heptameric Sm protein complex (Seto et al. 1999). The 5' end of hTR is also modified by a TMG cap (Jady et al. 2004); however, unlike the yeast TR, hTR does not interact with Sm proteins, suggesting that it is not a member of the snRNA family (Lukowiak et al. 2001; Bachand et al. 2002). Proteins that are required for the accumulation of small nucleolar RNAs (snoRNAs) are required for human but not yeast TR stability when these TRs are expressed in yeast, further supporting the hypothesis that yeast and vertebrate TR accumulation are regulated differently (Dez et al. 2001). Little is known about TR processing and stability in ciliates.

Vertebrate TR localizes to both the nucleolus and Cajal bodies, which are small intranuclear foci that have been implicated in posttranscriptional RNA modification and ribonucleoprotein (RNP) assembly (Mitchell et al. 1999a; Narayanan et al. 1999; Zhu et al. 2004 and references therein). hTR is thought to be a member of the small Cajal-body RNA (scaRNA) family, whose members share some structural and functional similarities with RNAs from the snoRNA family (Jady et al. 2004). scaRNAs and snoRNAs direct pseudouridylation of rRNAs and snRNAs; however, there is no evidence that hTR mediates a similar function (Jady et al. 2004 and references therein). hTR stability, processing and localization *in vivo* are regulated by two vertebrate-conserved domains (H/ACA box and CR7 domains) that are not required for *in vitro* catalytic function or TERT interactions (Figure 1.7) (Mitchell et al. 1999a; Martin-Rivera and Blasco 2001; Fu and Collins 2003). As for both snoRNAs and scaRNAs, the H/ACA box domain is essential for TR stability, nuclear retention and nucleolar localization, and interaction with the dyskerin complex, which includes dyskerin, GAR1, NHP2 and NOP10 (Mitchell et al. 1999a; Mitchell et al. 1999b; Narayanan et al. 1999; Dragon et al. 2000; Pogacic et al. 2000; Lukowiak et al. 2001; Martin-Rivera and Blasco 2001). The H/ACA domain also directs processing of hTR transcripts to generate hTRs containing a precisely-defined 3' end (Mitchell et al. 1999a).

Several lines of evidence implicate the nucleolus in human telomerase assembly and/or regulation. First, both hTERT and hTR localize to the nucleolus (Mitchell et al. 1999a; Narayanan et al. 1999; Etheridge et al. 2002; Wong et al. 2002). Nucleolar localization of hTERT is dependent on N-terminal hTERT sequences that have been

implicated in hTR binding (RID2: section 1.5.4.3), and is regulated by the nucleolus-specific protein nucleolin, in a partially hTR-dependent fashion, though nucleolar hTERT localization does not require the presence of hTR (Etheridge et al. 2002; Yang et al. 2002; Khurts et al. 2004). Second, green fluorescent protein (GFP)-tagged hTERT is sequestered within the nucleolus in normal cells, and is released into the nucleoplasm only during S phase, whereas nucleolar sequestration is abolished in SV40-transformed or cancerous cells; these observations suggest that nucleolar sequestration may be important for telomerase regulation (Wong et al. 2002). Finally, the nucleolar protein PINX1, which inhibits telomerase activity and telomere elongation in human cells and yeast, likely prevents assembly of the TERT and TR telomerase components by competing with TR for interaction with the high affinity TR-binding domain of TERT (RID2: section 1.5.4.3) (Zhou and Lu 2001; Banik and Counter 2004; Lin and Blackburn 2004). Together, these observations suggest that the nucleolus may house unassembled or inactivated telomerase components.

A loop in the hTR CR7 domain contains a CAB box sequence motif that specifies localization of hTR and other scaRNAs to Cajal bodies (Figure 1.7) (Jady et al. 2004). A point mutation found in an adjacent helix of the hTR CR7 domain in DKC patients disrupts hTR-hTR interactions and the structural conformation of CR7, and impairs hTR accumulation *in vivo*; however, it is unknown if this mutation affects Cajal body localization (Fu and Collins 2003; Ren et al. 2003; Theimer et al. 2003). hTR is bound by PHAX, an snRNA export adaptor that has recently been implicated in transport of snoRNAs to Cajal bodies (Boulon et al. 2004). Human telomerase also interacts with SMN, a protein that is specific to Cajal bodies (Bachand et al. 2002). hTR localizes to Cajal bodies only in the presence of hTERT, which is also found in Cajal bodies, and hTR localization to Cajal bodies is enhanced during S phase, implying that Cajal bodies might be the site of final steps in telomerase assembly and/or activation (Jady et al. 2004; Zhu et al. 2004). This hypothesis may be supported by the observation that GFP-tagged hTERT is only released from the nucleolus into the nucleoplasm during S phase in non-transformed cells (Wong et al. 2002).

hTR also interacts with other proteins known to be involved in RNA processing, RNA stabilization and RNP assembly, including the human La autoantigen, human Staufen and L22; however the functions of these proteins in telomerase biogenesis have

not been determined (Le et al. 2000; Ford et al. 2001). Proteins that contain La-like motifs also associate with ciliate TRs, where they may contribute to RNP stability, and possibly also telomerase catalytic function (Aigner et al. 2000; Witkin and Collins 2004). Authentic La proteins usually interact with tracts of uridine residues at the 3' end of RNA polymerase III transcripts; however, the telomerase-associated La motif-containing protein in *Euplotes*, p43, interacts specifically with Stem I and adjacent sequences/structures of the *Euplotes* TR (which corresponds to the vertebrate TR P1b helix), and stimulates telomerase activity and repeat addition processivity (Aigner et al. 2003; Aigner and Cech 2004). Interestingly, a La motif-containing protein in *Tetrahymena*, p65, interacts with a similar structure in the *Tetrahymena* TR and regulates assembly of telomerase; however, p65 has not been implicated in telomerase catalytic function (Prathapam et al. 2005).

Other proteins that interact with the TR include p80 and p95 (in *Tetrahymena*), Est1p and Ku (in budding yeast), and TEP1, Ku70/80 and hnRNPs A1/UP1, A2, C1 and C2 (in mammals) (Collins et al. 1995; Steiner et al. 1996; Harrington et al. 1997a; Nakayama et al. 1997; Gandhi and Collins 1998; Ford et al. 2000; Zhou et al. 2000; Fiset and Chabot 2001; Peterson et al. 2001; Moran-Jones et al. 2005; Ting et al. 2005). None of these proteins have been demonstrated to play a role in TR processing, stability, subnuclear localization or RNP stability. TEP1 (the mammalian orthologue of ciliate p80) is a subunit of the cytoplasmic RNP vault complex, which may be involved in nuclear-cytoplasmic transport; however, deletion of the genes encoding p80 or TEP1 does not impair telomerase activity (Chugani et al. 1993; Harrington et al. 1997b; Kickhoefer et al. 1999; Liu et al. 2000b; Miller and Collins 2000). Instead, p80, but not TEP1, is a negative regulator of telomere length (Liu et al. 2000b; Miller and Collins 2000). hnRNPs A1/UP1 and A2, p95, Ku and Est1p can all interact with telomeric DNA as well as TR. (Collins et al. 1995; Virta-Pearlman et al. 1996; LaBranche et al. 1998; Fiset and Chabot 2001; Moran-Jones et al. 2005). hnRNP A1/UP1 and p95 are, respectively, positive and negative regulators of telomere length (LaBranche et al. 1998; Miller and Collins 2000). Ku and Est1p have both been implicated in recruitment and/or activation of telomerase at telomeres in yeast (sections 1.2.4.1.2 and 1.2.4.2.2), though similar functions have not been identified for hnRNP A1/UP1 or p95.

1.5.4. Telomerase reverse transcriptase

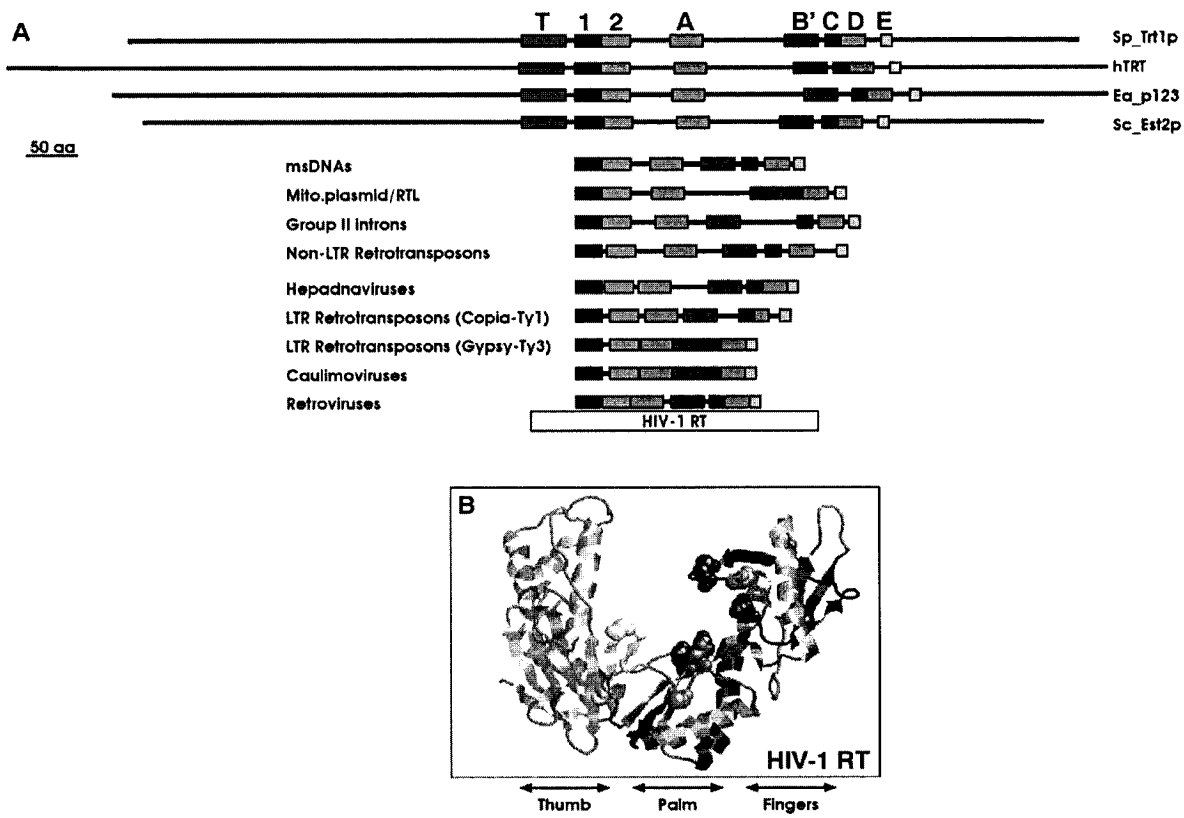
TERT is a reverse transcriptase (RT) that is phylogenetically more closely related to group II introns and non-LTR retrotransposons than viral reverse transcriptases, suggesting that it is evolutionarily ancient (Figure 1.11) (Eickbush 1997; Nakamura et al. 1997; Nakamura and Cech 1998). The TERT proteins are large, ranging in size from 103 kDa in *S. cerevisiae* to 134 kDa in *Tetrahymena* (Counter et al. 1997; Lingner et al. 1997b; Bryan et al. 1998; Collins and Gandhi 1998). TERT contains a central domain consisting of RT motifs (Figure 1.11) (Lingner et al. 1997b; Nakamura et al. 1997). The large size of TERTs is attributable to the presence of extensive N- and C-terminal sequences flanking the RT motifs that are conserved among TERTs but do not exhibit homology to any other known protein (N terminus and C terminus) (Figures 1.11 and 1.12) (Xia et al. 2000; Peng et al. 2001). As expected, these sequences make a major contribution to telomerase-specific catalytic functions (Figure 1.12) (Sections 1.5.4.2 and 1.5.4.3).

1.5.4.1. TERT polymerase domain

The central catalytic event of reverse transcription is the template-specified addition of a dNTP to the 3'OH group of a primer (nucleotidyl transfer reaction). This addition requires metal ion-coordinating residues and a basic active site architecture that are conserved in both RNA- and DNA-directed polymerases (reviewed in Sousa 1996; Jager and Pata 1999; Steitz and Yin 2004). Nucleic acid polymerases are modular enzymes containing a core polymerase domain, and frequently, accessory domains that confer enzyme-specific catalytic properties such as sequence-specific nucleic acid binding, proofreading, or other, nuclease-dependent functions (reviewed in Sousa 1996). The structure of the core polymerase domain of all nucleic acid polymerases resembles a cupped right hand, which is organized into "fingers", "palm" and "thumb" subdomains (Figure 1.11) (Kohlstaedt et al. 1992). The fingers subdomain contacts the template strand and incoming dNTPs, the palm is the site of nucleotidyl transferase activity and template-directed nucleotide addition, and the thumb interacts with the duplex products of nucleic acid synthesis (reviewed in Sousa 1996; Jager and Pata 1999; Steitz and Yin 2004). The structure of the palm subdomain is well-conserved among nucleic acid polymerases, whereas the sequences and structures of the fingers and thumb subdomains vary in different polymerase classes, according to the identity of their templates and products (i.e.

Figure 1.11: Structure and RT sequence motifs of telomerase proteins.

(**A**) Locations in TERT homologues of an N-terminal telomerase-specific motif T, and conserved RT motifs 1, 2, and A through E, are indicated by colored boxes (section 1.5.4). *S. pombe* (Sp_Trt1p), human (hTRT), *Euplotes* (Ea_p123) and *S. cerevisiae* (Sc_Est2p) TERTs are shown. The open rectangle labeled HIV-1 RT delineates the portion of this protein shown in (B). (**B**) The crystal structure of the p66 subunit of HIV-1 RT depicting the right-handed conformation common to the polymerase domains of all nucleic acid polymerases (section 1.5.4.1) (Brookhaven code 1HNV). Color-coding of RT motifs matches that in (A). The view is from the back of the right hand, which allows all motifs to be seen. Note that this figure is adapted from one of the earliest studies that reported the identification of TERT proteins, which predicted that the N-terminal telomerase-specific T motif might constitute the TERT thumb. More recent work has indicated that the T motif (which is part of RID2: see section 1.5.4.3) is instead important for assembly with the TR, and that the TERT thumb is likely encoded by the telomerase-specific C terminus (see Figure 1.12 and sections 1.5.4.2 and 1.5.4.3).


A
T 1 2 A B' C D E
Sp_Trt1p
hTRT
Ea_p123
Sc_Est2p
50 aa
msDNAs
Mito.plasmid/RTL
Group II introns
Non-LTR Retrotransposons
Hepadnaviruses
LTR Retrotransposons (Copia-Ty1)
LTR Retrotransposons (Gypsy-Ty3)
Caulimoviruses
Retroviruses
HIV-1 RT
B
HIV-1 RT
Thumb
Palm
Fingers

DNA vs RNA).

TERT contains RT motifs (1 and 2, and A-E) that are common to the core polymerase domains of all RTs and retrotransposons (Figure 1.11) (Lingner et al. 1997b; Nakamura et al. 1997). The HIV-1 RT has been successfully aligned with the RT portion of seven TERTs, and is often used as a model for TERT RT function because its crystal structures in complex with template, primer, nucleotide and inhibitors have previously been solved (Figure 1.11) (Kohlstaedt et al. 1992; Jacobo-Molina et al. 1993; Ren et al. 1995; Ding et al. 1998; Huang et al. 1998; Jaeger et al. 1998; O'Reilly et al. 1999). Many RT-conserved residues in the TERT RT motifs are implicated in telomerase catalytic function *in vitro* and *in vivo*, implying that the catalytic active site of TERT is likely similar to other RTs with respect to basic polymerase functions such as coordination of metal ions, nucleotide recognition and addition, and interactions with template and substrate in the active site itself (Counter et al. 1997; Harrington et al. 1997b; Lingner et al. 1997b; Weinrich et al. 1997; Beattie et al. 1998; Collins and Gandhi 1998; Greenberg et al. 1998; Bryan et al. 2000b; Haering et al. 2000; Miller et al. 2000; Peng et al. 2001).

One of the major differences between the TERT RT domain and other RTs is that TERT contains a telomerase-specific insertion between motifs A and B' in its putative fingers subdomain, which is referred to as the IFD (insertion in fingers domain) motif (Figure 1.11) (Lingner et al. 1997b; Nakamura et al. 1997). The fingers subdomain differs in RNA- and DNA-directed nucleic acid polymerases, and confers polymerase template specificity (reviewed in Sousa 1996). The telomerase-specific IFD is important for interaction with both the DNA substrate and TR template, and has been implicated in repeat addition processivity in *S. cerevisiae* telomerase (Lue et al. 2003). IFD mutations impair the ability of telomerase to extend primers that can hybridize to only a few nt in the TR template, suggesting that it may be important for recognition and/or stabilization of the RNA-DNA duplex formed at the beginning of the telomerase catalytic cycle and following each round of Type II translocation (Lue et al. 2003). Mutation of a ciliate TERT-specific residue in RT motif 1 of *Tetrahymena* TERT results in a similar phenotype, implying that this region of the putative TERT fingers subdomain may interact with the same region of RNA-DNA duplexes as the IFD (Miller et al. 2000). Interestingly, though repeat addition processivity is reduced in *S. cerevisiae* IFD mutants, nucleotide addition processivity is enhanced, implying that TERT-dependent recognition and/or stabilization of

template/substrate duplexes slows the translocation of telomerase along its template, at least in yeast telomerase (Lue et al. 2003). This hypothesis is supported by the observation that the nucleotide addition processivity of *K. lactis* telomerase is inversely proportional to the potential length of the duplex formed by primer base-pairing to the TR template (Fulton and Blackburn 1998).

The TERTs also differ from other RTs at a residue in motif C of the palm subdomain located two amino acids before the invariant metal ion-coordinating aspartate residues. In the HIV-1 RT this residue is a tyrosine whose sidechain interacts with the terminal and penultimate 3' nucleotides of the primer substrate; however, no TERT examined to date has a tyrosine at this position (Bryan et al. 2000a; Peng et al. 2001 and references therein). This tyrosine is invariant in viral RTs, is important for enzyme processivity, and is thought to enable precise positioning of the primer substrate in the active site (Bryan et al. 2000a; Peng et al. 2001 and references therein). Substitution of the corresponding residue in *Tetrahymena* TERT with tyrosine enhances telomerase affinity for the DNA substrate and repeat addition processivity (Bryan et al. 2000a). In contrast, substitution of the corresponding residue in *S. cerevisiae* TERT with tyrosine impairs nucleotide addition processivity (Peng et al. 2001). Since in these studies nucleotide and repeat addition processivity were not measured for the *Tetrahymena* and yeast mutants, respectively, it remains possible that inhibition of nucleotide addition processivity might in fact enhance repeat addition processivity, as has been observed for mutation of the IFD motif (Lue et al. 2003). Reduction of nucleotide addition processivity might be important for slowing telomerase movement along 5' sequences of the template, thus facilitating the stalling at the 5' template boundary that likely precedes the translocation step of repeat addition processivity. Alternatively, the apparent conflict between these results may instead reflect the profoundly different levels of repeat addition processivity normally observed for yeast and *Tetrahymena* telomerases. It is unclear why a tyrosine is not present at this position in the TERTs, though it is evidently not required for telomerase activity. One proposal is that telomerase-specific modes of primer interaction, via the anchor site and/or IFD sequences might stabilize enzyme-substrate interactions sufficiently to obviate the need for the RT-invariant tyrosine in motif C (Bryan et al. 2000a; Lue et al. 2003).

1.5.4.2. TERT C-terminal putative 'thumb' and telomerase processivity

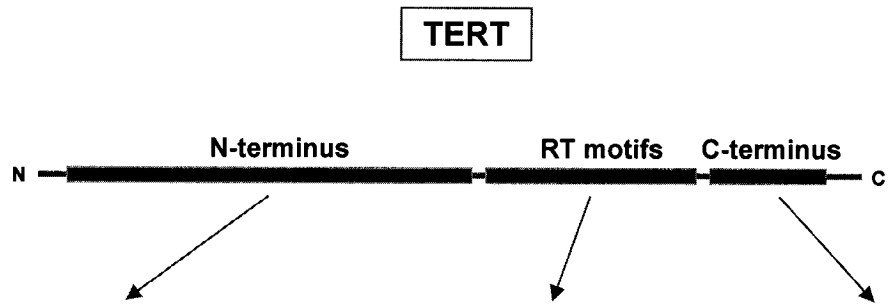
In RTs, nucleotide addition-type processivity along a template is regulated by motif C, motif E ("primer grip") and the motif E-adjacent thumb subdomain (reviewed in Sousa 1996; Jager and Pata 1999; Peng et al. 2001). Motifs C and E position the terminal 3' OH group of the primer substrate in the catalytic active site (Jacobo-Molina et al. 1993; Ding et al. 1997; Ding et al. 1998). Mutation of RT-conserved primer grip residues in Motif E of *Tetrahymena* and yeast TERTs reduces telomerase activity (Bryan et al. 2000b; Miller et al. 2000; Peng et al. 2001). In yeast telomerase, such mutations impair nucleotide addition processivity, suggesting that motif E may be the primer grip of TERT (Peng et al. 2001).

The flexible thumb subdomain of polymerases interacts with duplex template-primer products, and is thought to be important for Type I translocation along the template, perhaps by a ratchet-like motion; however, the mechanism by which the thumb regulates nucleotide addition processivity remains unclear (Ding et al. 1997; Ding et al. 1998). Most of the telomerase-specific C-terminal domain of *S. cerevisiae* and human TERTs has been implicated in nucleotide addition processivity and affinity for DNA substrates, suggesting that the TERT C terminus likely constitutes the thumb subdomain of TERT (Figure 1.12) (Chapter 4; Peng et al. 2001; Hossain et al. 2002; Huard et al. 2003; Lee et al. 2003). The hTERT C terminus also contributes to repeat addition processivity (Huard et al. 2003).

Polymerase thumb subdomains interact with primer/template duplexes. However, mutation or deletion of human and yeast TERT C-terminal residues does not appear to significantly impair TERT-TR interactions in coimmunoprecipitations, implying that the C terminus does not associate stably with the TR (Beattie et al. 2000; Bachand and Autexier 2001; Banik et al. 2002; Hossain et al. 2002; Huard et al. 2003). The TERT C terminus has been implicated in functional and physical TERT multimerization, and interacts *in vitro* with two domains in the TERT N terminus, RID1 and RID2 (section 1.5.4.3) (Chapter 3; Beattie et al. 2001; Arai et al. 2002; Wang et al. 2002).We have recently found using competitive quantitative RNA binding assays that mutation of different hTERT C-terminal sequences can enhance hTR interactions, suggesting that the C-terminus might regulate other hTR interaction sites in hTERT (e.g. RID1 or RID2: section 1.5.4.3), or could associate with hTR transiently in the WT enzyme (Moriarty et al. 2005). Interestingly, the

Figure 1.12: Function of telomerase-specific N-terminal and C-terminal TERT sequences.

Schematic depiction of the major regions of the telomerase reverse transcriptase, and some of the functions that have been ascribed to these regions in diverse telomerases. This summary includes observations that are reported in this thesis, as well as data reported in the literature. These functions are described in greater detail in sections 1.5.4.1 to 1.5.4.3.



Telomerase-specific

- Telomerase RNA interactions (all TERTs)
- Assembly of telomerase RNP (all TERTs)
- DNA substrate interactions? (yeast, human)
- Anchor site-type functions (human)
- Repeat addition processivity (human)
- 5' template boundary definition (*Tetrahymena*)
- Template alignment and positioning (*Tetrahymena*)
- Nucleolar localization (human)
- Functional and physical TERT multimerization (human, *Euplotes*)
- *In vivo* function independent of catalytic activity? (yeast and human)
- Akt and c-Abl phosphorylation sites? (human)
- Interactions with molecular chaperones (human)
- Interactions with PINX1 telomerase inhibitor (yeast and human)
- Interactions with Est3p and Est1p? (yeast)

Conserved with HIV-1 and other RTs

- Polymerization (all TERTs)
- Nucleotide binding (*Tetrahymena*)
- Nucleotide addition processivity (yeast)
- Primer grip? (yeast)
- Repeat addition processivity: telomerase-specific sequences (yeast, *Tetrahymena*)
- Template alignment and positioning in active site (*Tetrahymena*, yeast)
- Physical TERT multimerization (human and *Euplotes*)
- Akt and Src kinase phosphorylation sites regulating activation, nuclear export? (human)

Telomerase-specific

- Putative thumb of TERT (nucleotide addition processivity) (yeast and human)
- DNA substrate interactions? (yeast and human)
- Repeat addition processivity (human)
- 14-3-3-dependent nuclear localization and CRM1-dependent nuclear export (human)
- Functional and physical TERT multimerization (human, *Euplotes*)
- *In vivo* function independent of catalytic activity? (human)
- Interactions with hnRNPs C1 and C2? (human)

presence of an RNA pseudoknot inhibitor in the HIV-1 RT active site inhibits movement of the polymerase thumb, in addition to competing for primer/template duplex binding (Kensch et al. 2000). This suggests the intriguing possibility that the TR pseudoknot might similarly affect the DNA binding and processivity functions of the putative TERT thumb, perhaps by disrupting Type I translocation and DNA interactions prior to Type II translocation at the 5' template boundary.

The hTERT C terminus contains a 14-3-3 protein binding site adjacent to a nuclear export signal motif, and mutation of the 14-3-3-binding site or expression of a dominant-negative 14-3-3 variant redistributes hTERT from the nucleus to the cytoplasm in a CRM1-dependent fashion (Seimiya et al. 2000). It has recently been demonstrated that the hEST1 proteins contain a 14-3-3-like domain, and it will be interesting to determine if these proteins interact with the hTERT C terminus (Fukuhara et al. 2005). The hTERT C terminus was also previously thought to be important for biological functions unrelated to catalysis, since mutation of the most C-terminal hTERT residues or addition of a variety of different C-terminal tags prevents hTERT-mediated immortalization of hTERT-negative cells, without apparently affecting telomerase activity or nuclear localization (Counter et al. 1998; Banik et al. 2002; Kim et al. 2003). Recent evidence indicates that such C-terminal hTERT modifications in fact impair catalytic function and affinity for the DNA substrate; however, these observations do not preclude the possibility that C-terminal sequences mediate both catalytic and non-catalytic functions (Chapter 4; Lee and Blackburn 1993).

1.5.4.3. TERT N terminus: TR interactions, template boundary definition and repeat addition processivity

Telomerase differs from other RTs in several important ways. First, most RTs interact transiently with their template RNA in a fashion that promotes mobility along the entire length of the long template. In contrast, TR is stably associated with TERT, and only a small, strictly defined portion of TR is used as a template. Second, telomerase exhibits a unique, repeat addition form of processivity. Though the TERT RT motifs are required for basic polymerase function, they are not sufficient for high-affinity binding of the TR or repeat addition processivity (Chapter 3; Beattie et al. 2000; Bachand and Autexier 2001; Lai et al. 2001). In the last five years, we and several other groups have

determined that the TERT N terminus is a major site of telomerase-specific catalytic functions, including repeat addition processivity, anchor site-type functions and interactions with the DNA substrate, association with the TR, and TR template boundary definition (Figure 1.12). As later chapters of this thesis discuss TERT N-terminal functions extensively, the following paragraphs provide a simplified, brief introduction to this TERT region.

The N terminus constitutes approximately half of TERT, and varies in length in different organisms, due to differences in the length of a poorly conserved, catalytically inessential 'linker' region joining two large domains of conserved sequences that are important for telomerase catalytic function and telomere length maintenance (Figure 2.1) (Chapters 2 and 3; Weinrich et al. 1997; Friedman and Cech 1999; Bryan et al. 2000b; Miller et al. 2000; Xia et al. 2000; Armbruster et al. 2001; Bosoy et al. 2003; Friedman et al. 2003). Each of these domains, which we refer to as RNA interaction domains 1 and 2 (RID1 and RID2), interacts with TR and confers distinct, telomerase-specific catalytic functions (Figure 2.1) (Chapters 2 to 4). Several functional- and sequence motif-based nomenclatures have been used to define various elements in the N terminus, and these nomenclatures will be described in greater detail in later chapters. Despite a proliferation of alternative nomenclatures, all groups who have mapped the N terminus by functional mutagenesis and sequence comparison have identified similar TERT-conserved sequence regions that are important for telomerase activity (Chapter 2; Lingner et al. 1997b; Nakamura et al. 1997; Bryan et al. 1998; Friedman and Cech 1999; Malik et al. 2000; Miller et al. 2000; Xia et al. 2000; Armbruster et al. 2001). Therefore, with a few exceptions, most nomenclatures can be used interchangeably.

The RID2 domain is located immediately N-terminal of the RT motifs, and is separated from RID1 by a non-conserved, catalytically-inessential linker region (Figure 2.1). Most non-conservative single amino acid substitutions of conserved residues in RID2 impair telomerase activity and TR interactions (Chapters 2 and 3; Weinrich et al. 1997; Bryan et al. 2000b; Miller et al. 2000). Mutations that alter or delete small groups of contiguous conserved RID2 sequences greatly reduce or eliminate TR association, and completely or nearly abolish telomerase activity, indicating that this domain is essential for telomerase catalytic function and TR interactions (Chapters 2 and 3; Friedman and Cech 1999; Bryan et al. 2000b; Armbruster et al. 2001; Bosoy et al. 2003). In human and

Tetrahymena TERTs, RID2 alone is sufficient for high affinity association with TR, implying that it is the major TERT site mediating telomerase RNP assembly (Chapter 3; Lai et al. 2001; O'Connor et al. 2005). The human RID2 is functionally inseparable from the immediately adjacent core hTERT polymerase domain, suggesting that it might constitute a large extension of the polymerase fingers subdomain (Chapter 3). The high affinity TR interaction domain of *Tetrahymena* TERT is unusual because it includes a ciliate-specific motif (CP2) that is located C-terminal of RID1 and is separated from the remainder of RID2 by the linker (Miller et al. 2000; Lai et al. 2001). CP2 is important for 3' template boundary definition, and also regulates 5' template boundary definition together with the remainder of the high affinity *Tetrahymena* TR interaction domain (Miller et al. 2000; Lai et al. 2002). It has been impossible to examine the role of hTERT RID2 in template boundary definition because mutation of conserved RID2 residues completely abolishes telomerase activity (Chapters 2 and 3; Armbruster et al. 2001). In *Tetrahymena* and human telomerases, RID2 interacts with family-specific TR stem-loop structures that are also important for telomerase activity; hTERT RID2 interacts with the P6.1 helix of the CR4/CR5 domain (Chapter 3; Lai et al. 2001; O'Connor et al. 2005). Isolated RID2 sequences from *Euplotes* and human TERTs can also coimmunoprecipitate WT TERT *in vitro* in a TR-independent fashion, suggesting that RID2 may constitute a TERT-TERT interaction site (Arai et al. 2002; Wang et al. 2002). hTERT RID2 coimmunoprecipitates an hTERT fragment containing the C terminus and RT motif E, which can itself coimmunoprecipitate WT hTERT (Arai et al. 2002). Motifs C to E of *Euplotes* TERT also interact with full-length TERT, but cannot coimmunoprecipitate RID2 sequences in the absence of the TERT C terminus, implying that RID2-RT motif interactions may not be conserved, or could require the TERT C terminus (Wang et al. 2002). The RID2 region involved in TERT-TERT interactions in *Euplotes* telomerase maps to a sequence motif (CP) that is present in all TERTs (Wang et al. 2002). Interestingly, alteration of CP motif sequences has a relatively small effect on the activity of the monomeric *Tetrahymena* telomerase, whereas mutations in the yeast and human CP motifs strongly impair or abolish telomerase activity, suggesting that TERT-TERT interactions at this site might be important for the catalytic function of some telomerases (Chapters 2 and 3; Friedman and Cech 1999; Bryan et al. 2000b; Miller et al. 2000; Armbruster et al. 2001; Bosoy et al. 2003).

The RID1 domain is located at the extreme N terminus of TERT, and is separated from RID2 in most TERTs by the non-conserved linker region (Figure 2.1). Deletion of RID1 does not greatly impair TR interactions, though RID1 mutations do reduce this association (Chapters 2 and 3; Friedman and Cech 1999; Beattie et al. 2000; Bachand and Autexier 2001; Lai et al. 2001). RID1 sequences constitute a proteolytically-stable domain of yeast TERT, and the hTERT RID1 is functionally and physically separable from a core hTERT polymerase domain consisting of RID2, the RT motifs and the C terminus (Chapter 3; Xia et al. 2000; Beattie et al. 2001). hTERT RID1 is also essential for repeat addition processivity, and interacts with the processivity-regulating pseudoknot/template domain of hTR, and with the hTERT C terminus (Chapter 3). Recent evidence indicates that *Tetrahymena* TERT sequences that correspond to hTERT RID1 are also physically separable from the remainder of hTERT, and can interact independently with a processivity-regulating region of the TR (O'Connor et al. 2005). Yeast and human RID1 sequences have been implicated in interactions with and affinity for the DNA substrate, and we have determined that RID1 may be part of the hTERT anchor site (Chapter 4; Xia et al. 2000; Lee et al. 2003). Together these observations imply that RID1 is a bona fide accessory domain of TERT that confers several telomerase-specific functions. A detailed review of our own and others' work characterizing RID1 functions will be provided in the following chapters of this thesis.

1.5.4.4. TERT-associated proteins

Like the TR, a number of proteins have been shown to interact with TERT. These include the previously discussed PINX1 telomerase inhibitor (in yeast and human) and the 14-3-3 protein (in human), which are respectively implicated in telomerase RNP assembly and nuclear localization (sections 1.5.3.4 and 1.5.4.2). The chaperones HSP90, p23 and HSP70 interact with hTERT and promote assembly of active telomerase *in vitro* and *in vivo* by an undefined mechanism probably related to their functions as chaperones; HSP90 and p23, but not HSP70, remain stably associated with human telomerase after *in vitro* assembly, and interact with hTERT RID1 (Holt et al. 1999; Forsythe et al. 2001). hTERT also interacts with the human homologues of the yeast Est1p protein, hEST1A and hEST1B, and with Ku (sections 1.2.4.1.2 and 1.2.4.2.2). Finally, the Est3p protein, which is an essential component of the telomerase-dependent telomere length maintenance

pathway in *S. cerevisiae*, is also thought to associate with telomerase via TERT interactions (Lendvay et al. 1996; Lingner et al. 1997a; Hughes et al. 2000). It is hypothesized that Est3p might be involved in recruiting telomerase to the telomere, though its role in telomere length maintenance is not yet understood (Hughes et al. 2000). Interestingly, overexpression of Est1p partially suppresses the telomere shortening phenotypes of yeast expressing TERT proteins with mutations in RID1 or RID2, whereas Est3p overexpression partially but specifically suppresses the telomere shortening phenotypes of cells expressing TERT RID1 mutants; furthermore, a yeast RID1 mutant is impaired in the ability to interact with both Est1p and Est3p (Friedman et al. 2003). These observations imply that the TERT N terminus interacts directly or indirectly (via the TR) with additional cellular proteins that are important for its function *in vivo*.

1.5.5. Human telomerase regulation

As described in previous sections, human telomerase is regulated at the level of RNP assembly and nuclear and subnuclear localization, and is also likely regulated by proteins that control its access to and possible activation at the telomere itself. Recent evidence indicates that hTERT ubiquitination and proteolysis are important for regulation of telomerase stability and telomere length (Kim et al. 2005). Human telomerase activity is also modulated posttranslationally by phosphorylation, though the mechanisms and function of this form of regulation is not yet well understood (reviewed in Cong et al. 2002). Telomerase activity is enhanced by Akt kinase and several isoforms of protein kinase C (PKC), and is inhibited by protein phosphatase 2A and the c-Abl kinase; phosphorylation-dependent telomerase upregulation often correlates with increased cell survival and proliferation, though it is unknown if this is a direct result of enhanced telomerase activity (Bodnar et al. 1996; Ku et al. 1997; Li et al. 1997; Li et al. 1998; Kang et al. 1999; Kharbanda et al. 2000; Breitschopf et al. 2001; Yu et al. 2001; Akiyama et al. 2002). Akt kinase phosphorylates peptides corresponding to hTERT residues 220-229 or 817-826 (from the N-terminal linker and RT domain, respectively), while c-Abl-dependent phosphorylation may require a c-Abl interaction site in the N-terminal linker (Kang et al. 1999; Kharbanda et al. 2000). Akt-dependent signaling upregulates telomerase activity in response to a number of growth factors, including estrogen and cytokines, and may also promote NFκB-mediated nuclear import of hTERT (Akiyama et al. 2002; Akiyama et al.

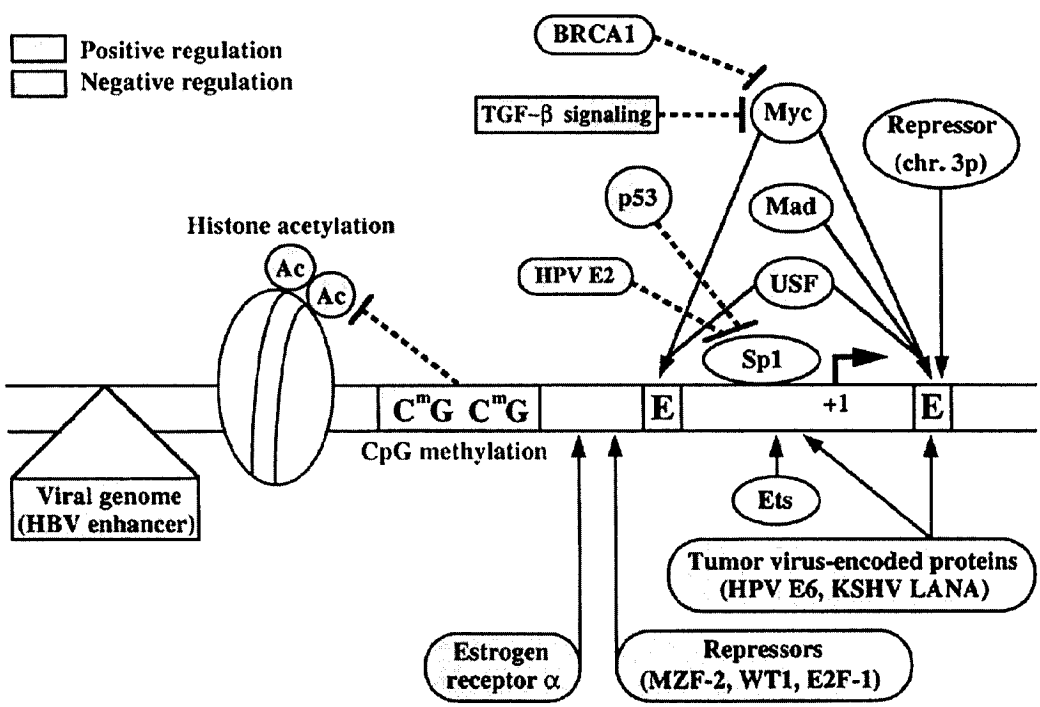
2003; Kawagoe et al. 2003; Kimura et al. 2004). Src kinase family-dependent phosphorylation of hTERT tyrosine residue 707 (in the RT domain) results in Ran GTPase-dependent export of hTERT from the nucleus in response to oxidative stress (Haendeler et al. 2003). Together, these observations imply that phosphorylation may play an important role in controlling the subcellular localization of telomerase. The functions of phosphorylation-dependent upregulation and inhibition of telomerase activity are unknown.

The most important form of telomerase regulation in human cells is transcriptional (Figure 1.13). hTR is constitutively expressed in most telomerase-negative somatic cells, whereas hTERT mRNA expression in is stringently repressed in telomerase-negative cells, and is upregulated in telomerase-positive cells (reviewed in Horikawa and Barrett 2003). A complex network of factors regulates *hTERT* transcription. A detailed discussion of *hTERT* transcription is beyond the scope of this thesis, but the major regulators are summarized schematically in Figure 1.13. Telomerase expression promotes the immortalization of human cells. It is not surprising, therefore, that one of the central positive regulators of *hTERT* expression is the proto-oncogene c-Myc, or that c-Myc binding to E-boxes in the *hTERT* promoter is enhanced by oncogenic viral proteins (reviewed in Horikawa and Barrett 2003). Similarly, two negative regulators of *hTERT* transcription are the tumor suppressor gene product p53 and the TGFβ tumor suppressor pathway; TGFβ likely regulates *hTERT* transcription by controlling expression of c-Myc, but may also influence alternative splicing of hTERT mRNAs (Cerezo et al. 2002; reviewed in Horikawa and Barrett 2003). Though the hTERT promoter contains a CpG methylation island, no direct correlation has been observed been hTERT mRNA expression status and promoter methylation (reviewed in Horikawa and Barrett 2003). In contrast, histone acetylation/deacetylation is likely important for *hTERT* trans-activation/repression in malignant and normal, somatic cells, respectively (Cong and Bacchetti 2000; reviewed in Liu et al. 2004b).

Figure 1.13: Transcriptional regulation of *hTERT*.

Multiple mechanisms of transcriptional regulation of the *hTERT* gene via its promoter and downstream sequences. Sites of actions within the promoter are not depicted in scale. Some activators (e.g. Myc) and repressors (e.g. Mad) may function through recruitment of HAT (histone acetyl transferases) and HDAC (histone deacetylases), respectively. Though Sp1 may act as an activator of *hTERT* transcription in cancer cells, it may recruit HDAC to repress hTERT transcription in normal cells (not shown in this figure). E: two canonical E-box (CACGTG) elements upstream and downstream of the transcription initiation site that are central to transcriptional regulation.

This figure is adapted from an article published by Horikawa and Barrett, "Transcriptional regulation of the telomerase hTERT gene as a target for cellular and viral oncogenic mechanisms," in the journal Carcinogenesis (2003) Vol.24 (7): 1167-1176, by permission of Oxford University Press.


Positive regulation
Negative regulation
BRCA1
TGF-β signaling
Myc
Repressor
(chr. 3p)
p53
Mad
Histone acetylation
Ac
Ac
HPV E2
USF
Sp1
C^mG C^mG
E
+1
E
CpG methylation
Viral genome
(HBV enhancer)
Ets
Tumor virus-encoded proteins
(HPV E6, KSHV LANA)
Estrogen
receptor α
Repressors
(MZF-2, WT1, E2F-1)

THESIS OBJECTIVES

The general subject of this thesis is the biochemical and functional characterization of the human telomerase catalytic mechanism. The specific aim of my doctoral work has been to characterize the roles of the telomerase-specific TERT N terminus in the human telomerase catalytic mechanism and in nucleic acid interactions. Such work has potential implications for the rational design of specific anti-telomerase therapeutic inhibitors, and is also important for understanding the mechanistic relationship of telomerase to other nucleic acid polymerases. To map and characterize hTR interaction sites in the hTERT N-terminus, we constructed a panel of hTERT mutants bearing small internal substitutions and deletions, and examined their ability to interact with hTR using a quantitative immunoprecipitation-based RNA binding assay that we developed for *in vitro*-reconstituted human telomerase (Chapter 2). As described in Chapter 3, we characterized the specific catalytic properties mediated by the two distinct N-terminal hTR interaction regions (RID1 and RID2) defined in Chapter 2, and identified their respective interaction sites in hTR. We found that RID1 was essential for the telomerase-specific catalytic property of repeat addition processivity, and that processivity-regulating sequences of RID1 interacted with a processivity-determining region of hTR (Chapter 3). In Chapter 4 we identified and characterized an additional role for RID1 in an anchor site-type affinity for DNA substrates that was important for repeat addition processivity. In Chapters 2 to 4, we also investigated the role of hTERT-hTERT and hTR-hTR interactions in regulating repeat addition processivity.

2. CHAPTER 2: Functional multimerization of human telomerase requires an RNA interaction domain in the N terminus of the catalytic subunit

2.1. Preface

The catalytic protein subunit of telomerase (TERT) contains extensive telomerase-specific N-terminal sequences. At the beginning of this study, the functions of some, but not most conserved sequences in the N terminus had been mapped for the TERTs from *S. cerevisiae* and *Tetrahymena*; the functions of this region in human TERT (hTERT) had been crudely mapped by using N-terminally-truncated hTERT variants. These previous reports implicated the TERT N terminus in telomerase RNA (TR) interactions and catalytic function, but the sites mediating catalytic function and association with the TR had not been precisely mapped or characterized, especially in hTERT. In this study, we used internal deletion and substitution mutants to identify groups of hTERT residues implicated in telomerase catalytic activity and hTR interactions. During the course of this study, another group reported that the hTERT N-terminus is involved in functional hTERT multimerization. Therefore, we also examined the requirements for functional and physical multimerization of hTERT.

2.2. Abstract

Functional human telomerase complexes are minimally composed of the human telomerase RNA (hTR) and a catalytic subunit (hTERT) containing reverse transcriptase (RT)-like motifs. The N-terminus of TERT proteins is unique to the telomerase family, has been implicated in catalysis, telomerase RNA binding and telomerase multimerization, and conserved motifs have been identified by alignment of TERT sequences from multiple organisms. We studied hTERT proteins containing N-terminal deletions or substitutions to identify and characterize hTERT domains mediating telomerase catalytic activity, hTR binding and hTERT multimerization. Using multiple sequence alignment, we identified two vertebrate-conserved TERT N-terminal regions containing vertebrate-specific residues that were required for human telomerase activity. We identified two RNA interaction domains, RID1 and RID2, the latter containing a vertebrate-specific RNA binding motif (VSR). Mutations in RID2 reduced the association of hTR with hTERT by 50-70%. Inactive mutants defective in RID2-mediated hTR binding failed to complement an inactive hTERT mutant containing an RT motif substitution to reconstitute activity. Our

results suggest that functional hTERT complementation requires intact RID2 and RT domains on the same hTERT molecule, and is dependent on hTR and the N-terminus.

2.3. Introduction

The telomerase enzyme is a ribonucleoprotein (RNP) that extends the 3' ends of linear eukaryotic chromosomes. It is minimally composed of a protein catalytic subunit, TERT (telomerase reverse transcriptase: hTERT in humans), and a telomerase RNA (TR: hTR in humans) that contains a short template used for *de novo* synthesis of telomeric DNA repeats (reviewed in Blackburn 1999). Telomerase activity is associated with an increased capacity for cellular proliferation in immortal unicellular eukaryotes, and in most immortalized human cancer cells (Oulton and Harrington 2000). Identifying the mechanisms of telomerase assembly and catalytic function is essential to understand the role of telomerase in immortalization.

The human telomerase holoenzyme is large (~1000 kDa) (Schnapp et al. 1998), and mammalian telomerase activity is associated with a number of proteins that may be implicated in RNP assembly, processing and stability (Harrington et al. 1997a; Nakayama et al. 1997; Holt et al. 1999; Mitchell et al. 1999b; Le et al. 2000). Auxiliary proteins identified in yeast also mediate the access of telomerase to telomeres (Evans and Lundblad 1999; Hughes et al. 2000). However, human telomerase affinity-purified under stringent salt conditions has a molecular mass of 600 kDa, consistent with a minimal complex composed of two hTERTs and two telomerase RNAs (Wenz et al. 2001). Recent studies indicate that hTERT proteins functionally (Beattie et al. 2000; Armbruster et al. 2001; Beattie et al. 2001) and physically (Armbruster et al. 2001) multimerize *in vivo* and *in vitro*. Functional telomerase multimerization refers to the functional complementation of two distinct, inactive hTERT mutants to reconstitute telomerase activity (Beattie et al. 2001). Physical hTERT multimerization has been demonstrated by the coimmunoprecipitation of rabbit reticulocyte lysate (RRL)-synthesized hTERT proteins with GST-hTERT (Armbruster et al. 2001). However, the association of hTERT proteins in immunoprecipitates may be indirect, and could be mediated by other proteins or RNAs present in RRL. Though yeast and human telomerases contain more than one telomerase RNA (Prescott and Blackburn 1997a; Wenz et al. 2001), the stoichiometry of TERT molecules in telomerase complexes is unknown.

The TERT component of telomerase is limiting and required for telomerase function *in vivo* and *in vitro* (Counter et al. 1997; Weinrich et al. 1997; Beattie et al. 1998; Bodnar et al. 1998; Liu et al. 2000a). TERT is a reverse transcriptase (RT) containing motifs (1 and 2, and A-E) that are common to all RTs (reviewed in Nakamura and Cech 1998). Mutations of most residues conserved between TERTs and members of the broader RT family abolish telomerase activity (for review, see Bryan et al. 2000b). The TERT RT domain is essential for catalytic activity, but is not sufficient for efficient binding of the *Tetrahymena* and human telomerase RNA components (Beattie et al. 2000; Bryan et al. 2000b; Bachand and Autexier 2001; Lai et al. 2001), nor for complementation of full-length inactive hTERT containing a substitution in the RT domain (Beattie et al. 2000; Beattie et al. 2001). The N- and C-terminal TERT regions are not conserved among RT family members, and therefore may mediate telomerase-specific functions. Most of the C-terminus of *Tetrahymena* and human TERTs is required for *in vitro* catalytic activity, though this region is not essential for telomerase RNA binding (Beattie et al. 2000; Bachand and Autexier 2001; Lai et al. 2001). The C-terminus of Est2p (*S. cerevisiae* TERT) influences telomerase processivity (Peng et al. 2001), and the hTERT C-terminus is also implicated in functional multimerization with other hTERT molecules (Beattie et al. 2001).

The TERT N-terminus is larger and more highly conserved than the C-terminus (Xia et al. 2000; Peng et al. 2001). Most of the N-terminus of *Tetrahymena*, *S. cerevisiae* and human TERTs is required to reconstitute wild-type levels of telomerase catalytic activity (Weinrich et al. 1997; Friedman and Cech 1999; Beattie et al. 2000; Bryan et al. 2000b; Miller et al. 2000; Xia et al. 2000; Armbruster et al. 2001; Bachand and Autexier 2001; Lai et al. 2001). Portions of the *Tetrahymena* and human TERT N-termini are essential for efficient binding of the telomerase RNA (Beattie et al. 2000; Bachand and Autexier 2001; Lai et al. 2001), and a newly-identified ciliate-specific motif (CP2) in *Tetrahymena* TERT is one element that defines the enzyme's *in vitro* 5' RNA template boundary (Miller et al. 2000). The hTERT N-terminus is also implicated in functional multimerization with other hTERT molecules (Beattie et al. 2000; Beattie et al. 2001), and a recently identified DAT (dissociates activities of telomerase) domain is required for telomere length maintenance, but not for *in vitro* catalytic activity (Armbruster et al. 2001).

Six major regions in the N-terminus have been identified by sequence alignment of ten TERT family members (Figure 2.1): the non-conserved extreme N-terminus (N), motif GQ (also identified as Region I, motif T2 or motif N) (Friedman and Cech 1999; Malik et al. 2000; Miller et al. 2000; Xia et al. 2000), motif CP (Bryan et al. 1998), a poorly conserved putative linker region between motifs GQ and CP (Xia et al. 2000), motif QFP (also termed Region III) (Friedman and Cech 1999; Xia et al. 2000) and motif T (also termed Region IV) (Lingner et al. 1997b; Nakamura et al. 1997; Friedman and Cech 1999). The CP motif, first identified in ciliates and corresponding to Region II of Est2p (Friedman and Cech 1999), contains residues that are also conserved in non-ciliate TERTs (Xia et al. 2000). An additional ciliate-specific motif, CP2, is located in the linker region of *Tetrahymena* TERT near the GQ motif boundary (Miller et al. 2000). The hTERT DAT domain is encoded by the first half of the GQ motif (Armbruster et al. 2001). The roles for most of the conserved regions in the TERT N-terminus remain to be elucidated.

The TERT N-terminus has been implicated in a number of telomerase-specific functions. However, the domains and mechanisms mediating these functions have not been completely characterized. In this study recombinant human telomerases containing deletions and single amino acid substitutions were expressed in *S. cerevisiae* and *in vitro* transcription/translation (RRL) reactions to identify and characterize the regions of the hTERT N-terminus involved in *in vitro* reconstitution of human telomerase activity, telomerase RNA binding and multimerization with other hTERTs.

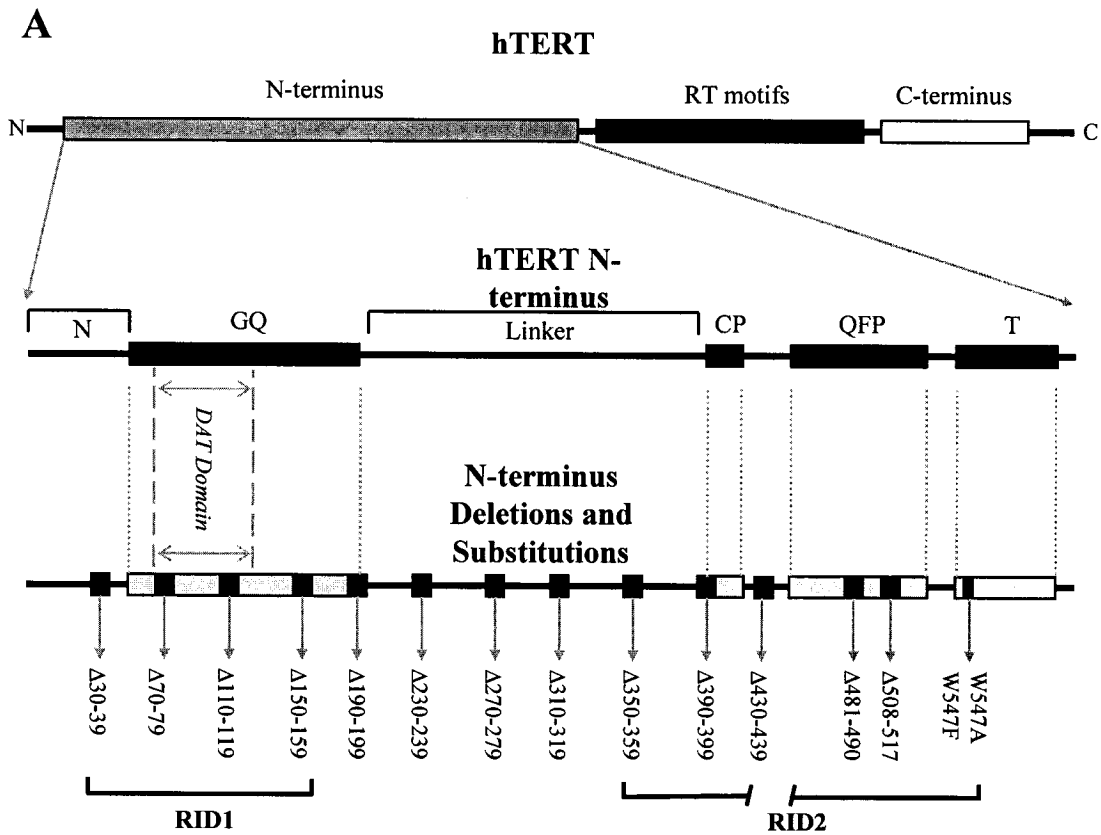
2.4. Materials and methods

2.4.1. Expression constructs

The construction of the pET28b hTERT, pET28b hTERT D868N, and pET28a GST-hTERT rabbit reticulocyte lysate (RRL) expression plasmids, and the pEGKT GST-hTERT and pEGKT GST-hTERT D868N yeast expression plasmids was previously described (Bachand and Autexier 1999). pET28a GST-hTERT D868N was constructed by replacing a 1435-bp fragment from *Xho*I-digested pET28a GST-hTERT by a similar fragment from *Xho*I-digested pET28b hTERT D868N. The construction of *in vitro* transcription and yeast expression plasmids for hTR was previously described (Autexier et al. 1996; Bachand and Autexier 1999).

Figure 2.1: Map of hTERT N-terminus, location of N-terminal mutations, and sequence alignment of the extreme N-terminus and linker of vertebrate TERTs.

(A) A schematic illustration of hTERT and the location of conserved N-terminal subregions previously identified by multiple sequence alignment (Xia et al. 2000). Alternative nomenclature for N-terminal subregions is described in the Introduction. Mutations performed in this study are indicated by black boxes on the linear map of the hTERT N-terminus. The DAT domain is a recently identified region (Armbruster et al. 2001). RID1 and RID2 refer to RNA interaction domains 1 and 2 identified in this study. **(B)** Sequence alignment of the extreme N-terminus (N) of the vertebrate TERTs demonstrating that this region is conserved among vertebrate TERTs. Alignment of human, *M. musculus, M. auratus* and *Xenopus laevis* TERT sequences (Harrington et al. 1997b; Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Greenberg et al. 1998; Martin-Rivera et al. 1998; Guo et al. 2001; Kuramoto et al. 2001) was performed using the BLAST® program. The symbol '+' indicates non-identical conserved residues. Residues conserved in all vertebrate TERTs are underlined. **(C)** Sequence alignment of the vertebrate TERT linker indicating that the vertebrate linker contains conserved subregions, specifically at the C-terminal end. Alignment was performed as described above. Residues conserved in all vertebrate TERTs are underlined. The C-terminal end of the illustrated sequences is continuous with sequences of the CP motif as defined by Xia et al.(Xia et al. 2000). A highly conserved vertebrate-specific motif (VSR motif, for vertebrate-specific RNA binding motif) identified in this study is shown in bold.



B

BLAST Alignment of the Extreme N-terminus Region (N) of Vertebrate TERTs

```
hTERT          1  MPRAPRCRAVRSLLRSHYREVLPLATFVRRLGPQGWRLVQRGDPAAFRALVAQCLVC  57
M. auratus     1  MPRAPRCRAVR+LLRS YR+V PLATFVRRLGP+G +LVQ GDP  FR LVA+CLVC  57
M. musculus    1  M RAPRC AVRSLLRS YREV PLATFVRRLGP+G RLVQ GDP  +R LVAQCLVC  57
Xenopus laevis 1  MP       + S+L+  Y +VL +  +   L P G +++  GD   FR+ VA+ +VC  60
```

C

BLAST Alignment of the Linker Region of Vertebrate TERTs

```
hTERT          195  RRLGCERAWNHSVREAGVPLGLPAPGARRRGGSASRSLPLPKRPRRGAAPEPERTPVGQ  253
M. auratus     196  R   +G     R       N  S    +EA                      P  Q  262
M. musculus    196  R   +G +++                                          PV +  254
Xenopus laevis

hTERT          254  GSWAHPGRTRGPSDRGFCVVSPARPAEEATSLEGALSGTRHSHPSVGRQHHAGPPSTSR  312
M. auratus     263       +T  P+      V  +R  +E  S      G   +  SV  +H   P STS+  319
M. musculus    255     + R   P+  +  V SPAR AE+  S +G +S    S  SV  +H   P STS   315
Xenopus laevis

hTERT          313  PPRPWDTPCPPVYAETKHFLYSSGDKEQLRPSFLLSSLRPSLTGARRLVETIFLGSRPW  371
M. auratus     320            Y ETK FLYS G +E+              TGARRLVE +FLG RP  379
M. musculus    316            + ET+HFLSYSGD+E+              TGARRLVE IFLGSRP  376
Xenopus laevis 386                        K+    SF+L+SL  +  +G+++LVETIFL +    419
                                                            VSR motif

hTERT          372  MPGTPRRLPRLPQRYWQMRPLFLEL  396
M. auratus     380    G     RL +RYWQMRPLF +L   404   CP motif
M. musculus    377    G   R RL +RYWQMRPLF +L   401
Xenopus laevis 420      P+R  +LP+RYW+M+P + EL  450
```

Site-directed mutagenesis, based on the Quik™ Change Site-Directed Mutagenesis kit from Stratagene, was used to generate pET28b hTERT constructs coding for N-terminal deletions and substitutions. Deletions and substitutions were confirmed by restriction enzyme digest and/or sequencing using a T7 DNA polymerase sequencing kit (USB Corporation, Cleveland). Yeast pEGKT GST-hTERT expression constructs bearing hTERT N-terminal deletions and substitutions were generated by PCR amplification of pET28b hTERT mutation constructs, using the 5' and 3' primers 5'-TGCTCTAGACCCGCGCGCTCCCCGC-3' and 5'-CCCAAGCTTTCAGTCCAGGATGGTCTTG-3', containing *Xba*I and *Hind*III restriction sites respectively. *Xba*I-*Hind*III-digested PCR products were cloned into the pEGKT vector digested with the same enzymes.

2.4.2. Recombinant telomerase production

The TnT® T7-Coupled Reticulocyte Lysate System (Promega) was used to generate hTERT proteins from pET28b hTERT and pET28a GST-hTERT expression constructs, as per the manufacturers' instructions, using 0.8 μCi/μl ^{35}S-methionine (Perkin Elmer). Human telomerase RNA was synthesized from an *Fsp*I-linearized phTR+1 plasmid, as previously described (Autexier et al. 1996). Purified hTR was included in the *in vitro* transcription/translation reactions at a concentration of 80 fmoles/μl, in the presence of 8 fmoles/μl pET-hTERT plasmid DNA.

Recombinant human telomerase was generated in GST-hTERT- and hTR-expressing yeast as previously described (Bachand and Autexier 1999), except that yeast were grown in selective medium containing 1% raffinose prior to induction with 4% galactose. Cell pellets were lysed in 3 to 4 volumes of lysis buffer (0.25 mM deoxycholic acid, 10 mM Tris-HCl pH 8, 1.2 mM $MgCl_2$, 15% glycerol, 0.1 mM EDTA pH 8, 0.1 mM EGTA pH 8, 1.5 mM DTT, 5 mM β-mercaptoethanol, 1 μM pepstatin A, 1 μM leupeptin, 0.2 mM 4-(2-aminoacyl) benzene sulfonyl fluoride hydrochloride (AEBSF), 38 U/ml RNAguard Amersham Pharmacia), using a previously described glass bead lysis method (Bachand and Autexier 1999). Expression of GST-hTERT proteins was confirmed by Western blot analysis using 0.3 μg/ml of an affinity-purified hTERT antibody (described below).

2.4.3. Immunoprecipitations

Immunoprecipitations were performed with antibodies specific to GST (Amersham Pharmacia) or hTERT. In some experiments, BSA and/or *E. coli* tRNA were added to the immunoprecipitation buffer (10 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 1% NP-40, 10% glycerol, 150 mM NaCl, 0.2 mM AEBSF, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, 38 U/ml RNAguard Amersham Pharmacia) as blocking agents (each at 100 ng/ml). Rabbit preimmune serum or GST or hTERT antibodies were prebound to pre-equilibrated protein A-sepharose beads (Amersham Pharmacia) by incubation (from 2-h to overnight) at 4°C. RRL and yeast lysates containing hTERT proteins were precleared with rabbit preimmune serum for 1 hour, followed by 2 to 3 hours of immunoprecipitation with immobilized GST or hTERT antibodies. Four washes were performed in immunoprecipitation buffer containing 150 mM (washes 1 and 4) or 300 mM (washes 2 and 3) NaCl. Beads suspended in immunoprecipitation buffer were used directly in subsequent assays to detect hTR, hTERT, GST-hTERT and telomerase activity. hTERT antibody was used at 0.9 to 1.35 μg/ml in immunoprecipitations; efficiency was >25%.

2.4.4. Affinity-purified polyclonal hTERT antibody

An hTERT peptide-directed polyclonal hTERT antibody was generated in rabbits and affinity-purified using the immunogenic peptide N-SEAEVRQHREARPALLTSRLRFIPKC-C. The peptide used to generate and purify the hTERT antibody was synthesized at the Sheldon Biotechnology Centre of McGill University, and the hTERT antibody was raised and purified by Strategic Biosystems, California, USA. This peptide is a variant of a peptide previously designed for hTERT antibody generation (Harrington et al. 1997b), and falls within Motif 1 of the hTERT RT domain. The hTERT antibody detected recombinant GST-hTERT or hTERT expressed in yeast and RRL. The specificity of anti-hTERT was confirmed by Western blot analysis of yeast-expressed GST-hTERT immunoprecipitated by a GST antibody.

2.4.5. Telomerase activity assays and quantification of telomerase activity

Telomerase activity was detected by a modified, two-step version of the telomeric repeat amplification protocol (Autexier et al. 1996), with minor modifications. Five to 10% of immunoprecipitates, 15 μg of total protein from yeast crude lysates, or 0.5-1 μl of

RRL were assayed for telomerase activity in a 20 µl final volume during the elongation step of the reaction. Five to 10 µl of this elongation reaction were amplified by PCR. Each 50 µl PCR reaction contained 20 pmoles each of TS, NT and ACX primers, and 1 x 10^{-19} moles of the TSNT primer. Amplification of TSNT by TS and NT primers generates an internal control (IC). These primers have been described previously (Kim and Wu 1997). The PCR reaction mix was composed of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 50 µM dNTPs, and each reaction contained 2 U Taq DNA polymerase and 5 µCi ^{32}P dGTP (800 Ci/mmol, Perkin Elmer). PCR reactions were performed for 25 cycles of 95°C for 30 sec, 50°C for 30sec, and 72°C for 1 min 30 sec. Telomerase activity was quantified from RRL samples expressing equal amounts of hTERT protein. The telomerase elongation product signal generated by individual hTERT mutants was normalized to the PCR internal control signal, and the obtained ratio was expressed as a fraction of the ratio calculated for the elongation products from wild-type telomerase that was always included with each experiment.

2.4.6. Telomerase RNA binding assay and quantification of RNA binding

hTERT N-terminal mutants were synthesized and ^{35}S-labelled in RRL in the presence of ^{32}P-labelled hTR, using a modified version of a method previously developed to detect telomerase RNA binding to *Tetrahymena* TERT (Bryan et al. 2000b). hTR was synthesized from the *Fsp*I-linearized phTR+1 plasmid in the presence of ^{32}P-UTP (800 Ci/mmol). Linearized plasmid (2.5 µg) was combined with T7 RNA polymerase buffer (40 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT), 250 U T7 RNA polymerase (NEB) and 250 µCi ^{32}P UTP (Perkin Elmer) in a 50 µl reaction mixture containing 0.5 mM each of ATP, GTP and CTP. Labelled hTR was diluted to a final concentration of 1 pmol/µl with unlabelled, *in vitro*-synthesized hTR. The specific activity of diluted labelled hTR was 1 x 10^6 to 1 x 10^7 cpm/pmol, and 0.5 pmol of hTR were included in a 12.5 µl RRL reaction. hTERT-hTR complexes were immunoprecipitated using hTERT antibody. After washing, 100% of beads were resuspended in SDS-PAGE loading buffer (100 mM DTT, 1.67% SDS, 5% glycerol, 0.83% β-mercaptoethanol, 58 mM TrisHCl pH 6.8), and immunoprecipitated hTERT and coprecipitated hTR were resolved on 7.5% SDS-PAGE gels and detected as previously described (Bryan et al.

2000b). Bands corresponding to hTR and hTERT were quantified using a Molecular Dynamics Densitometer and ImageQuant software. The hTR signal in each lane was divided by the hTERT signal in the same lane, and this ratio was expressed relative to the same ratio calculated for immunoprecipitated wild-type hTERT and coprecipitated hTR loaded on the same gel. As labelled hTR generated significant levels of non-specific background in each lane, background signal was subtracted from the signal obtained for hTERT bands, to more accurately quantify the hTERT signals present in each lane.

2.4.7. Multimerization assays

In all mixing experiments, GST-hTERT proteins expressed in yeast or RRL were mixed with hTERT proteins expressed in RRL, then incubated on ice for 1 hour. GST-hTERT/hTERT complexes were immunoprecipitated with a GST antibody (Amersham Pharmacia). Five to 10% of immunoprecipitates were examined for telomerase activity by TRAP, and the remainder was loaded on 7.5% SDS-PAGE gels. Immunoprecipitated GST-hTERT proteins and coprecipitated hTERT proteins were detected by Western blot analysis using hTERT antibody, and/or by visualization of ^{35}S-labelled proteins on fixed, dried SDS-PAGE gels exposed to Kodak XAR sensitive film.

For the mixing experiments in which yeast-expressed GST-hTERT mutants were mixed with RRL-synthesized WT or D868N hTERT (Figure 2.4A,B), 90 µg of total yeast lysate proteins were mixed with 6 µl of RRL. For the mixing experiments in which yeast-expressed D868N GST-hTERT was mixed with hTERT mutants synthesized in rabbit reticulocyte lysates (Figure 2.5A), two quantification steps were performed before mixing. First, 0.5 µl aliquots of RRL-synthesized ^{35}S-labelled hTERT mutants were subjected to electrophoresis on 7.5% SDS-PAGE gels and quantified relative to wild-type hTERT loaded on the same gel. Second, yeast-generated D868N GST-hTERT was quantified relative to RRL-synthesized wild-type hTERT using Western blot analysis. Yeast-expressed D868N GST-hTERT and RRL-synthesized hTERT were mixed in a 1:1 ratio (~1 µl RRL for every 20 µl yeast lysate). Quantification of input proteins prior to mixing was performed similarly for RRL-based mixing experiments (Figures 2.5B, 2.6A). Approximately 5-10 µl each of RRL-synthesized GST-hTERT and hTERT were mixed in these experiments. Mixes were diluted to 20 µl with yeast lysis buffer prior to incubation on ice.

In the experiments where D868N GST-hTERT was cosynthesized with hTERT mutants, equal quantities of plasmids were added to rabbit reticulocyte lysates (20 ng/μl for each plasmid) and synthesis was carried out in the presence of hTR as described above. Immunoprecipitations were performed using 20 μl of each synthesis reaction.

2.5. Results

We generated a series of recombinant telomerases containing single amino acid substitutions and 10 amino acid deletions (Figure 2.1A) to characterize the roles of the hTERT N-terminus in telomerase activity, telomerase RNA binding and multimerization with other hTERT molecules. Deletions were regularly spaced at 40 amino acid intervals, with the exception of Δ481-490 and Δ508-517, which were designed to overlap with previously characterized Est2p mutations (Est2p residues 303-312 and 330-339, respectively) (Friedman and Cech 1999). Non-conservative and conservative substitutions of the tryptophan at position 547 in the hTERT T motif to alanine (W547A) or phenylalanine (W547F), respectively, were also generated (Figure 2.1A). The *Tetrahymena* TERT encodes a phenylalanine at this location, in contrast to all other TERTs, which contain a tryptophan residue. Deletion of hTERT residues 70-79, 150-159, 481-490 and 508-517 removed highly conserved amino acids previously identified by the alignment of TERT sequences from yeasts, ciliates, vertebrates and plants (Table 2.1) (Xia et al. 2000).

Alignment of TERT sequences from diverse organisms reveals substantial divergence in sequence composition and length in the linker and at the extreme N-terminus (N) (Xia et al. 2000). We aligned four vertebrate TERT sequences (Harrington et al. 1997b; Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997) using the NCBI BLAST program (Altschul et al. 1997) to determine if any portions of the N and linker regions are conserved (Figure 2.1: B and C). The extreme N-terminus (hTERT residues 1-57) was conserved among vertebrates TERTs (Figure 2.1B). The N-terminal two thirds of the linker were weakly conserved, but the C-terminal end of the linker contained two blocks of highly conserved amino acids (Figure 2.1C). One of these conserved linker regions (R381-L396 in hTERT) was continuous with the previously identified CP motif (Figure 2.1C). The second conserved block of amino acids in the linker (T355-L366 in

Table 2.1: Telomerase activity, hTERT/hTR association and functional multimerization of hTERT N terminus mutants

Mutation	Identity and Conservation of Deleted Residues [a,b]	Region of N-terminus [c]	Telomerase Activity [d]			hTERT/hTR Association [e]			RNA Interaction Domain [f]	Physical Multimerization with GST-hTERT D868N [g]	Functional Complementation of GST-hTERT D868N [h]
			Avg	*SD*	*n*	*Avg*	*SD*	*n*			
WT			1.00	0	5	1.00	0	4		+	+
D868N			0	0	4	1.05	0.12	3		+	-
Δ30-39	***RL***GPQ***G***W***RLV***	N	0.10	0.02	4	0.81	0.10	3	RID1	+	+/-
Δ70-79	***SF***R***QVS***CL***K***<u>***E***</u>	GQ motif	0.38	0.18	5	0.87	0.13	3	RID1	+	+
Δ110-119	GPPEAF***TTSV***	GQ motif (DAT domain)	0.99	0.06	3	0.83	0.11	3	RID1	+	+
Δ150-159	VH<u>LL</u>AR***CALF***	GQ motif	0	0	4	0.91	0.02	3	RID1	+	+
Δ190-199	ASGPRRRLGC	GQ/ linker	0.92	0.16	5	1.11	0.19	3		+	+
Δ230-239	RSLPLPKRPR	Linker	0.87	0.25	4	0.90	0.25	3		+	+
Δ270-279	FCVVSPARPA	Linker	1.16	0.18	5	1.01	0.26	3		+	+
Δ310-319	TSRPPRPWDT	Linker	0.70	0.41	4	1.07	0.07	2		+	+
Δ350-359	LRPSL***TGARR***	Linker (VSR motif)	0.01	0.01	3	0.55	0.08	2	RID2	+	-
Δ390-399	***RP***L***FLELLGN***	Linker/ CP motif	0.04	0.05	4	0.52	0.11	3	RID2	+	-
Δ430-439	KPQGSVAAPE		1.14	0.18	4	0.95	0.03	3		+	+
Δ481-490	<u>***RHNERRF***L***RN***</u>	QFP motif	0.04	0.04	3	0.41	0.1	4	RID2	+	-
Δ508-517	<u>***LT***W***K***M***S***V***R***D***C***</u>	QFP motif	0.15	0.18	3	0.27	0.07	4	RID2	+	-
W547A		T motif	0.01	0.01	3	0.43	0.10	4	RID2	+	-
W547F		T motif	1.00	0.02	3	0.77	0.13	4	RID2	+	+

a) ***Italics*** indicate 100% conservation among vertebrate TERTs (*H. sapiens*, *M. auratus*, *M. musculus*, *Xenopus*) (Harrington et al. 1997b; Kilian et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Greenberg et al. 1998; Martin-Rivera et al. 1998; Guo et al. 2001; Kuramoto et al. 2001). <u>***Underline + italics***</u> indicates > or = 70% conservation among all TERTs (*H. sapiens*, *M. auratus*, *M. musculus*, *Xenopus*, *Arabidopsis*, *S. pombe*, *Euplotes*, *Oxytricha*, *Tetrahymena*, *S. cerevisiae*, *Candida albicans*) (Xia et al. 2000). <u>**Underline with no italics**</u> indicates >70% conservation of a residue among all TERTs, but <100% conservation among vertebrate TERTs.

b) Alignments were performed with NCBI-BLAST, and using the multiple sequence alignment published by Xia et al., 2000 (Xia et al. 2000). See Figure 2.1 for sequence alignments of the extreme N-terminus (N) and linker regions of the vertebrate TERTs.

c) Motifs identified by multiple sequence alignment (Xia et al. 2000). Additional domains/motifs identified in hTERT are indicated in parentheses.

d) Telomerase activities of mutant telomerases reconstituted in RRL were expressed relative to the activity of wild-type enzyme.

e) hTR association with hTERT mutants generated in RRL was expressed relative to hTR association with wild-type hTERT.

f) <u>R</u>NA <u>i</u>nteraction <u>d</u>omain 1 (RID1). <u>R</u>NA <u>i</u>nteraction <u>d</u>omain 2 (RID2).

g) The physical association of hTERT mutants with GST-hTERT D868N *in vitro* was evaluated by SDS-PAGE using: 1) immunoprecipitated mixtures of yeast- or RRL-synthesized GST-hTERT D868N and RRL-synthesized hTERT mutants (see Figures 2.4C and 2.5A, respectively); and 2) immunoprecipitated RRL co-synthesis reactions (Figure 2.6).

h) The ability of hTERT mutants to functionally complement inactive GST-hTERT D868N co-synthesized in RRL was evaluated by TRAP assay. The symbol '+' indicates functional complementation to reconstitute telomerase activity. The symbol '-' indicates an inactive mutant that does not complement GST-hTERT D868N. '+/-' indicates weak complementation.

hTERT) was flanked on either side by non-conserved residues (Figure 2.1C) that are not required for *in vitro* telomerase activity (Armbruster et al. 2001). We named this second conserved linker region the VSR motif (for vertebrate-specific RNA binding motif; see below) (Figure 2.1C). Deletion of hTERT residues 30-39, 350-359 and 390-399 removed vertebrate-specific amino acids in the extreme N-terminus and linker (Table 2.1). Deletion of hTERT residues 190-199, 230-239, 270-279, 310-319 and 430-439 removed sequences that were not conserved among any of the TERTs (Table 2.1).

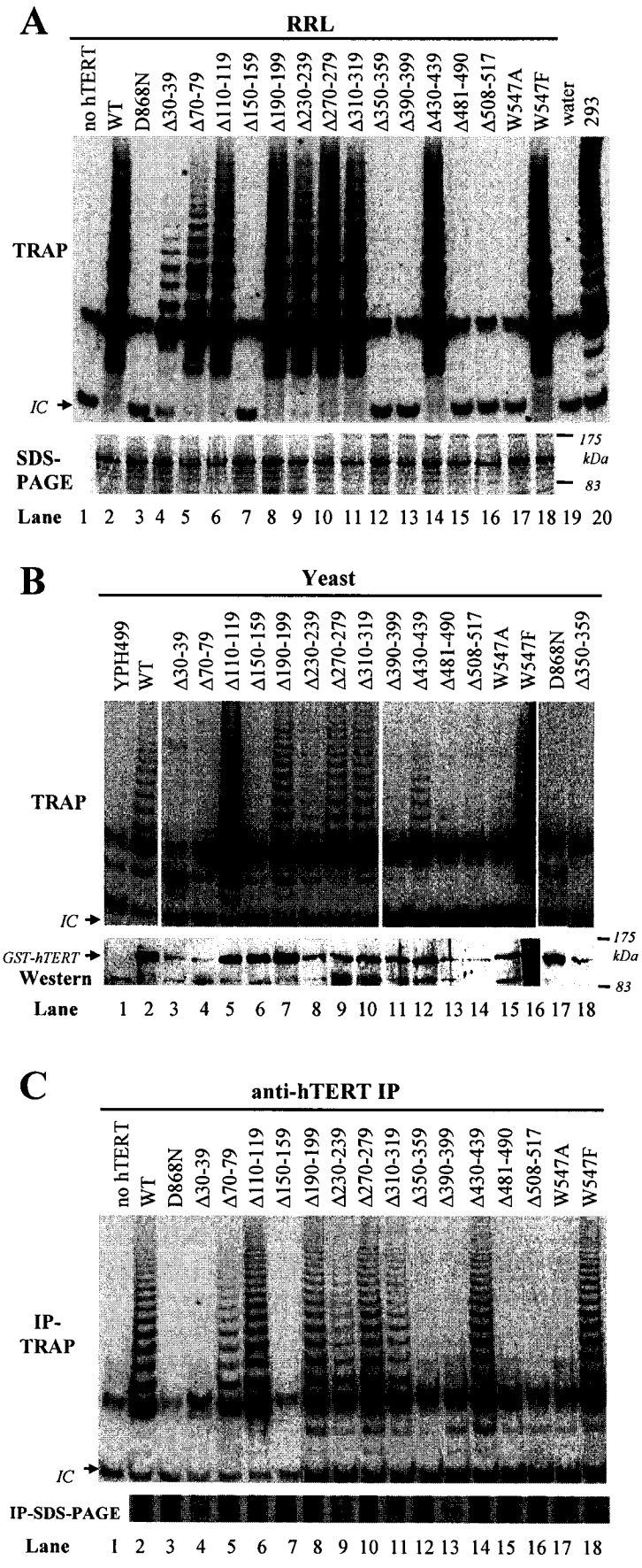
2.5.1. Telomerase activity of hTERT N-terminal mutants expressed in *S.cerevisiae* and *in vitro* transcription/translation systems

hTERT N-terminal mutants were expressed in RRL or as GST fusion proteins in *S. cerevisiae*, in the presence of the human telomerase RNA (Figure 2.2: A and B, respectively). The catalytically inactive RT domain point mutant, D868N, (Bachand and Autexier 1999) was also synthesized in both systems as a negative control for telomerase activity (Figure 2.2A, B, C: lanes 3, 17 and 3, respectively, top panels). All of the mutant proteins were stably expressed in RRL (Figure 2.2A, bottom panel), and were immunoprecipitated using an hTERT antibody (Figure 2.2C, bottom panel). However, GST-hTERT mutant protein expression levels varied in yeast. Specifically, GST-hTERTs containing mutations Δ30-39, Δ70-79, Δ481-490 and Δ508-517 were poorly expressed (Figure 2.2B: lanes 3, 4, 13 and 14, bottom panel). Three of these mutations partially or fully overlap with mutations associated with Est2pP protein instability (Friedman and Cech 1999).

The catalytic activities of reconstituted telomerases were assayed by the TRAP technique, using RRL-expressed enzyme (Figure 2.2A: top panel), yeast protein extracts (Figure 2.2B: top panel) or immunoprecipitated telomerase from RRL (Figure 2.2C: top panel). The relative levels of telomerase activity for mutant enzymes reconstituted in RRL are shown in Figure 2.2A. The Δ30-39 mutant, which reconstituted low levels of telomerase activity in crude RRL (Figure 2.2A: lane 4), was nearly inactive when expressed in yeast (Figure 2.2B: lane 3), or following immunoprecipitation from RRL (Figure 2.2C: lane 4). The Δ70-79 mutant, which was active in crude RRL and following immunoprecipitation from RRL (Figure 2.2A and C: lane 5), was inactive in yeast (Figure

Figure 2.2: Reconstitution of telomerase activity in *S. cerevisiae* and *in vitro* transcription/translation systems.

(A) Telomerase activity of hTERT N-terminal mutants expressed in RRL. Top panel: Reconstituted telomerase activity of different hTERT mutants (Figure 2.1) was detected using the PCR-based TRAP assay. A PCR amplification control is indicated by the label 'IC'. Control reactions were performed using RRL containing only hTR (lane 1), no lysate (lane 19: water), or partially-purified human 293 cell extracts (lane 20). D868N is a mutation in the RT domain of hTERT that abolishes telomerase activity. Bottom panel: Expression of hTERT mutants synthesized in RRL in the presence of ^{35}S-methionine was detected by SDS-PAGE. **(B)** Telomerase activity of GST-hTERT N-terminal mutants expressed in *S. cerevisiae*. Top panel: TRAP assays were performed using different GST-hTERT mutants, as indicated. A control reaction was performed using an extract of the parental yeast strain YPH499 not expressing GST-hTERT (Lane 1). Bottom panel: GST-hTERT fusion proteins expressed in *S. cerevisiae* were detected by Western blotting using a polyclonal hTERT antibody. **(C)** Immunoprecipitation of human telomerase from rabbit reticulocyte lysates expressing hTERT N-terminal mutants. Anti-hTERT immunoprecipitates from RRL expressing different hTERT mutants were analyzed for telomerase activity using TRAP (top panel), and for immunoprecipitation of ^{35}S-hTERT by SDS-PAGE (bottom panel).


A
RRL
no hTERT
WT
D868N
Δ30-39
Δ70-79
Δ110-119
Δ150-159
Δ190-199
Δ230-239
Δ270-279
Δ310-319
Δ350-359
Δ390-399
Δ430-439
Δ481-490
Δ508-517
W547A
W547F
water
293
TRAP
IC
SDS-PAGE
175 kDa
83
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B
Yeast
YPH499
WT
Δ30-39
Δ70-79
Δ110-119
Δ150-159
Δ190-199
Δ230-239
Δ270-279
Δ310-319
Δ390-399
Δ430-439
Δ481-490
Δ508-517
W547A
W547F
D868N
Δ350-359
TRAP
IC
GST-hTERT
Western
175 kDa
83
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
C
anti-hTERT IP
no hTERT
WT
D868N
Δ30-39
Δ70-79
Δ110-119
Δ150-159
Δ190-199
Δ230-239
Δ270-279
Δ310-319
Δ350-359
Δ390-399
Δ430-439
Δ481-490
Δ508-517
W547A
W547F
IP-TRAP
IC
IP-SDS-PAGE
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

2.2B: lane 4). Overall, the relative telomerase activities of the panel of mutants in yeast and crude or immunoprecipitated RRL were similar (Figure 2.2: compare A, B and C). However, the catalytic activity of wild-type and mutant telomerases reconstituted in yeast was generally weaker than the activity observed in crude and immunoprecipitated RRL samples (compare Figure 2.2B to Figures 2.2A and C, respectively). One exception was the GST-hTERT mutant Δ110-119, which reproducibly reconstituted more active telomerase than wild-type GST-hTERT when expressed in yeast (Figure 2.2B: lanes 5 and 2, respectively). This increased activity was not observed when the mutant enzyme was expressed in RRL (Figure 2.2A: lane 6). As all hTERTs were expressed at equal levels in RRL, the reconstituted telomerase activities of mutants relative to wild-type were quantified from crude and immunoprecipitated RRL. The average relative telomerase activities of the mutants are indicated in Table 2.1.

2.5.2. Residues unique to vertebrate TERTs are required for human telomerase activity

Deletions that removed residues highly conserved among all TERTs (Δ70-79, Δ150-159, Δ481-490, Δ508-517) severely impaired reconstitution of human telomerase activity (<40% of reconstituted wild-type telomerase activity) (Table 2.1). The non-conservative W547A substitution abolished telomerase activity, whereas a conservative change to a residue naturally encoded in the *Tetrahymena* TERT (W547F) had no detectable effect on telomerase activity using the TRAP assay (Table 2.1). All deletions that removed vertebrate-specific conserved residues, except Δ110-119, which is located within the catalytically non-essential DAT domain of hTERT (Armbruster et al. 2001), resulted in inactive or weakly active recombinant enzymes (<10% of reconstituted wild-type telomerase activity) (Table 2.1: Δ30-39, Δ350-359, Δ390-399). In contrast, deletion of non-conserved residues (Δ190-199, Δ230-239, Δ270-279, Δ310-319, Δ430-439) had a negligible effect on telomerase activity (>70% of reconstituted wild-type activity) (Table 2.1). These results demonstrate a functional role for conserved N-terminal domains previously identified by sequence alignment of TERTs from multiple organisms (Xia et al. 2000), as all hTERT mutations that altered highly conserved residues impaired *in vitro* catalytic activity. These and previously published results also suggest that some regions of

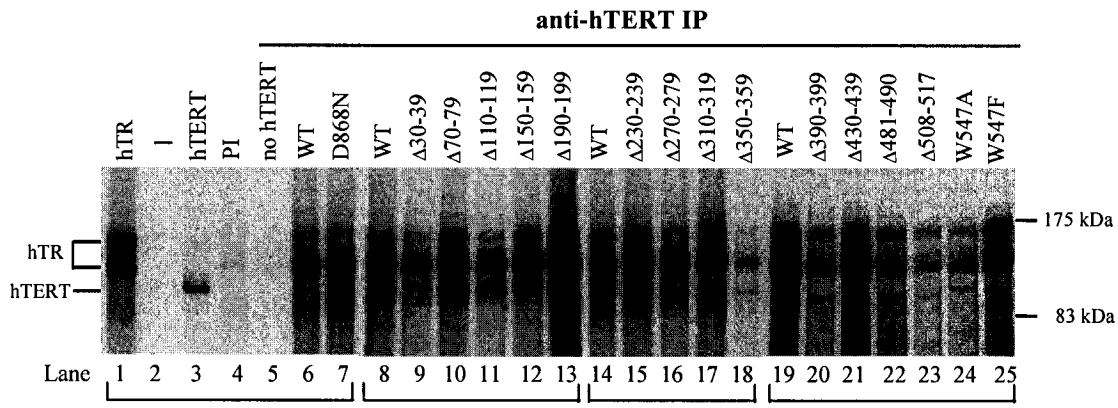
the TERT N-terminus may be implicated in organism-, vertebrate- or ciliate-specific *in vitro* catalytic function (Miller et al. 2000).

2.5.3. Vertebrate-specific conserved regions in hTERT are required for association with the telomerase RNA

Sequence alignment identified vertebrate-conserved segments of TERT (Figure 2.1). These regions may represent domains involved in protein-protein or protein-RNA interactions. Vertebrate telomerase RNAs share a common structure, including three conserved domains (CR4-CR5, CR7 and Box H/ACA domains) that are not present in the ciliate telomerase RNAs (Chen et al. 2000) and which may associate with TERT sequences unique to vertebrates. The association of ^{32}P-labelled hTR with RRL-synthesized hTERT mutants was examined following immunoprecipitation of hTERT/hTR complexes using an hTERT polyclonal antibody. The hTERT antibody immunoprecipitated all hTERT mutants expressed in RRL (Figure 2.2C). Representative results are shown in Figure 2.3, and quantification of hTERT-hTR binding from multiple experiments is summarized in Table 2.1. The catalytically-inactive D868N hTERT mutant bound hTR as efficiently as wild-type hTERT (Figure 2.3: lanes 7 and 6, respectively; and Table 2.1). Deletions in the extreme hTERT N-terminus and N-terminal two-thirds of the GQ motif, including the DAT domain, resulted in modest (10-20%), but reproducible reductions in hTR association with hTERT (Figure 2.3 and Table 2.1: Δ30-39, Δ70-79, Δ110-119, Δ150-159). We defined the region between amino acids 30 and 159 as the hTERT RNA interaction domain 1 (RID1). However, deletion of residues 230-239 also resulted in a 10% reduction in hTR association with hTERT, indicating that the RID1 domain might extend to residue 240. Deletion of portions of the hTERT linker that were only weakly conserved among the vertebrate TERTs did not affect telomerase RNA binding (>90% of wild-type hTERT-hTR binding) (Figure 2.3 and Table 2.1: Δ190-199, Δ230-239, Δ270-279, Δ310-319). Deletion of vertebrate-conserved residues in the linker VSR motif and at the CP motif/linker junction resulted in a 50% reduction in hTR binding (Figure 2.3 and Table 2.1: Δ350-359 and Δ390-399, respectively). Mutations in highly conserved residues of the CP, QFP and T motifs caused a 50-70% reduction in hTR

Figure 2.3: Association of the human telomerase RNA and hTERT N-terminal mutants.

^{35}S-labelled hTERT was synthesized in RRL in the presence of ^{32}P-labelled hTR. hTERT/hTR complexes were immunoprecipitated using an hTERT polyclonal antibody, and were resolved on 7.5% SDS-PAGE gels. hTR or hTERT present in crude RRL prior to immunoprecipitation are shown in lanes 1 and 3, respectively. Lane 2 is empty. Control reactions were performed to detect non-specific immunoprecipitation of wild-type hTERT/hTR complexes by rabbit preimmune serum (lane 4) and to detect the non-specific levels of hTR immunoprecipitated in the absence of hTERT (lane 5). Lanes 1 to 7, 8 to 13, 14 to 18 and 19 to 25 represent independent autoradiographs (indicated by brackets below the lane numbers). Levels of hTR coimmunoprecipitated with hTERT mutants are compared to the amounts of coimmunoprecipitated hTR and wild-type hTERT (lanes 6, 8, 14 and 19) loaded on the same gel.


anti-hTERT IP
hTR
I
hTERT
PI
no hTERT
WT
D868N
WT
Δ30-39
Δ70-79
Δ110-119
Δ150-159
Δ190-199
WT
Δ230-239
Δ270-279
Δ310-319
Δ350-359
WT
Δ390-399
Δ430-439
Δ481-490
Δ508-517
W547A
W547F
hTR
hTERT
175 kDa
83 kDa
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

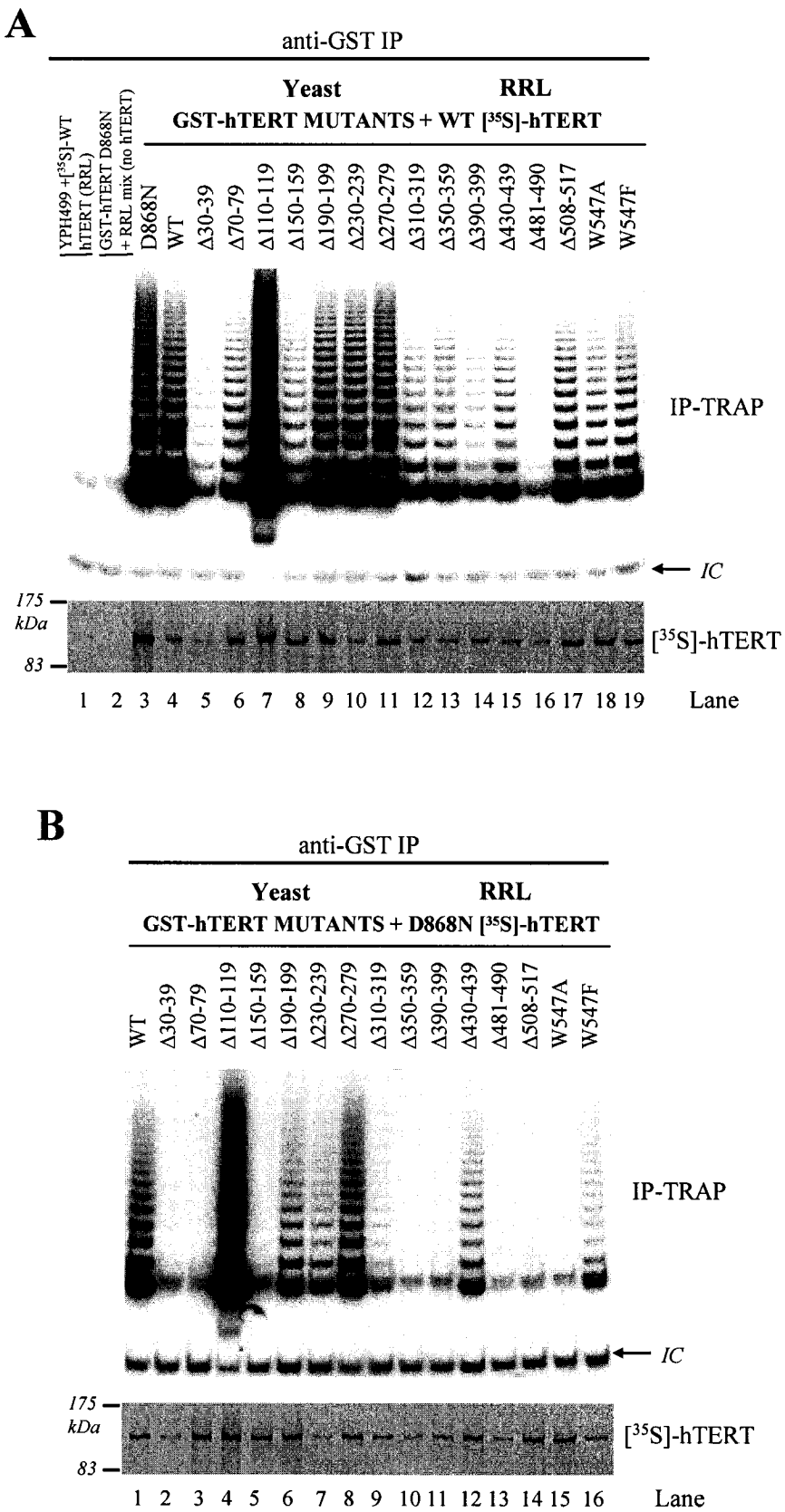
association with hTERT (Figure 2.3 and Table 2.1: Δ390-399, Δ481-490, Δ508-517 and W547A), indicating that these regions are likely involved in telomerase RNA binding. The conservative substitution W547F resulted in a more modest 25% reduction in hTR/hTERT association (Figure 2.3 and Table 2.1), and had no effect on telomerase activity (Table 2.1). We defined the hTR-interacting region containing the VSR, CP, QFP and T motifs (residues 350-547) as hTERT RNA interaction domain 2 (RID2). However, hTERT containing a deletion in a poorly conserved region between the CP and QFP motifs was catalytically active and bound hTR as efficiently as wild-type hTERT (Figure 2.3 and Table 2.1: Δ430-439), indicating that RID2 is divided into two non-contiguous regions, defined by residues 350-399 and 481-547, respectively. Mutants demonstrating a 50-70% reduction in RID2-mediated hTR binding exhibited a 5-fold or greater reduction in telomerase activity. Therefore, efficient RID2-mediated hTERT/hTR associations appear to be important for catalytic activity.

2.5.4. GST-hTERT N-terminal mutants associate with wild-type hTERT

Previous work suggests that human telomerase functions as a multimer (Beattie et al. 2001; Wenz et al. 2001). The physical association of hTERT proteins *in vitro* was demonstrated recently by the coimmunoprecipitation of hTERT with GST-hTERT (Armbruster et al. 2001). In an effort to determine the role of the N-terminus in multimerization, we investigated the interactions of GST-hTERT N-terminal mutants with wild-type hTERT. We mixed yeast lysates expressing GST-hTERT mutants and the human telomerase RNA with ^{35}S-labelled wild-type hTERT synthesized in RRL in the presence of hTR. If an interaction occurred between GST-hTERT mutants and wild-type hTERT, immunoprecipitation of inactive GST-hTERT mutants would coprecipitate wild-type hTERT and telomerase activity. Immunoprecipitation of GST protein alone does not coprecipitate hTERT (Armbruster et al. 2001, and data not shown). Immunoprecipitation of both active and inactive GST-hTERT mutants using a GST antibody coprecipitated ^{35}S-labelled wild-type hTERT and telomerase activity (Figure 2.4A, and compare Figures 2.2B and 2.4A: D868N, deletions 30-39, 70-79, 150-159, 350-359, 390-399, 481-490 and 508-517, and W547A). Therefore, certain N-terminal regions of hTERT deleted in this study may not be essential for the association of hTERT proteins. Some inactive N-

Figure 2.4: Association of GST-hTERT mutants with wild-type and D868N hTERT.

Yeast whole cell extracts were made from *S. cerevisiae* expressing GST-hTERT fusion proteins (D868N, WT and mutants, as indicated) in the presence of hTR. These extracts were mixed with RRL containing ^{35}S-labelled hTERT proteins synthesized in the presence of hTR. hTERT/GST-hTERT complexes were immunoprecipitated using a GST antibody, and were analyzed for telomerase activity by TRAP (top panels), and for coprecipitation of ^{35}S-labelled hTERT by SDS-PAGE (bottom panels). **(A)** GST-hTERT fusion proteins associate with wild-type hTERT. Extracts prepared from yeast expressing GST-hTERT N-terminal mutants, or from the parental YPH499 strain, and containing equal amounts of total cellular proteins were mixed with RRL expressing WT hTERT. Control reactions were performed to detect non-specific immunoprecipitation of WT hTERT in the absence of GST-hTERT (lane 1), and to determine whether RRL containing only hTR could complement GST-hTERT D868N to reconstitute telomerase activity (lane 2). **(B)** GST-hTERT fusion proteins physically associate with D868N hTERT, but inactive N-terminal mutants cannot functionally complement the inactive D868N mutant to reconstitute telomerase activity. Extracts prepared from yeast expressing GST-hTERT mutants were mixed with ^{35}S-labelled D868N hTERT synthesized in RRL, and analyzed as described above.


A
anti-GST IP
Yeast
RRL
GST-hTERT MUTANTS + WT [35S]-hTERT
YPH499 +[35S]-WT hTERT (RRL)
GST-hTERT D868N + RRL mix (no hTERT)
D868N
WT
Δ30-39
Δ70-79
Δ110-119
Δ150-159
Δ190-199
Δ230-239
Δ270-279
Δ310-319
Δ350-359
Δ390-399
Δ430-439
Δ481-490
Δ508-517
W547A
W547F
IP-TRAP
IC
175 kDa
83
[35S]-hTERT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Lane
B
anti-GST IP
Yeast
RRL
GST-hTERT MUTANTS + D868N [35S]-hTERT
WT
Δ30-39
Δ70-79
Δ110-119
Δ150-159
Δ190-199
Δ230-239
Δ270-279
Δ310-319
Δ350-359
Δ390-399
Δ430-439
Δ481-490
Δ508-517
W547A
W547F
IP-TRAP
IC
175 kDa
83
[35S]-hTERT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Lane

terminal mutants appeared to coprecipitate wild-type hTERT and telomerase activity less efficiently than others (Figure 2.4A: lanes 5 and 16). However, these proteins were poorly expressed in yeast compared to most GST-hTERT mutants (Figure 2.2B: lanes 3 and 13).

Similar mixing experiments were performed using D868N hTERT (Figure 2.4B) to determine if inactive GST-hTERT mutants could functionally complement this catalytically inactive RT domain mutant to reconstitute telomerase activity. Specifically, we mixed yeast lysates expressing GST-hTERT mutants and the human telomerase RNA with ^{35}S-labelled D868N hTERT synthesized in RRL in the presence of hTR. If functional complementation between two inactive proteins occurred, immunoprecipitation of an inactive GST-hTERT N-terminal mutant would coprecipitate D868N hTERT and telomerase activity. Though all GST-hTERT mutants coprecipitated ^{35}S-labelled D868N hTERT, inactive GST-hTERT mutants did not functionally complement D868N hTERT to reconstitute telomerase activity (compare Figures 2.2B and 2.4B). Therefore, we concluded that the physical association of hTERT proteins in coimmunoprecipitations is not sufficient for functional complementation of two distinct inactive proteins.

2.5.5. N-terminal mutations do not prevent physical association of hTERT proteins

Certain GST-hTERT mutants coimmunoprecipitated reduced amounts of ^{35}S-labelled hTERT and low levels of telomerase activity (Figure 2.4A: Δ30-39 and Δ481-490), suggesting that some hTERT N-terminal regions may be implicated in the physical association of hTERT proteins (Figure 2.4A). However, some GST-hTERT mutants (including Δ30-39 and Δ481-490) were poorly expressed in yeast (Figure 2.2B). Reduced expression levels of certain mutants would affect the amounts of GST-hTERT mutant proteins immunoprecipitated from yeast extracts, resulting in the coprecipitation of less ^{35}S-labelled hTERT. Thus, similar experiments to those described in Figure 2.4 were performed by mixing equal amounts of D868N GST-hTERT expressed in yeast with equal quantities of RRL-synthesized ^{35}S-labelled hTERT mutants (GST-hTERT and hTERT inputs equalized by Western blotting of crude extracts/RRL with antibody against hTERT), to determine if certain hTERT N-terminal regions are required for efficient physical multimerization. hTR was present in both RRL and yeast lysates. If an interaction occurred between D868N GST-hTERT and ^{35}S-labelled hTERT mutants,

immunoprecipitation of D868N GST-hTERT would coprecipitate ^{35}S-labelled hTERT mutant proteins. When equal amounts of GST-hTERT were immunoprecipitated (Figure 2.5A: middle panel), similar levels of coprecipitated mutant proteins were detected (Figure 2.5A: bottom panel), and inactive N-terminal mutants did not functionally complement the D868N GST-hTERT mutant to reconstitute activity (Figure 2.5A: top panel).

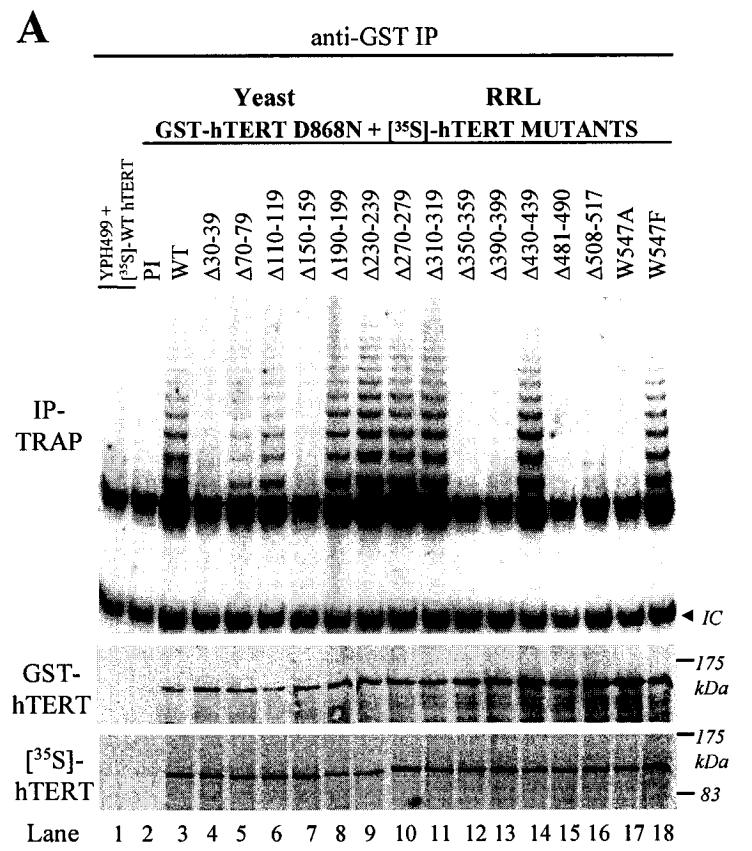
Similar experiments were performed using GST-hTERT D868N and N-terminal mutants synthesized in RRL (Figure 2.5B), to eliminate the possible effect of yeast lysate components on interactions between hTERT proteins. Again, the levels of coprecipitated hTERT N-terminal mutants were not grossly different (Figure 2.5B), and inactive mutants did not complement GST-hTERT D868N to reconstitute telomerase activity. Therefore, we concluded that these N-terminal mutations did not prevent the physical association of hTERT proteins in coimmunoprecipitates.

2.5.6. Physical association of hTERT molecules is hTR-independent

All preceding mixing experiments were performed with hTERT and GST-hTERT proteins synthesized in the presence of hTR, and therefore did not address whether hTR is required for the physical and functional association of hTERT proteins. We designed an experiment to determine if physical and functional interactions between hTERT proteins were dependent on the presence of hTR. GST-hTERT, GST-hTERT D868N, hTERT and D868N hTERT were synthesized separately in RRL in the presence or absence of hTR. GST-hTERT and hTERT proteins were mixed in different combinations, and GST-hTERT/hTERT complexes were immunoprecipitated with a GST antibody (Figure 2.6A). Only GST-hTERT/hTERT complexes containing a wild-type hTERT or GST-hTERT synthesized in the presence of hTR were active following immunoprecipitation (Figure 2.6A: compare lanes 4, 6, 10 and 11 to lanes 5, 7, 9 and 12). The addition of hTR after GST-hTERT and hTERT proteins were synthesized and mixed did not reconstitute telomerase activity (Figure 2.6A: lanes 7 and 12). However, coimmunoprecipitation of hTERT proteins did not require hTR (Figure 2.6A: SDS-PAGE, lanes 3 and 8). The independent synthesis of both GST-hTERT and hTERT proteins in the presence of hTR (Figure 2.6A: lanes 6 and 11), or the addition of hTR to protein mixtures (Figure 2.6A: compare lanes 7 and 12 to lanes 3 and 8) did not increase the efficiency of hTERT

Figure 2.5: N-terminal mutants are not defective in physical multimerization.

Equal amounts of GST-hTERT D868N and hTERT mutants synthesized separately in RRL or yeast in the presence of hTR were mixed, incubated on ice, and telomerase complexes were immunoprecipitated using a GST antibody. Immunoprecipitated hTERT/GST-hTERT complexes were examined for telomerase activity by TRAP (top panels), and for immunoprecipitation of hTERT and GST-hTERT proteins. **(A)** N-terminal mutants interact equally with GST-hTERT D868N expressed in yeast. Equal amounts of yeast-expressed GST-hTERT D868N and RRL-expressed hTERT mutants were mixed and immunoprecipitated. Control reactions were performed to detect non-specific immunoprecipitation of WT hTERT in the absence of GST-hTERT (lane 1), and in the absence of a GST antibody (lane 2). Middle panel: Immunoprecipitated GST-hTERT D868N was detected by Western blot with anti-hTERT. Bottom panel: Coimmunoprecipitated ^{35}S-labelled hTERT N-terminal mutants were detected by SDS-PAGE. **(B)** N-terminal mutants interact equally with GST-hTERT D868N expressed in RRL. Equal amounts of GST-hTERT D868N and hTERT mutations synthesized separately in RRL in the presence of hTR and ^{35}S-methionine were mixed and immunoprecipitated. Control reactions (lanes 3 and 4) were performed as described in Figure 2.5A. Bottom panel: Immunoprecipitated GST-hTERT D868N and coprecipitated hTERT mutants were detected by SDS-PAGE. GST-hTERT D868N and WT hTERT present in crude RRL prior to immunoprecipitation are shown in lanes 1 and 2, respectively.


A
anti-GST IP
Yeast
RRL
GST-hTERT D868N + [35S]-hTERT MUTANTS
YPH499 + [35S]-WT hTERT
PI
WT
Δ30-39
Δ70-79
Δ110-119
Δ150-159
Δ190-199
Δ230-239
Δ270-279
Δ310-319
Δ350-359
Δ390-399
Δ430-439
Δ481-490
Δ508-517
W547A
W547F
IP-TRAP
IC
GST-hTERT
175 kDa
[35S]-hTERT
175 kDa
83
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

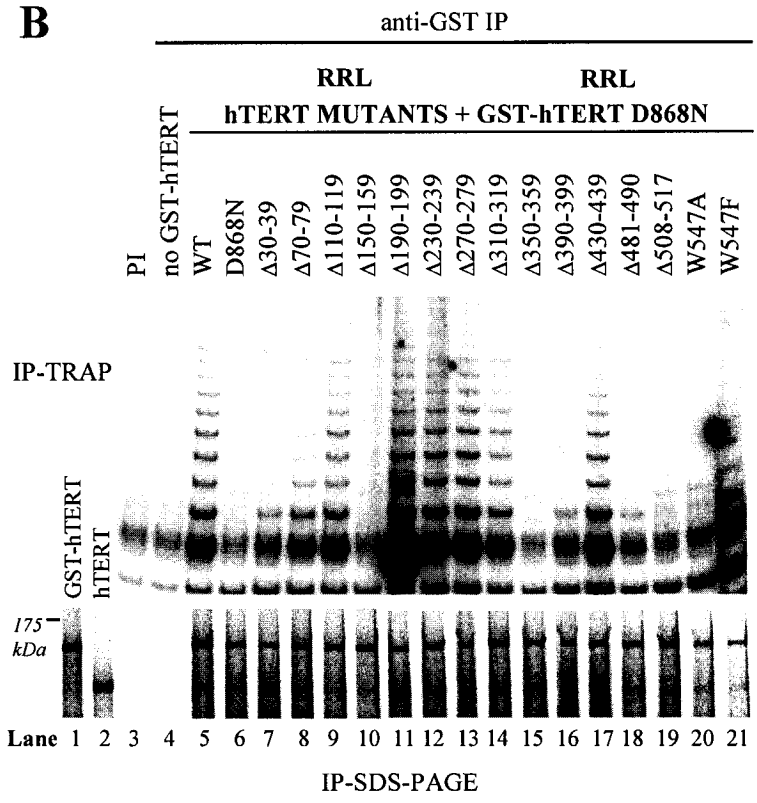
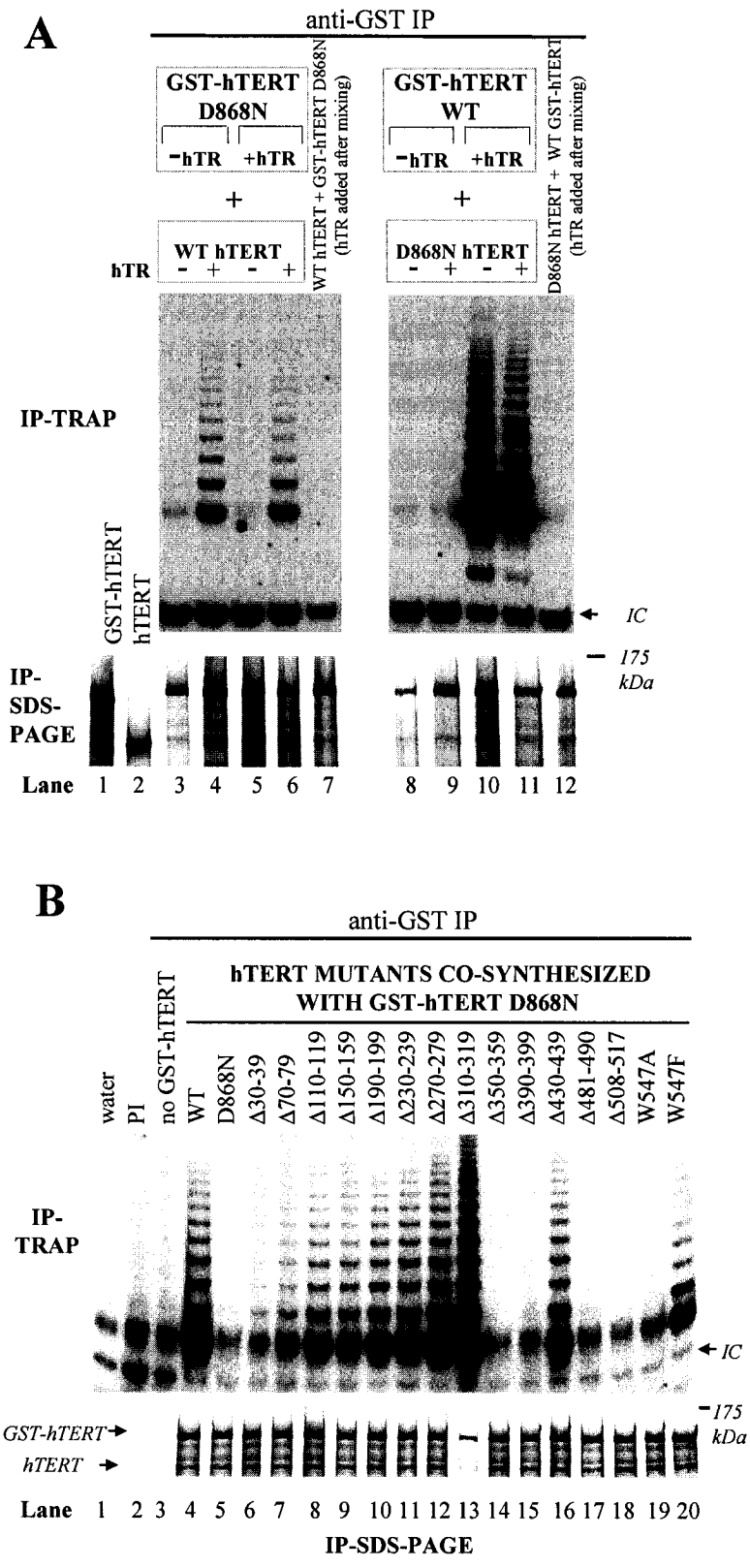

B
anti-GST IP
RRL
RRL
hTERT MUTANTS + GST-hTERT D868N
PI
no GST-hTERT
WT
D868N
Δ30-39
Δ70-79
Δ110-119
Δ150-159
Δ190-199
Δ230-239
Δ270-279
Δ310-319
Δ350-359
Δ390-399
Δ430-439
Δ481-490
Δ508-517
W547A
W547F
IP-TRAP
GST-hTERT
hTERT
175 kDa
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
IP-SDS-PAGE

Figure 2.6: Functional but not physical multimerization of hTERT proteins is hTR-dependent.

(A) Functional and physical interactions of WT and D868N hTERT in the presence or absence of hTR. Left panels: GST-hTERT D868N and wild-type (WT) hTERT proteins were independently synthesized in RRL in the absence or presence of hTR and ^{35}S-methionine, then mixed in different combinations, as indicated. Right panels: Wild-type (WT) GST-hTERT and D868N hTERT proteins were independently synthesized in RRL in the absence or presence of hTR, then mixed in different combinations, as indicated. Lanes 7 and 12 show reactions in which GST-hTERT and hTERT proteins were synthesized separately in the absence of hTR, and were then mixed with hTR prior to immunoprecipitation. Equal amounts of each protein were mixed, incubated on ice, and immunoprecipitated with a GST antibody. Top panels: Telomerase activity of the coimmunoprecipitates was measured by TRAP assay. Bottom panels: Immunoprecipitated GST-hTERT fusion proteins and coprecipitated hTERT proteins were detected by SDS-PAGE. GST-hTERT D868N and WT hTERT present in crude RRL prior to immunoprecipitation are shown in lanes 1 and 2, respectively. **(B)** N-terminal mutants defective in RID2-mediated hTR interactions cannot functionally complement an inactive RT domain mutant to reconstitute telomerase activity. GST-hTERT D868N and hTERT N-terminal mutants were cosynthesized in RRL in the presence of hTR and ^{35}S-methionine, and telomerase complexes were immunoprecipitated using a GST antibody. Control reactions (lanes 2 and 3) were performed as described in Figure 2.5A. Top panel: Telomerase activity was measured by TRAP assay. Bottom panel: Immunoprecipitated GST-hTERT D868N and coprecipitated hTERT mutants were detected by SDS-PAGE.


A
anti-GST IP
GST-hTERT D868N
-hTR +hTR
+
WT hTERT
hTR - + - +
WT hTERT + GST-hTERT D868N (hTR added after mixing)
GST-hTERT WT
-hTR +hTR
+
D868N hTERT
- + - +
D868N hTERT + WT GST-hTERT (hTR added after mixing)
IP-TRAP
GST-hTERT
hTERT
IC
175 kDa
IP-SDS-PAGE
Lane 1 2 3 4 5 6 7 8 9 10 11 12
B
anti-GST IP
hTERT MUTANTS CO-SYNTHESIZED WITH GST-hTERT D868N
water
PI
no GST-hTERT
WT
D868N
Δ30-39
Δ70-79
Δ110-119
Δ150-159
Δ190-199
Δ230-239
Δ270-279
Δ310-319
Δ350-359
Δ390-399
Δ430-439
Δ481-490
Δ508-517
W547A
W547F
IP-TRAP
IC
175 kDa
GST-hTERT
hTERT
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
IP-SDS-PAGE

coimmunoprecipitation. GST-hTERT proteins seemed to be immunoprecipitated less efficiently in the absence of hTR, and the amounts of coprecipitated hTERT decreased correspondingly (Figure 2.6A: lanes 3 and 8). In conclusion, physical association of hTERT proteins was hTR-independent, but coimmunoprecipitation of active enzyme required the synthesis of wild-type hTERT molecules in the presence of hTR. These results suggest that the physical association of mixed hTERT proteins in coimmunoprecipitates is not sufficient for functional interactions, and that hTR molecules bound to inactive RT mutants cannot be used by another, wild-type hTERT molecule.

2.5.7. N-terminal mutants defective in RID2-mediated hTERT/hTR interactions cannot functionally complement an inactive RT domain mutant

We found that functional interactions between wild-type and D868N hTERTs required the synthesis of wild-type hTERT proteins in the presence of hTR, and that the addition of hTR to hTERT after protein synthesis could not reconstitute telomerase activity (Figure 2.6A). Thus, we reasoned that cosynthesis of GST-hTERT and hTERT proteins in the presence of hTR might facilitate the functional complementation between some inactive N-terminal mutants and the inactive GST-hTERT D868N to reconstitute telomerase activity. GST-hTERT D868N and N-terminal mutants were cosynthesized in RRL in the presence of the telomerase RNA, and immunoprecipitated telomerase complexes were examined for telomerase activity and the coprecipitation of N-terminal mutants (Figure 2.6B). All N-terminal mutant proteins, including those defective in RID2-mediated hTR binding, were coimmunoprecipitated equally (Figure 2.6B: lanes 6-20), supporting our previous conclusions that the physical association of hTERT molecules does not depend on the telomerase RNA, and that the N-terminal mutants we examined were not defective in physical multimerization. Coexpression of weakly active N-terminal mutants (Δ30-39 and Δ70-79) with GST-hTERT D868N reconstituted increased telomerase activity compared to the activity reconstituted by Δ30-39 or Δ70-79 mutants alone (compare Figure 2.5 to Figure 2.6B). The inactive mutant Δ150-159 functionally complemented GST-hTERT D868N to reconstitute telomerase activity (Figure 2.6B: lane 9). However, inactive N-terminal mutants Δ350-359, Δ390-399, Δ481-490, Δ508-517 and W547A did not functionally complement GST-hTERT D868N to reconstitute telomerase

activity (Figure 2.6B). These N-terminal mutants were defective in RID2-mediated hTR binding (Table 2.1 and Figure 2.3). Therefore, we concluded that functional complementation of hTERT molecules was dependent on RID2-mediated telomerase RNA interactions, and required the presence of intact RT and RID2 domains on the same molecule.

2.6. Discussion

2.6.1. Conserved regions of the hTERT N-terminus are required for *in vitro* catalytic activity

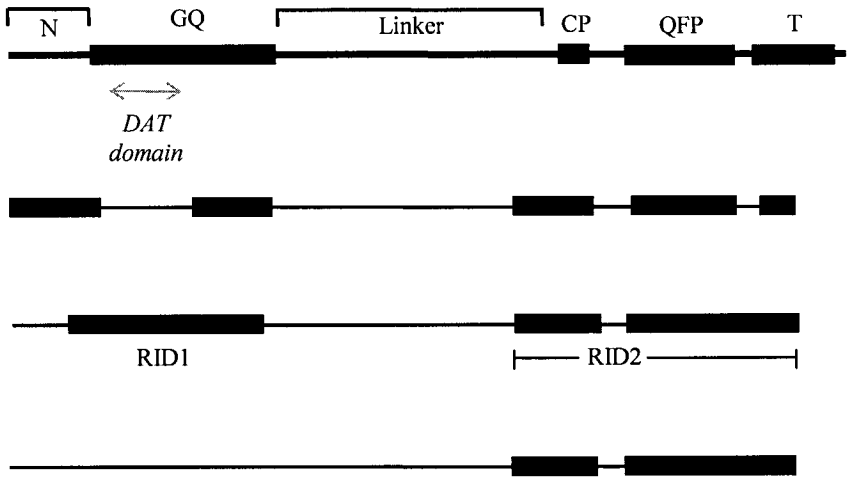
We expressed recombinant human telomerases containing N-terminal mutations in RRL and *S. cerevisiae*. Our results demonstrate a catalytic role for conserved N-terminal domains previously identified by sequence alignment of TERTs from multiple organisms (Xia et al. 2000), as all hTERT mutations that altered highly conserved residues impaired *in vitro* catalytic activity (Table 2.1, Figure 2.7). Mutation of some of these residues in *Tetrahymena* and yeast TERTs has also been shown to affect telomerase activity (Friedman and Cech 1999; Miller et al. 2000). Conversely, non-conserved residues were dispensable for *in vitro* catalytic activity. In addition, alignment of multiple vertebrate TERT sequences revealed vertebrate-specific conserved residues in the extreme N-terminus and linker that were essential for human telomerase activity. Though the extreme N-terminus and linker are not conserved among all TERTs, they are also implicated in yeast and *Tetrahymena* telomerase catalytic function (Miller et al. 2000; Xia et al. 2000).

2.6.2. hTERT N-terminal RNA interaction domain 1 (RID1)

Mutations in the extreme N-terminus and part of the hTERT GQ motif (residues 30-159) resulted in modest reductions in association of hTR with hTERT (Table 2.1, Figure 2.7). N-terminal truncations that delete this region of *Tetrahymena* and human TERTs appear to cause small reductions in telomerase RNA binding (Beattie et al. 2000; Lai et al. 2001). The corresponding sequences of Est2p are implicated in non-specific binding of single-stranded nucleic acids (Xia et al. 2000) and in telomerase RNA binding (Friedman and Cech 1999). Additionally, secondary structure predictions show that the hTERT extreme N-terminus and GQ motif are likely to form a continuous, highly

Figure 2.7: Schematic summary of functional domains of the hTERT N-terminus.

Conserved N-terminal subregions previously identified by alignment of multiple TERT sequences (Xia et al. 2000) are depicted on a linear map of the hTERT N-terminus. Also shown is the recently identified DAT domain (Armbruster et al. 2001). *In vitro* telomerase activity data were derived from this study and (Armbruster et al. 2001). Detailed mapping of hTERT N-terminal regions implicated in telomerase catalytic activity can be found in (Armbruster et al. 2001). Data identifying hTR interaction domains (RID1 and RID2) and regions required for *trans* complementation of the catalytically-inactive RT domain mutant GST-hTERT D868N were derived from this study.



hTERT N-terminus

Regions required for wild-type levels of *in vitro* telomerase activity

hTR interaction domains

Regions required for *trans* complementation of GST-hTERT D868N

structured domain (Armbruster et al. 2001); parts of this domain are required for *in vivo* telomere length maintenance in yeast and human cells (Armbruster et al. 2001). Though involved in hTR interactions, our results suggested that RID1 is not the major hTR binding domain of hTERT.

2.6.3. hTERT N-terminal RNA interaction domain 2 (RID2)

In agreement with previous results implicating the CP, QFP and T motifs of yeast and *Tetrahymena* TERTs in telomerase RNA binding (Friedman and Cech 1999; Bryan et al. 2000b), mutations in these motifs of hTERT caused severe defects in the association of hTR. In addition, deleting part of a vertebrate-specific conserved motif in the hTERT linker (VSR motif) compromised hTR binding. RID2 (residues 350 to 547) overlaps with the hTERT RNA binding region previously defined by the analysis of hTERT N-terminal truncations (Beattie et al. 2000; Bachand and Autexier 2001; Lai et al. 2001). Our data further indicated that RID2 contained at least two distinct RNA interaction regions separated by non-conserved sequences between the CP and QFP motifs that are not essential for RNA binding or catalytic activity (Table 2.1, Figure 2.7). Our results suggested that RID2 is the major hTR binding domain in the hTERT N-terminus.

2.6.4. Vertebrate-specific RNA binding (VSR) motif

The VSR motif of hTERT (residues 355-366) may be functionally analogous to the ciliate-specific CP2 motif characterized in *Tetrahymena* TERT (Miller et al. 2000). Both the VSR and CP2 motifs are required for *in vitro* catalytic activity and interactions with telomerase RNA (Miller et al. 2000; Lai et al. 2001). However, the CP2 and VSR motifs are unrelated in sequence, and are located at opposite ends of their respective TERT linkers. The CP2 motif is located near the GQ motif boundary of the *Tetrahymena* TERT linker. The hTERT linker may contain an additional RNA binding motif near the GQ motif boundary. However, sequence alignments demonstrated that the first half of the linker (near the GQ motif boundary) is poorly conserved among vertebrate TERTs, and our data and others' indicate that this region is not required for human telomerase activity (Armbruster et al. 2001), or RNA binding. We hypothesize that differences in the VSR and CP2 motif sequences, and in their location with respect to more conserved elements of the RNA binding domain (RID2 in hTERT), may reflect the specific interaction requirements

of telomerase RNAs with different secondary structures. The observation that ciliate- and vertebrate-specific conserved TERT elements are required for interactions with telomerase RNAs warrants further comparative mapping of the TERT-TR interaction domains from different organisms.

2.6.5. Physical multimerization of hTERT proteins is hTR-independent

Recent studies indicate that i) the human telomerase complex contains two functional telomerase RNA molecules, ii) separately inactive hTERT fragments can functionally multimerize *in vitro* and *in vivo*, and iii) hTERT molecules physically multimerize *in vitro* (Armbruster et al. 2001; Beattie et al. 2001; Wenz et al. 2001). The hTERT N-terminus was previously proposed as one of the protein-protein interaction sites in telomerase multimers (Beattie et al. 2001). Interactions between hTERT molecules may be direct, or may be mediated by other members of the telomerase complex such as the telomerase RNA, or by other proteins such as molecular chaperones. Though our data demonstrated that hTERT proteins synthesized in yeast and RRL can interact with one another, the physical association of these proteins appeared to be independent of the N-terminus. Physical multimerization of hTERT proteins occurred in the absence of hTR, in agreement with the recently published results of Armbruster and colleagues (Armbruster et al. 2001), and appeared unaffected by mutations that caused defects in telomerase RNA association. Therefore, our data suggest that the physical association of hTERT proteins is not mediated by hTR, and may not be mediated by the N-terminus.

2.6.6. Functional complementation requires intact RNA binding and RT domains on the same hTERT molecule

Though hTR and hTR binding were not required for the physical association of hTERT molecules in coimmunoprecipitates, functional hTERT interactions were hTR-dependent. Two types of experiments were performed to study the multimerization requirements of human telomerase. First, hTERT proteins were synthesized separately in the presence or absence of hTR, and subsequently mixed. Active enzyme was immunoprecipitated by the inactive GST-hTERT D868N only when hTR was present during the synthesis of wild-type hTERT, suggesting that wild-type hTERT could not functionally interact with hTR bound to inactive GST-hTERT D868N. Our results support

the recent observation of Beattie and colleagues that functional complementation requires the association of telomerase RNA with only one hTERT subunit (Beattie et al. 2001). Second, hTERT proteins were cosynthesized with GST-hTERT D868N in the presence of the telomerase RNA to facilitate the assembly of functional telomerase multimers. Functional complementation that reconstituted telomerase activity occurred between the inactive RT domain mutant and all N-terminal mutants except those with defects in RID2-mediated RNA association (Table 2.1, Figure 2.7). The results from both types of experiments indicated that functional hTERT multimerization required the presence of intact RID2 and RT domains on the same hTERT molecule.

2.6.7. Role of the hTERT N-terminus in functional telomerase multimerization

The HIV-1 RT dimerizes in an assymetric, head-to-tail fashion and contains only one functional catalytic site (Restle et al. 1990; Kohlstaedt et al. 1992). Beattie and colleagues have proposed an analogous model of hTERT multimerization in which the N-terminus of one hTERT molecule acts *in trans* to allosterically mediate conformational changes in telomerase RNA(s) bound to another hTERT containing the RT domain and C terminus (Beattie et al. 2001). Our data indicate that a functional RID2 hTR interaction domain (residues 350-547) is also normally required on the same molecule as the RT domain and C terminus. Thus, potential *trans*-activating portions of the N-terminus may be located within the first 350 amino acids of hTERT, a region containing RID1 and the poorly conserved linker. This hypothesis is supported by recent results which demonstrate that the first 300-350 amino acids of hTERT can complement a non-overlapping hTERT protein containing intact RNA binding, RT and C-terminal domains to reconstitute telomerase activity (Beattie et al. 2001). In addition, our data and others' suggest that the extreme N-terminus and GQ motif of TERTs may constitute an independent domain (RID1) that interacts with telomerase RNA and possibly telomeric DNA (Friedman and Cech 1999; Beattie et al. 2000; Xia et al. 2000; Armbruster et al. 2001; Lai et al. 2001). These observations suggest a revised model of functional hTERT multimerization in which the extreme N-terminus and GQ motif (RID1) modulate the conformation of telomerase RNAs, perhaps *in trans*, whereas the RID2 domain mediates RNA binding interactions that functionally link it with the RT and C-terminal domains.

The hTERT N-terminus may also be involved in intramolecular N-terminal *cis* interactions. hTERT truncations lacking part of RID2 and all of the extreme N-terminus, GQ motif and linker (Beattie et al. 2001), or RID2 mutants (this study), do not functionally complement an RT-defective mutant. However, hTERT truncations lacking the entire N-terminus, including RID2, can complement hTERT mutants defective in the RT domain or C-terminus (Beattie et al. 2001). Though it is not clear why complementation is affected differently by partial and complete RID2 disruptions, these latter observations indicate that hTERT RID2 may act *in trans* with the RT domain and C terminus. This in turn suggests that independent domains of the hTERT N-terminus such as RID1 and RID2 may functionally interact *in cis*. Such an interaction could be mediated by the long, poorly conserved and catalytically non-essential linker of hTERT.

N-terminus-mediated *trans* interactions between hTERT proteins may occur via direct homomeric protein contacts, or be mediated by the telomerase RNA, telomeric DNA or other intervening proteins. None of the hTERT N-terminal mutants tested in our study were defective in physical association with other hTERT proteins. However, the small size of these mutations may be insufficient to disrupt protein associations. Interactions between hTERT proteins also occurred independently of hTR binding. We did not determine whether hTERT interactions might be altered in the presence of telomeric DNA. However, recent work by Armbruster and colleagues indicates that a telomeric oligonucleotide substrate is not essential for the physical association of hTERT proteins (Armbruster et al. 2001). These observations can be interpreted in a number of ways. First, the N-terminus may not interact with other hTERT molecules *in trans*. However, some inactive N-terminal mutants functionally complement RT domain mutants containing intact N-termini, supporting functional *trans* interactions (this study, and Beattie et al. 2001). Second, physical *trans* interactions may occur along an extensive surface of the hTERT N-terminus, and may be identified only by the analysis of larger deletions or substitutions. Third, the primary physical site of hTERT *trans* interactions may be located outside of the N-terminus. Mutations disrupting large subregions of hTERT, especially in the RT and C-terminal domains, will be required to identify and characterize the site(s) and mechanism mediating the physical and functional association of hTERT molecules.

We identified an RNA interaction domain in the hTERT N-terminus that was required for the functional association of hTERT proteins, suggesting a role for hTERT-hTR interactions and the N-terminus in telomerase multimerization. The potential roles of the RT and C-terminal domains, associated proteins and telomeric DNA in mediating the molecular mechanism of telomerase multimerization will require further investigation. A better understanding of the mechanisms regulating hTERT multimerization will help to elucidate the role of multimerization in the catalytic function of human telomerase.

2.7. Acknowledgments

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3. CHAPTER 3: Functional organization of repeat addition processivity and DNA synthesis determinants in the human telomerase multimer

3.1. Preface

In the study described in the preceding chapter, we identified two distinct hTR-interacting regions (RID1 and RID2) in the hTERT N terminus. In the study summarized here, we identified the respective RID1 and RID2 interaction sites in hTR and further defined the RID1 sequences required for association with hTR. The preceding chapter described the identification of hTERT N-terminal sequences that are important for human telomerase activity, using a PCR-based telomerase assay (TRAP assay) that does not permit visualization of all telomerase products. In the study summarized here, we examined in detail the catalytic properties of RID1, RID2 and hTR mutants using a direct primer extension telomerase assay that reveals all telomerase products at nucleotide resolution. We found that RID1 and RID2 mutations cause distinct catalytic phenotypes that mirror the phenotypes caused by mutations in their respective interaction regions in hTR. We identified RID1 as an independent hTERT domain that is essential for the telomerase-specific property of repeat addition processivity, and mapped the functional boundaries of this domain. Furthermore, we continued the mapping of hTERT sequences involved in functional multimerization that was begun in the preceding chapter, and examined the role of hTERT-hTERT interactions in repeat addition processivity. Finally, during the course of this study it was reported in the literature that hTR dimerizes, and in the study described here we examined the role of hTR-hTR interactions in repeat addition processivity.

3.2. Abstract

Human telomerase is a multimer containing two telomerase RNAs (hTR) and most likely two telomerase reverse transcriptases (hTERT). Telomerase synthesizes multiple telomeric repeats using a unique repeat addition form of processivity. We investigated hTR and hTERT sequences that were essential for DNA synthesis and processivity using a direct primer extension telomerase assay. We found that hTERT consists of two physically separable functional domains, a polymerase domain containing RNA interaction domain 2 (RID2), reverse transcriptase (RT) and C-terminal sequences, and a major accessory

domain, RNA interaction domain 1 (RID1). RID2 mutants defective in high affinity hTR interactions and an RT catalytic mutant exhibited comparable DNA synthesis defects. The RID2-interacting hTR P6.1 helix was also essential for DNA synthesis. RID1 interacted with the hTR pseudoknot/template domain and hTERT's RT motifs and putative thumb, and was essential for processivity, but not DNA synthesis. The hTR pseudoknot was essential for processivity, but not DNA synthesis, and processivity was reduced or abolished in dimerization-defective pseudoknot mutants. *Trans*-acting hTERTs and hTRs complemented RID1 and pseudoknot mutants' processivity defects, respectively. These data provide novel insight into the catalytic organization of the human telomerase complex, and suggest that repeat addition processivity is one of the major catalytic properties conferred by telomerase multimerization.

3.3. Introduction

Telomerase is a DNA polymerase that catalyzes the *de novo* addition of telomeric DNA repeats to the 3' ends of linear chromosomes, and is essential for the long term proliferation of most eukaryotic cells. Telomerase is minimally composed of two subunits, the telomerase reverse transcriptase (TERT) and the telomerase RNA (TR), which contains the template used to direct DNA synthesis (reviewed in Harrington 2003). Processive telomerases add multiple DNA repeats to a single substrate, by reiteratively copying the RNA template, a property referred to as reiterative, repeat addition or Type II processivity. This unusual form of processivity is unique to telomerase, and is distinct from the nucleotide addition (Type I) form of processivity shared by all polymerases. Repeat addition processivity entails: 1. initial alignment of the 3' ends of the DNA substrate and RNA template; 2. copying of the RNA template to its 5' boundary; and 3. translocation of the RNA template and/or DNA substrate within the active site so that the new DNA 3' end realigns with the 3' boundary of the RNA template. Repetitions of this cycle generate the periodic pattern of short DNA repeats that is characteristic of processive telomerase activity *in vitro*.

Many nucleic acid polymerases are functionally divided into two major domains, a core polymerase domain whose minimal function is template-directed (Type I) processive DNA synthesis, and a major accessory domain that confers the unique activities of individual polymerases (reviewed in Sousa 1996). Since repeat addition (Type II)

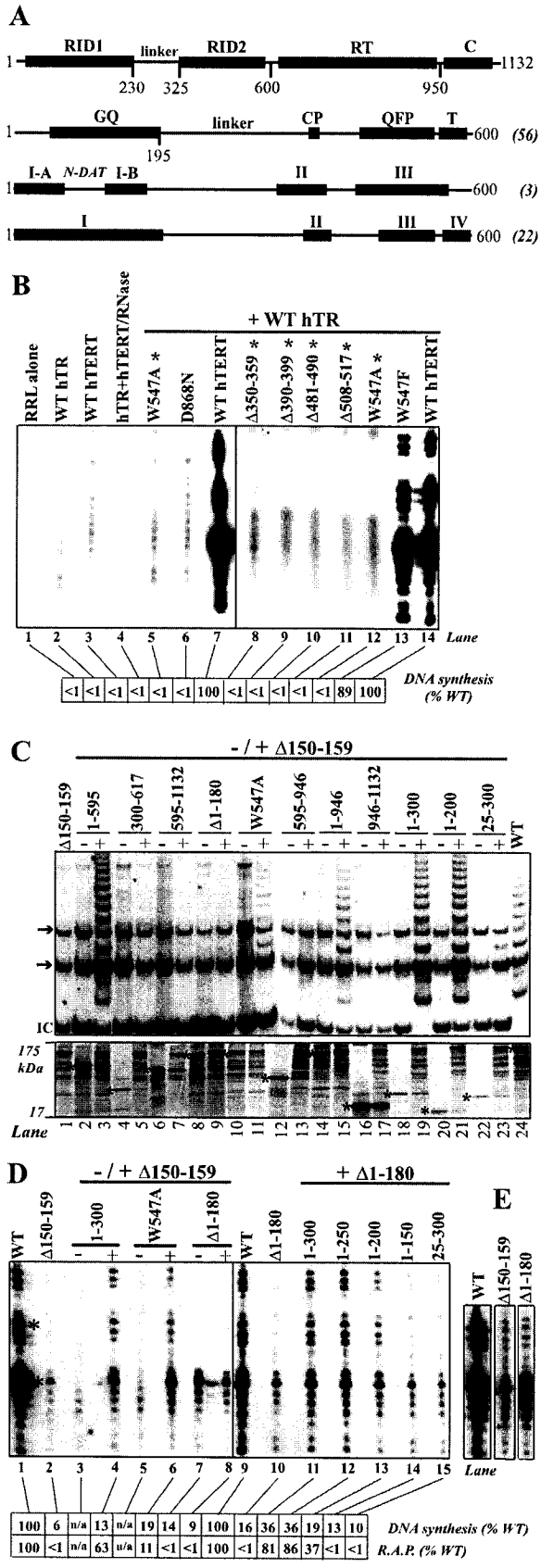
processivity is unique to telomerase, telomerase-specific elements in TERT and the TR may confer this specialized catalytic property. However, human telomerase elements that mediate the enzyme's polymerase function (DNA synthesis) and the telomerase-specific property of repeat addition processivity could not be identified until recently, when a direct primer extension assay was adapted for use with recombinant vertebrate telomerases reconstituted in rabbit reticulocyte lysates (RRL) (Chen and Greider 2003a; Huard et al. 2003). In this study we examined the role of different hTERT and hTR sequences in DNA synthesis and repeat addition processivity. We use the term "processivity" to refer to repeat addition (Type II or reiterative) processivity unless otherwise indicated.

The TRs from different organisms display limited sequence homology, but their secondary structures are conserved within families. Common features include the template and a pseudoknot structure (Romero and Blackburn 1991; Chen et al. 2000; Tzfati et al. 2003). Vertebrate telomerase RNAs contain two regions that are important for telomerase activity, the pseudoknot/template and CR4/CR5 domains (Figure 3.4A) (reviewed in Harrington 2003). mTR and hTR pseudoknot/template domain sequences regulate repeat addition processivity, and interact with TERT, though the specific site of interaction in TERT is unknown (Beattie et al. 2000; Bachand and Autexier 2001; Chen and Greider 2003a). Sequences in the hTR pseudoknot P3 helix mediate TR dimerization (Ly et al. 2003b). The P1b helix of the hTR pseudoknot/template domain also contributes to 5' template boundary definition (Chen and Greider 2003b). The mTR and hTR CR4/CR5 domains interact with TERT (Mitchell and Collins 2000; Bachand and Autexier 2001; Chen et al. 2002); specifically, hTR's CR4/CR5 interacts with hTERT's RID2 region (see below) (Lai et al. 2001). The hTR and mTR CR4/CR5 domains contain the P6.1 helix, which is important for human and mouse telomerase activity and TERT interactions (Mitchell and Collins 2000; Chen et al. 2002), though the specific site of P6.1 interaction in hTERT and mTERT has not been identified.

The TERT proteins are also phylogenetically conserved, and consist of central reverse transcriptase (RT) motifs flanked by telomerase-specific N- and C-terminal sequences (Figure 3.1A) (reviewed in Kelleher et al. 2002). The *Saccharomyces cerevisiae* and human TERT C termini functionally resemble the thumb of the human immunodeficiency virus type I (HIV-1) RT because of their contributions to Type I processivity, and the hTERT C-terminus is also implicated in Type II processivity

Figure 3.1: Distinct hTERT sequences are essential for DNA synthesis and repeat addition processivity.

hTERT variants were synthesized in RRL in the presence of wild-type hTR, and the activity of recombinant telomerases was analyzed by TRAP (C) or the direct primer extension assay (B, D, E). Repeat addition processivity (R.A.P.) and first telomere repeat DNA synthesis (DNA synthesis) values were expressed as a percent of the corresponding values for wild-type enzyme, and are indicated at the bottom of each lane. **(A)** Top panel: schematic depicting conserved hTERT regions. Numbers indicate the amino acid boundaries defined in this study and others (see text). Lower panels: schematics depicting functionally important TERT N-terminal regions identified in previous studies (references shown at right). Motifs/regions identified in *S. cerevisiae* TERT were mapped to hTERT N-terminal sequences using the alignment reported by Xia et al. (Xia et al. 2000). **(B)** Analysis of RID2 and RT mutants' elongation products. The two panels in this figure depict independent experiments. Asterisks indicate RID2 mutants defective in high affinity hTR interactions (Moriarty et al. 2002b). **(C)** hTERT variants were synthesized in the presence (+) or absence (-) of a RID1 mutant (Δ150-159) to test for their ability to complement the RID1 mutant's activity defects. Top panel: arrows indicate the position of non-specific PCR primer dimers. IC= PCR internal control. WT=wild-type hTERT. Expression of ^{35}S-labeled hTERT proteins (indicated by asterisks) was detected by SDS-PAGE (bottom panel). **(D)** hTERT variants were analyzed for the ability to complement the repeat addition processivity defects of RID1 mutants Δ150-159 and Δ1-180. Repeat addition processivity was calculated by comparing the normalized intensity of the major product in the first and second repeats (indicated by asterisks, and corresponding to the first G in the telomeric repeat TTAGGG). The processivity of each reconstituted enzyme, shown at the bottom of each lane, was expressed as a percentage of the processivity of wild-type (WT) telomerase. **(E)** Longer exposure permitted the identification and quantification of second repeat products generated by RID1 mutants.


A
RID1
linker
RID2
RT
C
1
1132
230
325
600
950
GQ
linker
CP
QFP
T
600
(56)
195
I-A
N-DAT
I-B
II
III
600
(3)
I
II
III
IV
600
(22)
B
+ WT hTR
RRL alone
WT hTR
WT hTERT
hTR+hTERT/RNase
W547A *
D868N
WT hTERT
Δ350-359 *
Δ390-399 *
Δ481-490 *
Δ508-517 *
W547A *
W547F
WT hTERT
Lane
DNA synthesis (% WT)
<1 <1 <1 <1 <1 <1 100 <1 <1 <1 <1 <1 89 100
C
- / + Δ150-159
Δ150-159
1-595
300-617
595-1132
Δ1-180
W547A
595-946
1-946
946-1132
1-300
1-200
25-300
WT
1C
175 kDa
17
Lane
D
- / + Δ150-159
+ Δ1-180
WT
Δ150-159
1-300
W547A
Δ1-180
WT
Δ1-180
1-300
1-250
1-200
1-150
25-300
E
WT
Δ150-159
Δ1-180
Lane
100 6 n/a 13 n/a 19 14 9 100 16 36 36 19 13 10
DNA synthesis (% WT)
100 <1 n/a 63 n/a 11 <1 <1 100 <1 81 86 37 <1 <1
R.A.P. (% WT)

(Peng et al. 2001; Hossain et al. 2002; Huard et al. 2003). The hTERT N terminus can be subdivided into two regions, RNA interaction domains 1 and 2 (RID1 and RID2), which are separated by a non-conserved linker that is not required for telomerase activity (Figure 3.1A) (Xia et al. 2000; Armbruster et al. 2001; Moriarty et al. 2002b). RID1 encompasses the previously identified GQ and T2 motifs, and is also referred to as Region I (for review of alternative nomenclature, see Kelleher et al. 2002) (Figure 3.1A). RID2 includes the CP, QFP and T motifs, alternatively referred to as Regions II, III and IV, respectively (for review, see Kelleher et al. 2002) (Figure 3.1A). Both RID1 and RID2 are required for wild-type levels of TR interaction and telomerase activity in yeast, *Tetrahymena* and human (for review, see Kelleher et al. 2002). The RT-adjacent RID2 is a site of high affinity TR binding (Lai et al. 2001). The catalytic function of the more N-terminal RID1 is poorly understood. The RID1 of *S. cerevisiae* TERT (Est2p) is important for telomerase activity and interacts non-sequence-specifically with both DNA and RNA (Xia et al. 2000). Deletion of the hTERT RID1 greatly reduces but does not abolish telomerase activity, and affects the ability of human telomerase to extend non-telomeric primers (Beattie et al. 2001). These observations suggest that although RID1 is not essential for catalysis, it may be an important site of DNA interaction in both yeast and human TERTs (Xia et al. 2000; Beattie et al. 2001). RID1 also contains residues that are not required for wild-type levels of *in vitro* telomerase activity, but which are important for telomere length maintenance in *S. cerevisiae* and human cells (Friedman and Cech 1999; Xia et al. 2000; Armbruster et al. 2001). N-terminal hTERT sequences that are important for *in vivo* function are referred to as the N-DAT domain (Figure 3.1A) (Armbruster et al. 2001). Though mutation of N-DAT sequences does not appear to affect catalytic function when telomerase activity is measured using the telomeric repeat amplification protocol (TRAP) (Armbruster et al. 2001; Moriarty et al. 2002b), recent work indicates that some N-DAT mutants are less active than wild-type hTERT when activity is measured using a direct primer extension (conventional) assay or non-telomeric DNA primers (Lee et al. 2003). Similarly, the direct primer extension assay reveals catalytic defects in hTERT C-terminal mutants that are undetected by the PCR-based TRAP assay (Huard et al. 2003).

The human telomerase enzyme appears to be an obligate dimer. Stringently purified human telomerase complexes contain two hTRs, and on the basis of size measurements are predicted to contain two hTERTs (Wenz et al. 2001). *S. cerevisiae*

telomerase also contains at least two functionally interacting TRs, but *Tetrahymena* telomerase is active as a monomer (Prescott and Blackburn 1997a; Bryan et al. 2003). hTR monomers are interdependent, and reconstitution of hTR-hTR interactions restores telomerase activity to dimerization-defective hTR mutants (Wenz et al. 2001; Ly et al. 2003b). Similarly, distinct, overlapping hTERT mutants can functionally complement each other's activity defects, indicating that hTERT monomers can function *in trans* (Beattie et al. 2001; Moriarty et al. 2002b). RT and RNA template dimerization are essential for replication of the double-stranded RNA genomes of retroviruses such as HIV-1 (Götte et al. 1999; Balakrishnan et al. 2003, and references therein). However, the catalytic functions of telomerase multimerization have not been defined.

Previous work indicates that the TERT C-terminal and RT sequences functionally resemble the thumb and RT motifs of the HIV-1 RT and other nucleic acid polymerases (Kelleher et al. 2002). However, telomerase contains a TERT-specific N terminus, and employs a stably integrated, structurally complex RNA as a template. Many telomerases also exhibit a unique, repeat addition form of processivity. In this study, we used a recently adapted direct primer extension assay and recombinant human telomerases reconstituted in RRL to characterize the telomerase-specific elements in hTERT and hTR that are essential for polymerase function (DNA synthesis) and the telomerase-specific property of repeat addition processivity. We also investigated the role of hTERT-hTERT, hTR-hTR and hTERT-hTR interactions in mediating these catalytic functions. Our results indicated that the functional domain organization of the entire hTERT protein resembles that of other nucleic acid polymerases, and that TR and a major non-polymerase domain of TERT (RID1) confer repeat addition processivity to human telomerase. Furthermore, TR and TERT multimerization played important roles in repeat addition processivity. These data provided novel insight into the catalytic organization of the human telomerase complex. We propose that the mechanism mediating repeat addition processivity may represent a variation on a catalytic mechanism shared by other reverse transcriptases.

3.4. Materials and methods

3.4.1. Constructs

pET28-hTERT wild-type and D868N (Bachand and Autexier 1999), 595-1132, Δ1-180, 595-946 and 1-946 (Bachand and Autexier 2001), Δ150-159, Δ350-359, Δ390-399,

Δ481-490, Δ508-517, W547A and W547F (Moriarty et al. 2002b), Δ936-945, Δ963-972, Δ993-1002, Δ1020-1029 and Δ1077-1086 (Huard et al. 2003) constructs were previously described. pET28-hTERT constructs 1-595 and 946-1132 were cloned by PCR using pET28-hTERT as a DNA template. The pCR3.1-Flag-hTERT wild-type construct was previously described (Beattie et al. 2000). pCR3.1-Flag-hTERT 1-300, 1-250, 1-200, 1-150, 25-300 and 300-617 were cloned by PCR using pCR3.1-Flag-hTERT as a template. The luciferase-expressing construct is provided with the RRL kit (Promega). phTR wild-type, hTR170, hTR180 and hTR190 constructs were previously described (Autexier et al. 1996). During the final revision of this manuscript, we discovered that phTR180 and phTR190 also contain a deletion of nucleotides 200-201. These deletions were inadvertently introduced into the constructs when they were made in 1995, as the oligonucleotides used for PCR mutagenesis were designed based on an early incomplete version of the hTR gene sequence. The phTR 1-209, 207-451, Δ1-64, Δ65-144 and Δ145-208 constructs were cloned by PCR with a T7 promoter from the pGRN33 construct into the puc119 vector using a previously described strategy (Autexier et al. 1996). phTR GC107/108AG, P6.1.302UCUC, P6.256GCGCC, P6.296GGCGC, 306AAAAA, 315UUCAUU, 316AGU, P6.1.311GAGA, P6.1.302UCUCcomp, 36-45 and 122-129 constructs were cloned by site-directed mutagenesis, using phTR+1 (wild-type) as a template. The identity of all hTERT and hTR constructs was confirmed by restriction digest and/or sequencing.

3.4.2. *In vitro* reconstitution of telomerase, TRAP and direct primer extension assays

hTERT proteins were expressed in RRL in the presence of *in vitro* synthesized and purified hTRs (Moriarty et al. 2002b). When hybrid telomerases were reconstituted using mixtures of hTR or hTERT variants, equal amounts (μg) of hTR or hTERT-encoding plasmid DNA were added to the RRL reactions. TRAP and direct primer extension assays and quantification of repeat addition processivity were performed as described (Huard et al. 2003), except that conventional assays were performed with 1 μM of biotinylated $(TTAGGG)_3$ primer. First repeat DNA synthesis was quantified by expressing the total signal of the first six telomerase products (+1 to +6) generated by any mutant as a percentage of the total signal of the first six telomerase products generated by wild-type telomerase tested in the same experiment. Repeat addition processivity (R.A.P.) was

quantified using a previously described method (Hardy et al. 2001). R.A.P. values for mutant telomerases were usually expressed as a percentage of the R.A.P. value for wild-type telomerase tested in the same experiment (Figure 3.1, 5 and 6). The products of wild-type and variant telomerases quantified by this method were loaded on the same gel.

3.4.3. Pulse-chase time course experiments

Pulse-chase time course reaction conditions were adapted from (Bryan et al. 2000a). Direct primer extension assays were performed as described above. Biotinylated $(TTAGGG)_3$ pulse primer and non-biotinylated $(TTAGGG)_3$ chase primer were included at a final concentration of 1 and 150 μM, respectively. Reactions were stopped with a buffer containing RNase A and EDTA, as previously described (Huard et al. 2003). Quantification of first repeat DNA synthesis and processivity were performed as described above.

3.4.4. *In vitro* RNA binding assay

RNA binding and quantification were performed as described (Moriarty et al. 2002b), except that 3000 Ci/mmol UTP was used for hTR synthesis, and 1 μg/ml competitor BSA and tRNA and 0.45 μg/ml of Flag M2 antibody (Sigma) were used in immunoprecipitations. RNA and protein signals were distinguished by exposing SDS-PAGE gels on 2 sheets of film simultaneously, as previously described (Bryan et al. 2000b). For competition experiments, protein A sepharose beads (Sigma) were blocked extensively with BSA and tRNA prior to addition of RRL-reconstituted telomerase complexes, but these competitors were not included during immunoprecipitation.

3.4.5. *In vitro* hTR dimerization assay

hTR dimerization experiments were performed as described (Ly et al. 2003b). Briefly, 300 ng unlabeled hTR were trace-labeled with 1000 cpm of ^{32}P-labeled hTR synthesized *in vitro* in the presence of 3000 Ci/mmol UTP (Perkin Elmer). In experiments where dimerization of mixtures of hTRs was examined, 300 ng of each hTR were included in each mixture. Ice-cold RNA Incubation Buffer (final concentration: 50 mM NaCl, 25 mM Tris pH 6.8, 10 mM $MgCl_2$) was added to hTR mixtures to make a final volume of 10 μl, then mixtures were divided into two aliquots of 5 μl each. hTRs were denatured for 5

min. at 95°C, followed by snap-cooling on ice. One of each pair of aliquots was subsequently incubated at 37°C for 2 h, whereas the second aliquot was incubated 2 h on ice. After incubation, 1 µl 5X loading dye (20% glycerol, bromophenol blue) was added to each mixture, and samples were electrophoretically separated on 5% acrylamide gels (prepared with 30/0.8% acrylamide stock) in a 1X TBM buffer (90 mM Tris, 90 mM borate, 1 mM $MgCl_2$) at 100V. Gels were dried and exposed to Molecular Dynamics PhosphorImager cassettes for quantification. Quantification was performed using ImageQuant software as follows. The signal corresponding to hTR monomers was measured after subtraction of background signal in the lane above (between monomers and dimers); similar measurements were performed for hTR dimers (with background measured at a position above the dimers). % total dimer was expressed as (adjusted dimer signal)/(adjusted monomer signal + adjusted dimer signal).

3.4.6. hTERT protein interaction assay

Flag-tagged, unlabeled hTERT1-200 was prebound to Flag M2 antibody-coupled protein G sepharose beads (Sigma), followed by extensive washing to remove unbound proteins. ^{35}S-labeled hTERT proteins were added to the immunoprecipitation mixture, with or without 300 ng of hTR, and immunoprecipitations were performed for 4-h or overnight. Immunoprecipitation buffers, competitors and wash conditions have previously been described (Moriarty et al. 2002b). All proteins were synthesized in RRL.

3.5. Results

3.5.1. The hTERT RID2, RT and C-terminal sequences function *in cis* as a single domain

The function of many multidomain proteins, such as lac permease or the HIV-1 RT, is reconstituted when different domains of the proteins are expressed from discontinuous mRNAs (Bibi and Kaback 1990; Hostomsky et al. 1991). Functional complementation experiments have been used to define the boundaries of lac permease domains (Zen et al. 1994). We examined the domain organization of hTERT using a functional complementation approach and a series of overlapping hTERT proteins containing small internal deletions and substitutions. This method minimizes potential artifacts caused by misfolding of truncated proteins or deletion of whole domains.

Furthermore, the use of overlapping proteins permits analysis of *trans*-acting sequences in the hTERT multimer. To distinguish the hTERT sequences that function cooperatively in the same domain (*in cis*) from those which can function *in trans*, we cosynthesized pairs of inactive hTERT variants in RRL in the presence of wild-type hTR. Hybrid enzymes were tested for the ability to reconstitute telomerase activity using the telomeric repeat amplification protocol (TRAP) (Table 3.1). We found that inactive RID2, RT and C-terminal variants could not complement other hTERT variants with distinct mutations in RID2, RT or C-terminal sequences, whereas all inactive RID2, RT and C-terminal mutants could complement the activity defects of a RID1 mutant (Table 3.1) (Moriarty et al. 2002b; Huard et al. 2003). In a multimeric protein, the ability of two distinct, overlapping mutants to complement each other's activity defects suggests that the complementing sequences in these proteins can function *in trans*. Conversely, the inability of two distinct mutants to reconstitute catalytic function in a multimeric complex can be interpreted in several ways. First, one or both mutations may disrupt the ability of monomers to physically interact. We previously reported that hTERT RID2 and C terminal mutants cannot function *in trans* with an RT catalytic mutant, though RID2 and C-terminal variants coprecipitate with the RT mutant as efficiently as wild-type hTERT (Table 3.1) (Moriarty et al. 2002b; Huard et al. 2003), suggesting that the inability of these variants to complement each other's activity defects is not attributable to loss of hTERT-hTERT interactions. Second, two distinct mutants may be unable to reconstitute catalytic function if more than one functional copy of either mutated region is required for activity. However, the ability of a RID1 mutant to complement the activity defects of RID2, RT or C-terminal variants suggests that multiple functional copies of RID2, the RT motifs or the C terminus are not required for telomerase activity (Table 3.1). A third explanation for the inability of distinct mutants to complement each other's activity defects is that the sequences altered in these mutants normally function *in cis*; that is, they are part of the same functional domain (Zen et al. 1994). Since the inactive RID2, RT and C-terminal hTERT variants we examined did not demonstrate defects in hTERT-hTERT protein interactions (Moriarty et al. 2002b; Huard et al. 2003), and since mutations in these regions could be complemented by a RID1 variant (Table 3.1), we concluded that RID2, the RT motifs and C terminus of hTERT likely function *in cis* as part of a single functional domain. The hTERT RID2, RT and C-terminal sequences are predicted by the PSIPRED

Table 3.1: Functional complementation of hTERT mutants' activity defects in cosynthesis experiments.

Mutated hTERT Region	Coexpressed hTERT Mutant [a]	Region	Complementation [e]
RID1 (*Δ150-159*)	Δ350-359 [b], Δ390-399 [b], Δ481-490 [b], Δ508-517 [b], W547A [b]	RID2	+
	D868N [c]	RT	+
	Δ936-945 [b], ***Δ963-972*** [b], Δ993-1002 [b], Δ1020-1029 [b], ***Δ1077-1086*** [b]	C	+
RID2 (*W547A*)	Δ150-159 [b]	RID1	+
	D868N [c]	RT	-
	Δ936-945 [b], ***Δ963-972*** [b], Δ993-1002 [b], Δ1020-1029 [b], ***Δ1077-1086*** [b]	C	-
RT (*D868N*)	Δ150-159 [c]	RID1	+
	Δ350-359 [c], Δ390-399 [c], Δ481-490 [c], Δ508-517 [c], W547A [c]	RID2	-
	Δ936-945 [d], ***Δ963-972*** [d], Δ993-1002 [d], Δ1020-1029 [d], ***Δ1077-1086*** [d]	C	-

[a] hTERT mutants were cosynthesized with RID1, RID2 or RT variants, as indicated in the left column. Repeat addition processivity-defective C-terminal mutants identified in a previous study (Huard et al. 2003) are italicized.

[b] Cosynthesis experiments performed for this study.

[c] Previously reported cosynthesis experiments (Moriarty et al. 2002b).

[d] Previously reported cosynthesis experiments (Huard et al. 2003).

[e] The ability of two distinct hTERT mutants to complement each others' activity defects when cosynthesized in RRL in the presence of wild-type hTR was evaluated by TRAP assay. '-' indicates absence of complementation. '+' indicates reconstitution of telomerase activity by complementation.

secondary structure prediction program (http://bioinf.cs.ucl.ac.uk) (McGuffin et al. 2000) to form a continuous and closely spaced series of alpha helices and beta sheets, whereas RID1 is separated from other catalytically-important regions of hTERT by a non-conserved linker of low sequence complexity (data not shown) (Xia et al. 2000; Armbruster et al. 2001). Collectively, these observations suggested that the RID2, RT and C-terminal regions of hTERT act cooperatively within the same hTERT molecule.

3.5.2. RID2, RT and C-terminal sequences constitute the core polymerase domain of hTERT

Though it has previously been established using the TRAP assay that RID2 and the RT motifs are important for telomerase activity (for review see Kelleher et al. 2002), the PCR-based TRAP technique does not always distinguish between catalytic defects caused by poor efficiency of nucleotide addition (DNA synthesis) and those that are attributable to defects in the telomerase-specific property of repeat addition processivity. The shortest PCR products detected using the TRAP assay are 40-50 nucleotides in length (including the 18 nt substrate primer) and contain at least three telomere DNA repeats (Kim et al. 1994; Kim and Wu 1997; Szatmari and Aradi 2001; Gavory et al. 2002). Therefore, when the TRAP technique is used, non-processive enzymes that synthesize less than three telomere repeats cannot be distinguished from enzymes with severe defects in DNA synthesis. To characterize the specific catalytic functions mediated by the hTERT RID2 and RT regions, we therefore examined the elongation products of reconstituted telomerases using a direct primer extension assay. The catalytic defects associated with hTERT C-terminal mutations were recently described (Huard et al. 2003). We previously demonstrated that most sequences in the hTERT RID2 are required for high affinity interaction with hTR (Moriarty et al. 2002b). We found that all RID2 mutations that disrupt hTR binding abolished DNA synthesis (Figure 3.1B, lanes 5, 8-12: <1% wild-type first repeat DNA synthesis), whereas a RID2 mutation that does not impair hTR interactions did not affect the ability of recombinant telomerase to synthesize DNA (Figure 3.1B, lane 13). Non-specific signals were occasionally detected, for example in reactions with hTR alone, hTERT alone or an hTR without a template (Figure 3.1B, lanes 2-3; Figure 3.6A, lane 11). As expected, substitution of a highly conserved aspartate that is essential for the catalytic activity of all RTs abolished DNA synthesis (Figure 3.1B, lane 6:

D868N, <1% wild-type first repeat DNA synthesis) (Kelleher et al. 2002). The DNA synthesis defects of RID2 variants and this RT catalytic mutant were comparable (Figure 3.1B: compare lane 6 to lanes 5, 8-12), supporting the conclusion that hTR-interacting RID2 sequences are essential for DNA synthesis. The core polymerase domain of nucleic acid polymerases is composed of the catalytically essential palm and fingers subdomains, and a thumb subdomain that regulates nucleotide addition (Type I) processivity (Sousa 1996). The function of the core polymerase domain is confined to template-directed Type I processive DNA synthesis (Sousa 1996). Since the hTERT C terminus is also implicated in nucleotide addition (Type I) processivity and is not physically separable from RID2 and the RT motifs (Table 3.1) (Huard et al. 2003), and as RID2 and the RT motifs are physically inseparable and catalytically essential (Table 3.1; Figure 3.1B), we propose that RID2, RT and C-terminal sequences constitute the core polymerase domain of hTERT, and that these sequences function catalytically *in cis*.

3.5.3. The hTERT RID1 domain functions *in trans*

Next, we examined the hTERT sequences that can function *in trans* in hTERT multimers. Cosynthesis of an inactive RID1 mutant (Δ150-159) with numerous, overlapping inactive RID2, RT and C-terminal mutants reconstituted telomerase activity, indicating that RID1 can function *in trans* (Table 3.1; Figure 3.1C). The hTERT N terminus alone was both necessary and sufficient for complementation of the RID1 mutant's activity defect (Figure 3.1C, lanes 3 and 7), and reconstitution of activity was not dependent on RID2 sequences (Figure 3.1C, lane 5). Deletion of the first 180 hTERT residues (RID1) prevented complementation, and amino acids 1-200 were sufficient to restore telomerase activity (Figure 3.1C, lanes 9 and 21). Deletion of RID1 residues 1-24 greatly reduced complementation (Figure 3.1C, lane 23). These data demonstrated that the RID1 region is responsible for functional complementation of the Δ150-159 mutant's activity defects, and that RID1 sequences can function *in trans*.

3.5.4. RID1 is an essential determinant of repeat addition processivity

In contrast to RID2, RT and C-terminal sequences, RID1 is physically separable from the remainder of hTERT and can fulfill its catalytic role *in trans* (Table 3.1, Figure 3.1C). Similarly, isolated accessory and polymerase domains can assemble *in vitro* to

reconstitute the HIV-1 RT's catalytic function (Hostomsky et al. 1991). Since major accessory domains confer unique catalytic properties to nucleic acid polymerases (Sousa 1996), we investigated whether RID1 was important for telomerase-specific catalytic functions. We examined the elongation products of RID1 mutants Δ150-159 and Δ1-180 using the direct primer extension assay, and compared them to the products generated by wild-type hTERT and RID2 and RT variants (Figure 3.1B, D-E). RID1 mutants synthesized DNA less efficiently than wild-type enzyme; however, unlike RID2 and the RT motifs, RID1 was not essential for DNA synthesis (compare Figure 3.1B, lanes 5, 8-12, <1% wild-type DNA synthesis, with Figure 3.1D, lanes 2 and 10, 6-16% wild-type DNA synthesis). Similarly, the major accessory domains of HIV-1 RT (RNase H) and other nucleic acid polymerases stimulate but are not required for DNA synthesis (Hostomsky et al. 1991). The pausing pattern of the first repeat elongation products generated by RID1 mutants resembled the wild-type profile (Figure 3.1D, lanes 1, 2 and 10). However, both variants synthesized predominantly only one telomere repeat. The second repeat addition products generated by these mutants could be reliably detected and quantified following longer exposure (Figure 3.1E). Repeat addition processivity values were determined by calculating the ratio of the intensity of the major second repeat telomerase product to the intensity of the corresponding first repeat telomerase product (Hardy et al. 2001). This ratio was then compared to the ratio for wild-type enzyme. Comparison of these ratios permits quantification of processivity independent of differences in DNA synthesis efficiency, and this quantification method detected 40-fold differences in processivity even in enzymes with similar, low levels of DNA synthesis (Figure 3.1D, lanes 10 and 13). Processivity quantification confirmed that both RID1 mutants were non-processive (<1% of wild-type processivity). The Δ1-180 and Δ150-159 mutants exhibited similar catalytic defects, implying that amino acids 150-159 play a critical role in RID1 function. RID1 mutations cause a small reduction in hTERT's ability to interact with wild-type hTR (Beattie et al. 2000; Moriarty et al. 2002b); however, RID1 mutations do not inhibit hTERT-hTR interactions as extensively as RID2 mutations (Beattie et al. 2000; Bachand and Autexier 2001; Moriarty et al. 2002b). Furthermore, complexes containing RID1 mutants were sufficiently stable to permit complementation of activity defects by an isolated *trans*-acting RID1 domain or a RID2 mutant that inhibits ribonucleoprotein (RNP) assembly (Figure 3.1C, lanes 19 and 11; Figure 3.1D),

suggesting that major defects in RNP assembly do not account for the catalytic properties of RID1 mutants. We concluded that the hTERT RID1 is essential for repeat addition processivity, and that it is important but not essential for DNA synthesis.

3.5.5. Repeat addition processivity can be conferred by a *trans*-acting RID1

The results of complementation experiments performed using the TRAP assay and overlapping hTERT mutants indicated that RID1 can function *in trans* (Table 3.1; Figure 3.1C). We coexpressed a panel of hTERT mutants with the Δ150-159 protein to determine if *trans*-acting hTERTs could confer repeat addition processivity to this RID1 variant. Addition of amino acids 1-300 *in trans* stimulated DNA synthesis 2-fold, but enhanced processivity more than 60-fold (Figure 3.1D, compare lanes 2 and 4). Deletion of the first 180 residues of hTERT prevented complementation (Figure 3.1D, lane 8). A RID2 point mutant and RT and C-terminal mutants also restored processivity to Δ150-159, though not as efficiently as residues 1-300 (Figure 3.1D, lane 6; Appendix 1A). The ability of these overlapping hTERT proteins to complement the Δ150-159 processivity defect indicated that RID1 can mediate repeat addition processivity *in trans*.

To map the sequences required for complementation of RID1 mutants' processivity defects, we cosynthesized the Δ1-180 mutant with fragments containing residues from the RID1 and linker regions. Fragment 1-250 restored processivity to nearly wild-type levels, but amino acids 1-150 and 25-300 were not sufficient for complementation (Figure 3.1D, lanes 12, 14 and 15). Residues 1-200 also restored processivity to hTERT Δ1-180, albeit less efficiently than fragment 1-250 (Figure 3.1D, compare lanes 12 and 13). These results suggested that RID1 functions as a single domain spanning amino acids 1 to 250. Fragment 1-250, but not 1-200, also stimulated the efficiency of DNA synthesis approximately two-fold (Figure 3.1D, compare lane 10 to lanes 12 and 13). Fragment 1-200's complementation defect was not detected when telomerase activity was measured by TRAP (Figure 3.1C, lane 21). Previous mutagenesis studies using the TRAP assay reported that hTERT residues 200-250 are not implicated in telomerase activity, though mutations in this region cause small reductions in hTR interaction (Armbruster et al. 2001; Moriarty et al. 2002b). In contrast, the direct primer extension assay suggests a role for these residues in both repeat addition processivity and efficient DNA synthesis. To determine whether sequences between residues 200-250 and also the N-DAT region were

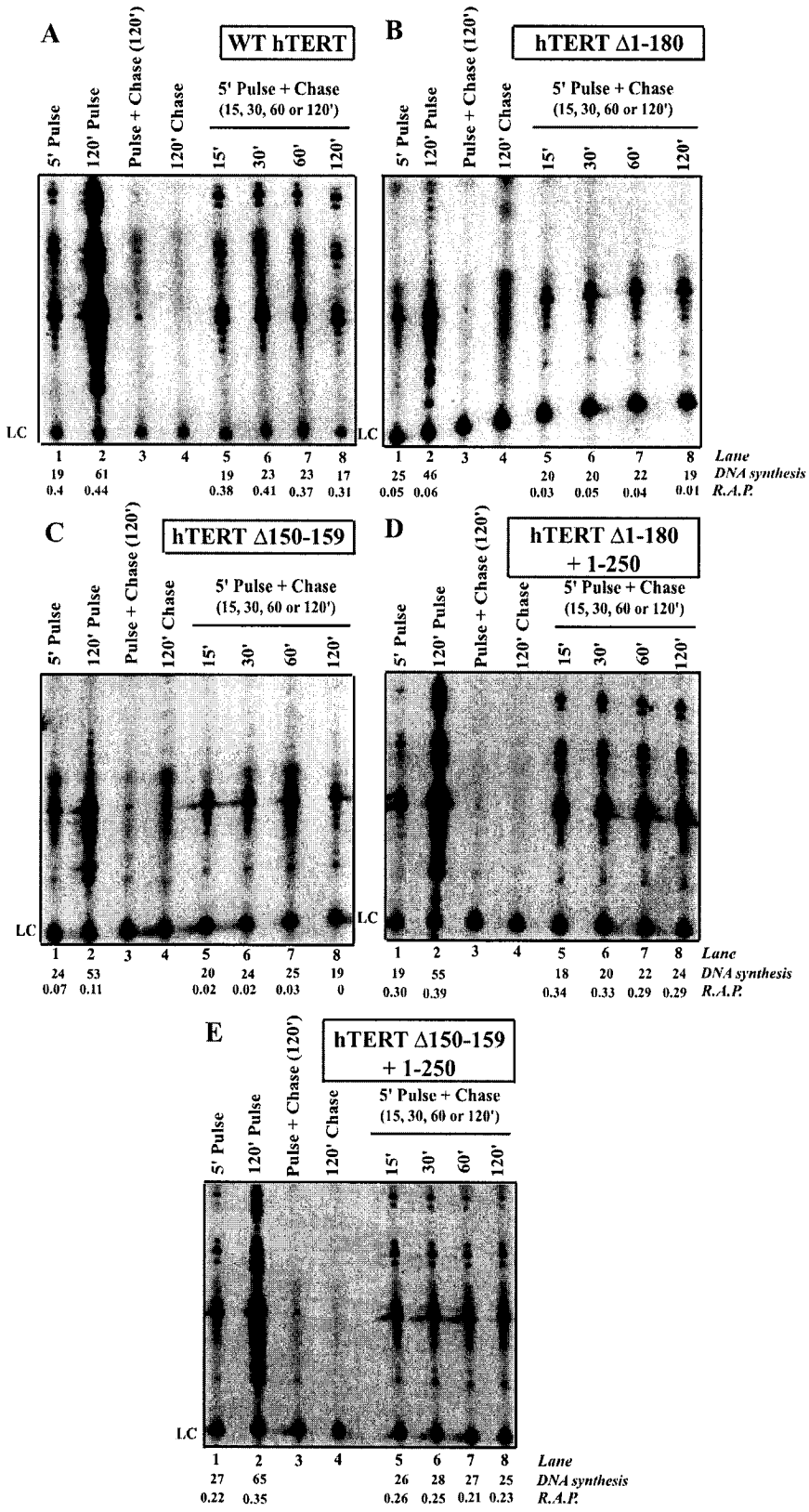
important for processivity, we examined the catalytic activity of two hTERT variants containing small internal deletions of amino acids 230-239 and 110-119, respectively (Appendix 1B) (Moriarty et al. 2002b). Both mutants exhibited wild-type levels of first repeat DNA synthesis and processivity when activity was detected using the direct primer extension assay and a telomeric primer (data not shown). These data indicate that this region of N-DAT and some residues between 200 and 250 are not required for processive elongation of telomeric primers. Secondary structure predictions for hTERT indicate that amino acids 206-250 are unlikely to form secondary structures (http://bioinf.cs.ucl.ac.uk). Furthermore, these sequences are not conserved, even among vertebrate TERTs (Xia et al. 2000; Moriarty et al. 2002b). Therefore, secondary structure predictions and the activity profiles of the Δ230-239 mutant and 1-200 and 1-250 RID1 fragments suggest that the functional C-terminal boundary of RID1 falls after hTERT residue 200, and likely does not include amino acids 230-250 (Figure 3.1A).

3.5.6. RID1 mutants' repeat addition processivity defects are not attributable to reduced elongation kinetics

To determine if the apparent processivity defects of the Δ1-180 and Δ150-159 RID1 mutants could be explained by reduced elongation kinetics, we examined the product profiles of these mutants in pulse-chase time course experiments (Figure 3.2). Fewer wild-type telomerase elongation products were generated during a 5 minute pulse-labeling compared to a longer pulse-labeling (Figure 3.2A, lanes 1 and 2). Since RID1 mutants were less active than wild-type hTERT (Figure 3.1D), we performed RID1 pulse-chase experiments using three times more RRL-reconstituted telomerase and primer extension reagents. Under these conditions, the elongation products of RID1 mutants were readily detectable following a 5 minute pulse-labeling (Figure 3.2B and C, lane 1). Like wild-type enzyme, the intensity of first repeat DNA synthesis products generated by RID1 mutants did not increase following addition of chase primer (Figure 3.2A-C, lanes 1, 5-8). Furthermore, the pattern of elongation products was the same after 0, 15, 30, 60 and 120 minutes of incubation with chase primer (Figure 3.2A-C, lanes 1, 5-8), indicating that RID1 mutations do not affect the kinetics of primer elongation. As previously observed for *Tetrahymena* telomerase (Bryan et al. 2000a), the high concentration of chase primer (150 μM) used in these assays did not prevent processive elongation of biotinylated pulse

Figure 3.2: RID1 mutants display specific defects in repeat addition processivity.

Pulse-chase time course experiments performed to determine if the product profiles of RID1 mutants were attributable to defects in repeat addition processivity or elongation kinetics. Wild-type hTERT **(A)** and RID1 variants **(B-E)** were synthesized in RRL in the presence of wild-type hTR, and in the absence (B, C) or presence (D, E) of DNA encoding the minimal RID1 region required for complementation of processivity defects (aa 1-250). Since RID1 mutants were less active than wild-type hTERT (Figure 3.1D), we performed RID1 pulse-chase experiments using three times more RRL-reconstituted telomerase and primer extension reagents (B-E). Experiments were performed with wild-type telomerase using standard assay conditions (A). Reconstituted telomerases were incubated with biotinylated $(TTAGGG)_3$ pulse primer for 5 min at 30°C in the presence of dATP, dTTP and α-^{32}PdGTP (direct primer extension assay). A 150-fold excess of non-biotinylated $(TTAGGG)_3$ chase primer was added and the reactions continued for 15, 30, 60 or 120 min. Elongated biotinylated pulse primers were separated from the products of chase primer elongation using magnetic streptavidin beads. Control 1 (lane 3): pulse and chase primers added simultaneously to demonstrate the efficiency of chase conditions. Control 2 (lane 4): reactions performed with chase primer alone to demonstrate that non-biotinylated chase primer elongation products are not purified by streptavidin beads. A 3' radio-labeled biotinylated TTAGGGT primer was added to stopped reactions prior to purification on streptavidin beads as a purification and loading control (LC). First repeat DNA synthesis and repeat addition processivity (R.A.P.) values for individual samples (expressed in arbitrary units) are indicated at the bottom of each lane. n=2 for all experiments.


A
WT hTERT
5' Pulse
120' Pulse
Pulse + Chase (120')
120' Chase
5' Pulse + Chase
(15, 30, 60 or 120')
15'
30'
60'
120'
LC
1 2 3 4 5 6 7 8
Lane
19 61 19 23 23 17
DNA synthesis
0.4 0.44 0.38 0.41 0.37 0.31
R.A.P.
B
hTERT Δ1-180
1 2 3 4 5 6 7 8
25 46 20 20 22 19
0.05 0.06 0.03 0.05 0.04 0.01
C
hTERT Δ150-159
1 2 3 4 5 6 7 8
24 53 20 24 25 19
0.07 0.11 0.02 0.02 0.03 0
D
hTERT Δ1-180
+ 1-250
1 2 3 4 5 6 7 8
19 55 18 20 22 24
0.30 0.39 0.34 0.33 0.29 0.29
E
hTERT Δ150-159
+ 1-250
1 2 3 4 5 6 7 8
27 65 26 28 27 25
0.22 0.35 0.26 0.25 0.21 0.23

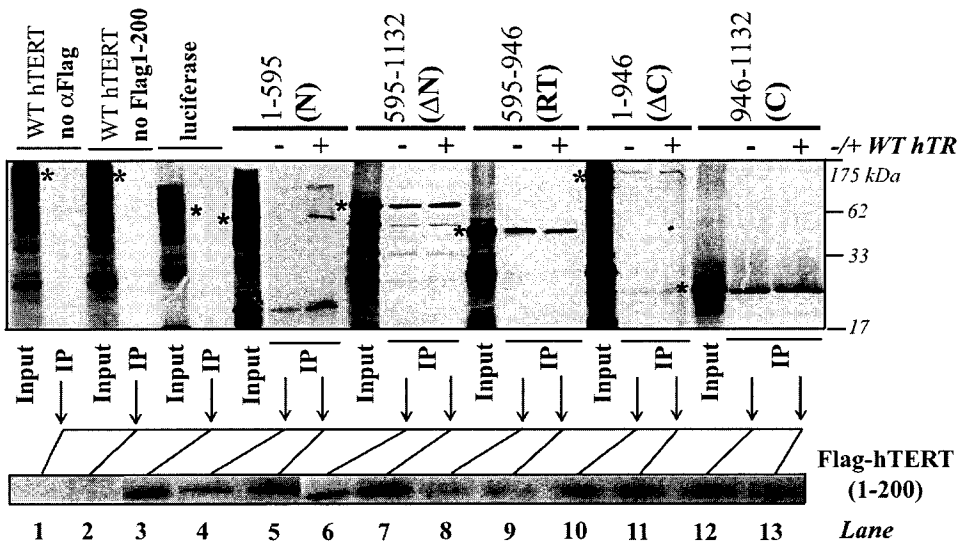
primers pre-bound to wild-type telomerase at a 1 μM final concentration (data not shown). Therefore, the inability of RID1 mutants to synthesize more than one telomere repeat under pulse-chase conditions is unlikely to be caused by non-specific telomerase inhibition at high concentrations of chase primer. Pulse-chase experiments also confirmed that the major property conferred by *trans*-acting RID1 sequences is repeat addition processivity (Figure 3.2D-E). Reconstitution of RID1 mutant telomerases in the presence of RID1 residues 1-250 strongly stimulated processivity and did not alter primer elongation kinetics (Figure 3.2: compare B to D, and C to E). Therefore, we concluded that deletion of RID1 residues 150-159 or 1-180 specifically impairs repeat addition processivity, and that *trans*-acting RID1 sequences confer this property to RID1 mutants.

3.5.7. RID1 interacts with the hTERT RT motifs and putative thumb

Since RID1 was physically separable from the hTERT polymerase domain and stimulated DNA synthesis and repeat addition processivity *in trans* (Figure 3.1D), we investigated whether RID1 associated with hTERT *in vitro*, and whether these interactions were dependent on hTR (Figure 3.3). RID1 associated most efficiently with fragments containing both the RT motifs and C terminus, and coprecipitation of these regions was not affected by hTR (Figure 3.3, lanes 8-13). Interactions were specific, since an unrelated protein, luciferase, did not coprecipitate with RID1 (Figure 3.3, lane 3). The hTERT N terminus associated less efficiently with RID1 than did variants containing RT and C-terminal sequences, and N terminus-RID1 interactions appeared to be partly dependent on hTR (Figure 3.3, lanes 4-5). We concluded that RID1 interacts with multiple sites in hTERT, but associates predominantly with the C terminus and RT motifs. The putative hTERT C-terminal thumb, motif E and a telomerase-specific insertion in the fingers region of *S. cerevisiae* TERT, and a telomerase-specific residue in the RT region of *Tetrahymena* TERT have previously been shown to influence repeat addition processivity (Bryan et al. 2000a; Huard et al. 2003; Lue et al. 2003; Bosoy and Lue 2004a). Interestingly, mutations in the HIV-1 RT's p51 thumb, which interacts with the p66 subunit's RNase H accessory domain, impair RNase H function (Cameron et al. 1997). These observations suggest that RID1's association with hTERT's RT and putative C-terminal thumb sequences might be important for repeat addition processivity. Interactions between RID1 and the hTERT RT

Figure 3.3: RID1 interacts with hTERT RT and C-terminal sequences.

^{35}S-labeled protein fragments corresponding to different hTERT regions (top panel, indicated by asterisks) were coimmunoprecipitated with unlabeled Flag-tagged hTERT1-200 (bottom panel) in the presence (+) or absence (-) of wild-type (WT) hTR. Flag-hTERT1-200 (RID1) was detected by immunoblotting with αFlag antibody.


WT hTERT no αFlag
WT hTERT no Flag1-200
luciferase
1-595 (N)
595-1132 (ΔN)
595-946 (RT)
1-946 (ΔC)
946-1132 (C)
-/+ WT hTR
175 kDa
62
33
17
Input
IP
Flag-hTERT (1-200)
1 2 3 4 5 6 7 8 9 10 11 12 13
Lane

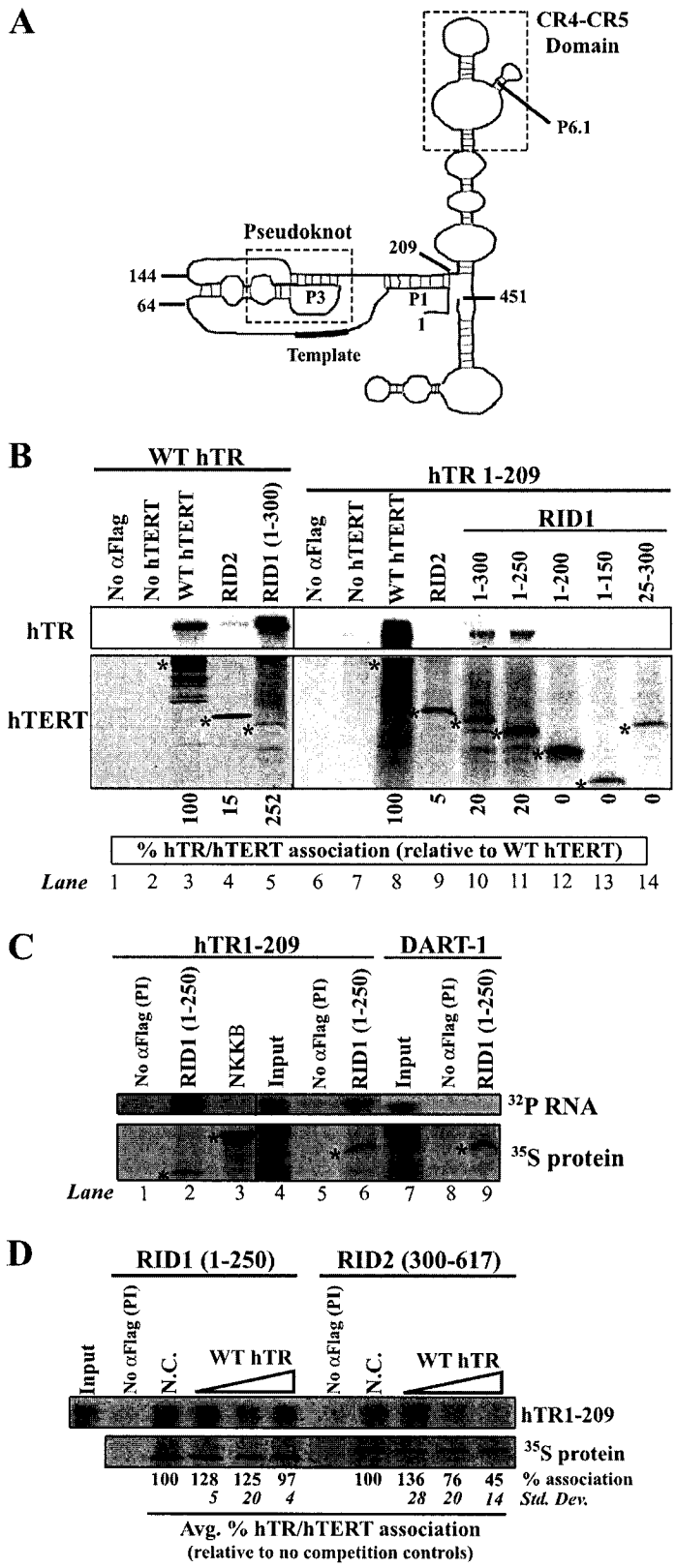
motifs and C terminus could also stimulate DNA synthesis efficiency as previously observed for the RNase H accessory domain of HIV-1 RT (Hostomsky et al. 1991).

3.5.8. RID1 sequences that mediate repeat addition processivity are required for *in vitro* association with the hTR template/pseudoknot domain

Est2p RID1 sequences interact non-sequence-specifically with DNA and RNA (Xia et al. 2000). Similarly, hTERT's RID1 can independently bind wild-type hTR, and RID1 mutations reduce hTR binding and abolished repeat addition processivity (Figure 3.1D) (Beattie et al. 2001; Moriarty et al. 2002b). The pseudoknot/template domain is an important mediator of vertebrate telomerase processivity (see below) (Chen and Greider 2003a). We investigated the *in vitro* interaction of this hTR region with RID1. Though the pseudoknot/template domain is known to associate with hTERT, the hTERT sequences mediating this interaction have not been identified (Beattie et al. 2000; Bachand and Autexier 2001). We found that the template/pseudoknot domain (nt 1-209) appeared to interact more efficiently with RID1 than RID2 (Figure 3.4B, lanes 9 and 10). On average, RID1 associated with hTR1-209 1.95 times more efficiently than with RID2 ($\rho<0.05$; n=5 independent experiments). Competition experiments performed with unlabeled competitor wild-type hTR and labeled hTR1-209 indicated that wild-type hTR inhibited binding of hTR1-209 to RID2 more efficiently than RID1 (Figure 3.4D). This result suggests that hTR1-209 associates more strongly with RID1 than RID2. Interestingly, lower concentrations of competitor wild-type hTR frequently stimulated the association of hTR1-209 with both RID1 and RID2 (Figure 3.4D). This stimulatory effect might be attributable to the ability of hTR 1-209 to dimerize with wild-type hTR (Ly et al. 2003b). RID1 bound nucleotides 1-209 less efficiently than full-length hTERT, implying that the template/pseudoknot domain may associate with additional hTERT regions (Figure 3.4B, lanes 8 and 10). More precise mapping of the RID1 sequences that interact with nucleotides 1-209 revealed that RID1 residues implicated in complementation of RID1 mutants' processivity and DNA synthesis defects were also required for pseudoknot/template interactions (compare Figure 3.1D, lanes 13-15 and Figure 3.4B, lanes 12-14). Interestingly, deleting amino acids 201-250 abolished hTR1-209 interactions, suggesting that the processivity- and DNA synthesis-regulating functions of these sequences may be mediated in part by hTERT-hTR interactions (Figure 3.4B, lanes

Figure 3.4: RID1 sequences that mediate repeat addition processivity are required for *in vitro* association with the hTR template/pseudoknot domain.

(A) Schematic of the predicted hTR secondary structure (Chen et al. 2000; Mitchell and Collins 2000; Chen et al. 2002). Numbers indicate nucleotide positions. The P1, P3 and P6.1 helices are indicated. **(B)** ^{32}P-labeled wild-type (WT) hTR or hTR nucleotides 1-209 (top panel) were coimmunoprecipitated with Flag-tagged, ^{35}S-labeled proteins (indicated by asterisks) corresponding to wild-type (WT) hTERT, RID2 (aa 300-617), or N- and C-terminally truncated RID1 variants (bottom panel). hTR association with RID1 and RID2 was expressed relative to hTR association with WT hTERT. **(C)** The specificity of hTR1-209-RID1 interactions was determined by examining the interaction of a non-specific Flag-tagged protein (NKKβ) or a non-specific Drosophila RNA (DART-1) with hTR1-209 and Flag-tagged RID1, respectively. One μl of a 1 in 500 dilution of input RRL reaction containing the appropriate ^{32}P-labeled RNA was loaded in input lanes. PI = preimmune serum. **(D)** Competition experiments. ^{35}S-labeled Flag-tagged RID1 and RID2 proteins were synthesized in RRL in the presence of equal amounts of ^{32}P-labeled hTR1-209, and increasing amounts of unlabeled wild-type competitor hTR (1.48×10^{-21} M, 1.48×10^{-20} M, 1.48×10^{-19} M). Ribonucleoprotein complexes were immunoprecipitated and visualized using the same method as other RNA-binding experiments. One μl of a 1 in 1000 dilution of input RRL reaction containing ^{32}P-labeled hTR1-209 was loaded in the input lane. PI = preimmune serum. N.C.= no competition (0 moles competitor hTR). After normalization to the amount of protein in each lane, hTR1-209 association with RID1 or RID2 in the presence of competitor hTR was expressed relative to the no competition (N.C.) control for each protein (% association). Average interaction and standard deviation values for different competitor hTR concentrations relative to N.C. controls are indicated at the bottom of each lane. $n \geq 3$ independent experiments for both RID1 and RID2.


A
CR4-CR5
Domain
P6.1
Pseudoknot
209
144
64
P3
P1
451
1
Template
B
WT hTR
hTR 1-209
No αFlag
No hTERT
WT hTERT
RID2
RID1 (1-300)
RID1
1-300
1-250
1-200
1-150
25-300
hTR
hTERT
100
15
252
100
5
20
20
0
0
0
% hTR/hTERT association (relative to WT hTERT)
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14
C
hTR1-209
DART-1
No αFlag (PI)
RID1 (1-250)
NKKB
Input
32P RNA
35S protein
Lane 1 2 3 4 5 6 7 8 9
D
RID1 (1-250)
RID2 (300-617)
Input
No αFlag (PI)
N.C.
WT hTR
hTR1-209
35S protein
100 128 125 97
5 20 4
100 136 76 45
28 20 14
% association
Std. Dev.
Avg. % hTR/hTERT association
(relative to no competition controls)

11 and 12; Figure 3.1D, lanes 12 and 13). Though fragment 1-200 was sufficient for complementation of the Δ1-180 mutant's processivity defect, the presence of residues 200-250 in hTERT Δ1-180 might rescue the hTR interaction defect of fragment 1-200 in hybrid enzymes (Figure 3.1D). Alternatively, the technical limitations of the RNA-binding assay may preclude identification of sequences that interact weakly with hTR, but are sufficient for minimal catalytic function. RID1 residues 1-24 were also essential for association with the pseudoknot/template (Figure 3.4B, lane 14), implying that the RID1 domain in its entirety is required for interaction with this hTR region. Since neither fragment 1-200 nor 25-300 bound hTR1-209 efficiently, it is unlikely that amino acids 1-250 interacted non-specifically with hTR. hTR1-209 did not interact with an unrelated protein, NKKβ (Figure 3.4C, lane 3), and an unrelated RNA, DART-1 (Boulanger et al. 2004), did not associate with RID1 (Figure 3.4C, lane 9). Furthermore, deletion of residues 150-159, which abolished repeat addition processivity, reduces the interaction of hTERT with wild-type hTR (Figure 3.1D) (Moriarty et al. 2002b). These results suggested that RID1's regulation of processivity may depend partly on its association with the hTR template/pseudoknot, though RID1-mediated hTERT-hTERT and DNA primer interactions could also contribute to this catalytic function.

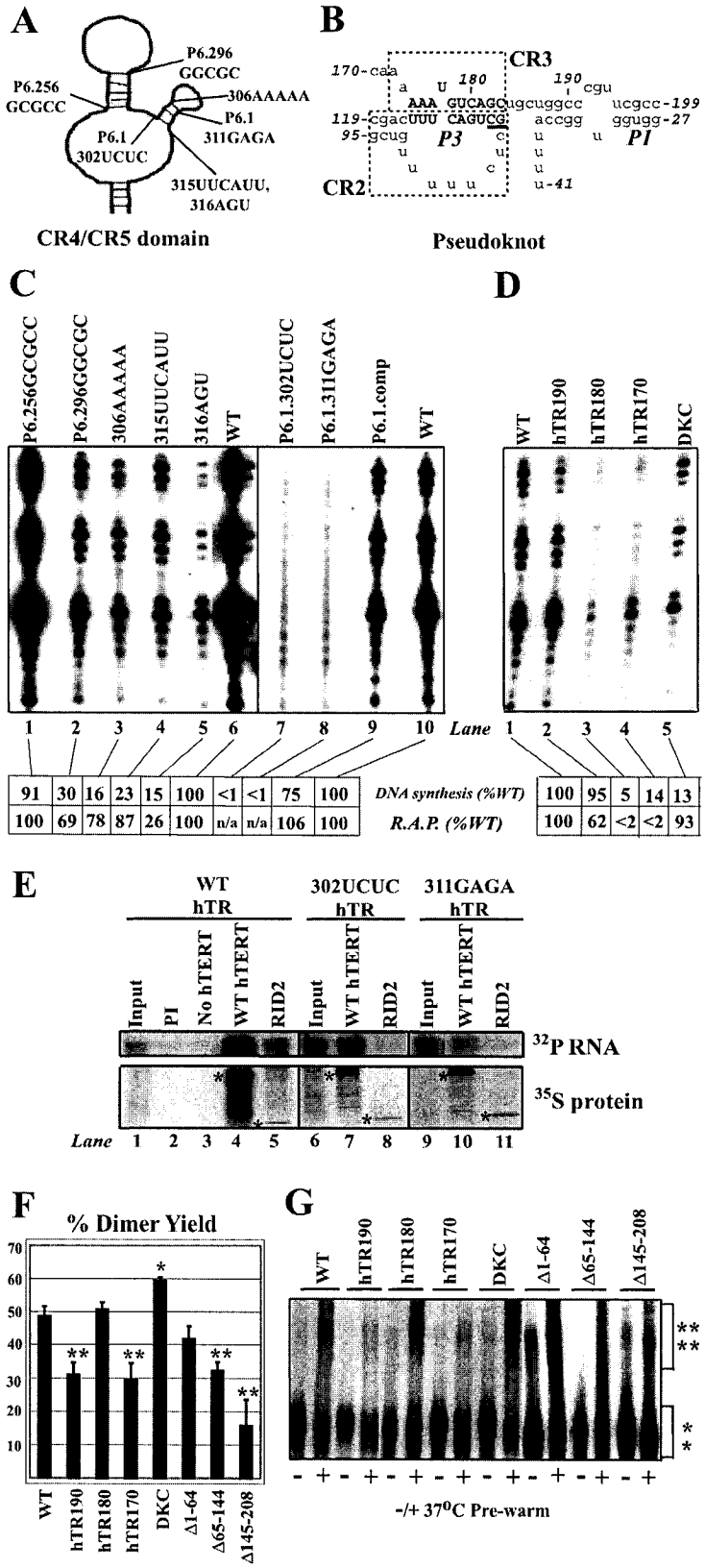
We found that the hTERT RID1 constitutes a single functional domain that is essential for repeat addition processivity. RID1 interacted with the processivity-regulating hTR pseudoknot/template domain, implying that RID1-hTR interactions might mediate processivity. In addition, RID2 sequences mediating high affinity hTR interactions were required for DNA synthesis. Since hTR-hTERT interactions were important for both DNA synthesis and processivity, we examined the roles of the hTR CR4/CR5 and pseudoknot/template domains in telomerase catalytic function.

3.5.9. RID2-interacting sequences in the hTR CR4/CR5 domain are essential for DNA synthesis

First we examined the effect of CR4/CR5 domain mutations on telomerase activity (Figure 3.5A, C). CR4/CR5-containing hTR sequences interact with hTERT RID2, and the stem structure of the mTR CR4/CR5 P6.1 helix is required for high affinity interaction with wild-type mTERT (Mitchell and Collins 2000; Lai et al. 2001; Chen et al. 2002). We found that disruption of base-pairing in the hTR P6.1 stem abolished DNA synthesis (<1%

Figure 3.5: Distinct hTR regions are essential for DNA synthesis and repeat addition processivity.

(A, B) Schematics of the predicted secondary structures of the hTR CR4/CR5 domain (A) and pseudoknot P3 base-pairing region (B) (Chen et al. 2000; Mitchell and Collins 2000; Chen et al. 2002). (A) The starting positions of mutations analyzed in this study are indicated. (B) The starting positions of the hTR170, hTR180 and hTR190 ten-nucleotide substitutions are numbered, and the location of the GC107/108AG (DKC) mutation is underlined. P3 residues implicated in hTR dimerization are indicated by upper-case letters (Ly et al. 2003b). Boxed nucleotides correspond to conserved regions 2 and 3 (CR2 and CR3). **(C-D)** The elongation products of telomerases reconstituted with wild-type hTERT and the indicated CR4/CR5 (C) or pseudoknot (D) substitution mutants were examined using the direct primer extension assay. Repeat addition processivity (R.A.P.) and first telomere repeat DNA synthesis (DNA synthesis) values were expressed as a percent of the corresponding values for wild-type enzyme, and are indicated at the bottom of each lane. **(E)** Interaction of wild-type hTR and P6.1 helix mutants with wild-type hTERT and RID2 (aa 300-617). The WT hTERT protein signal in lane 4 appears stronger than the WT hTERT protein signals in lanes 7 and 10 due to comigration of the efficiently coprecipitated WT hTR with hTERT in 12% SDS-PAGE gels. One µl of a 1 in 500 dilution of input RRL reaction containing the appropriate ^{32}P-labeled hTR variant was loaded in input lanes. PI = preimmune serum. **(F)** Quantification of *in vitro* hTR dimerization. n $\geq$3 independent experiments were performed for each hTR. Mean dimerization values that differed significantly from the values for wild-type (WT) hTR in a Student's t-Test are indicated by a single ($\rho<0.05$) or double ($\rho<0.01$) asterisk. (G) Representative hTR dimerization results. hTRs were incubated on ice (-) or at 37°C (+) for 2 hours prior to electrophoresis on 5% non-denaturing polyacrylamide gels. The positions of monomers and dimers are indicated by single and double asterisks, respectively.


A
P6.296
GGCGC
P6.256
GCGCC
306AAAAA
P6.1
311GAGA
P6.1
302UCUC
315UUCAUU,
316AGU
CR4/CR5 domain
B
CR3
CR2
P3
P1
Pseudoknot
C
P6.256GCGCC
P6.296GGCGC
306AAAAA
315UUCAUU
316AGU
WT
P6.1.302UCUC
P6.1.311GAGA
P6.1.comp
WT
1 2 3 4 5 6 7 8 9 10
91 30 16 23 15 100 <1 <1 75 100
100 69 78 87 26 100 n/a n/a 106 100
D
WT
hTR190
hTR180
hTR170
DKC
Lane 1 2 3 4 5
100 95 5 14 13
100 62 <2 <2 93
DNA synthesis (%WT)
R.A.P. (%WT)
E
WT hTR
302UCUC hTR
311GAGA hTR
Input
PI
No hTERT
WT hTERT
RID2
32P RNA
35S protein
Lane 1 2 3 4 5 6 7 8 9 10 11
F
% Dimer Yield
70
60
50
40
30
20
10
WT
hTR190
hTR180
hTR170
DKC
Δ1-64
Δ65-144
Δ145-208
G
WT
hTR190
hTR180
hTR170
DKC
Δ1-64
Δ65-144
Δ145-208
- + - + - + - + - + - + - + - +
-/+ 37°C Pre-warm

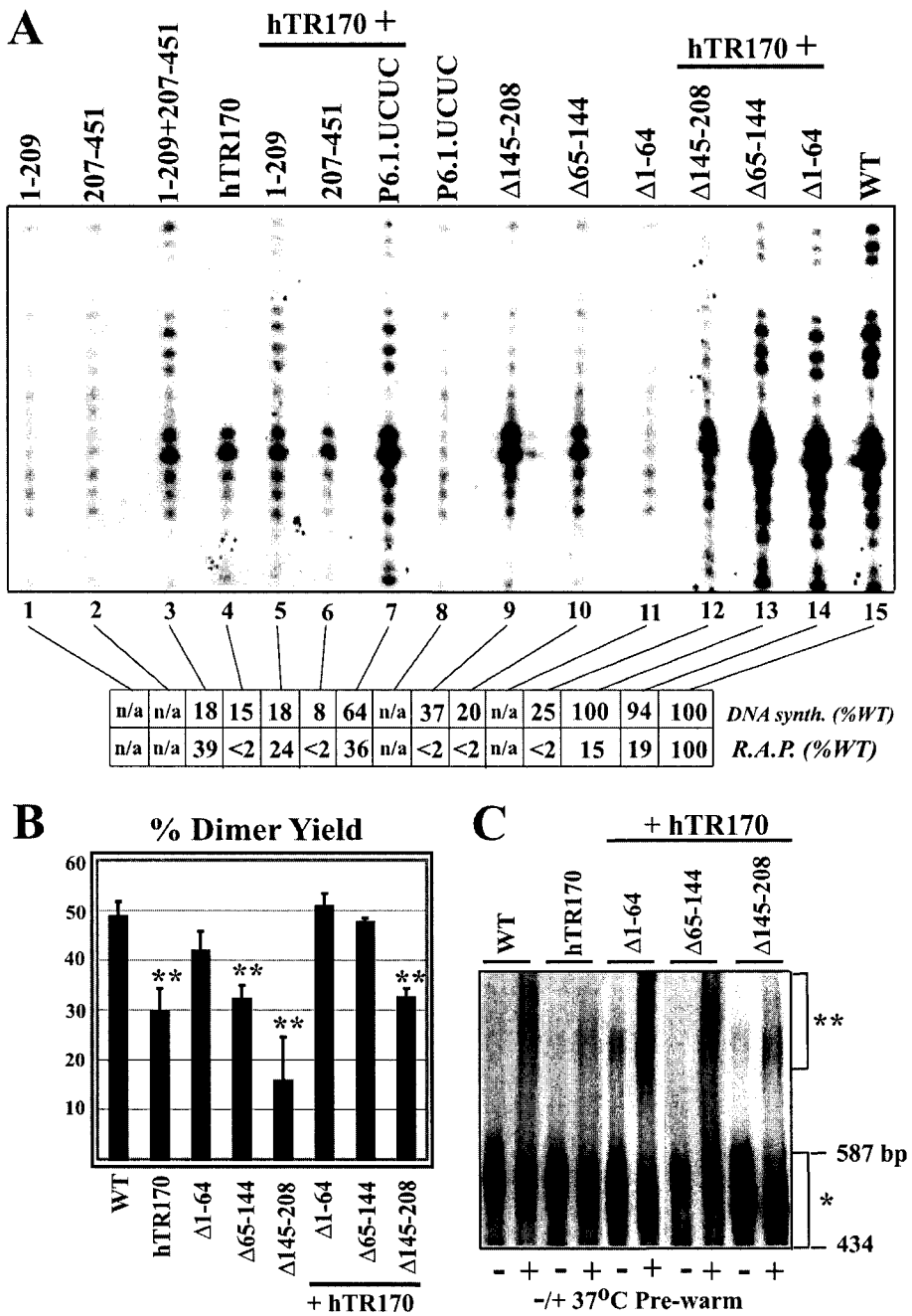
wild-type first repeat DNA synthesis), and compensatory mutations that reconstitute the P6.1 stem structure and rescue TERT interactions restored wild-type levels of catalytic activity (Figure 3.5C, lanes 7-9) (Chen et al. 2002). P6.1 base-pairing mutants were defective in interactions with RID2 (Figure 3.5E), indicating that the catalytic function of the P6.1 helix is likely mediated by binding to RID2. Mutation of other CR4/CR5 sequences had more modest effects on DNA synthesis (Figure 3.5C, lanes 1-5). Interestingly, an hTR variant containing altered sequences immediately 3' of the P6.1 stem loop also exhibited a 75% reduction in repeat addition processivity (Figure 3.5C, lane 5). We concluded that the RID2-interacting CR4/CR5 P6.1 stem loop is essential for DNA synthesis. Since hTR binding-defective RID2 mutants exhibited comparable DNA synthesis defects (Figure 3.1B), we concluded that high affinity interactions between the P6.1 stem loop and RID2 are likely required for the basic DNA synthesis function of telomerase.

3.5.10. Nucleotides in the hTR pseudoknot/template domain are essential for repeat addition processivity

Next, we investigated the effect of pseudoknot/template domain mutations on DNA synthesis and repeat addition processivity. We found that nucleotides outside the template were not essential for DNA synthesis, though deleting these sequences reduced the amountof DNA synthesized (Figure 3.6A, lanes 9-11, 15). In contrast, most mutations inhibited processivity, consistent with recent reports that the pseudoknot/template domain is important for this catalytic function (Figure 3.5D, lanes 2-4; Figure 3.6A, lanes 9-10) (also sub36-45, sub122-129; data not shown) (Chen and Greider 2003a; Lai et al. 2003; Mason et al. 2003). Interestingly, four hTR mutants, Δ65-144, Δ145-208, hTR170 and hTR180, exhibited elongation defects consistent with a complete loss of processivity (<2%) (Figure 3.5D, lanes 3 and 4; Figure 3.6A, lanes 9-10). hTR170 and hTR180 contain complementary substitutions at positions 170-179 (hTR170) and 180-189 (hTR180) in the P3 helix (Figure 3.5B). Substitution of hTR residues in this region with mTR sequences has been demonstrated to affect telomerase activity levels when chimeric TRs are assembled with hTERT, though a role in repeat addition processivity was not identified (Chen and Greider 2003a). We found that the hTR170 and hTR180 P3 mutants synthesized DNA less efficiently than wild-type enzyme, but were catalytically active and

Figure 3.6: Reconstitution of P3 base-pairing potential *in trans* restores repeat addition processivity and hTR dimerization to a P3 mutant.

(A) Recombinant telomerases were reconstituted in the presence or absence of hTR170, and elongation products were examined using the direct primer extension assay. Repeat addition processivity (R.A.P.) and first telomere repeat DNA synthesis (DNA synthesis) values were expressed as a percent of the corresponding values for wild-type enzyme, and are indicated at the bottom of each lane. **(B and C)** *In vitro* dimerization of hTR mixtures. Representative dimerization results are shown in (C). Monomers are indicated by single asterisks. Homo- and heterodimers comigrated extensively, and are collectively identified by a double asterisk. % dimer yield for mixtures of hTR mutants was calculated by measuring the combined homo- and heterodimer signal, which was expressed as a percentage of the total signal for monomers and dimers. Double asterisks in (B) indicate samples with significantly different mean dimerization values ($\rho<0.01$) compared to wild-type (WT) hTR. DNA Marker V (Roche) was used as a size indicator.


A
hTR170 +
hTR170 +
1-209
207-451
1-209+207-451
hTR170
1-209
207-451
P6.1.UCUC
P6.1.UCUC
Δ145-208
Δ65-144
Δ1-64
Δ145-208
Δ65-144
Δ1-64
WT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
n/a n/a 18 15 18 8 64 n/a 37 20 n/a 25 100 94 100 DNA synth. (%WT)
n/a n/a 39 <2 24 <2 36 n/a <2 <2 n/a <2 15 19 100 R.A.P. (%WT)
B
% Dimer Yield
60
50
40
30
20
10
**
WT
hTR170
Δ1-64
Δ65-144
Δ145-208
Δ1-64
Δ65-144
Δ145-208
+ hTR170
C
+ hTR170
WT
hTR170
Δ1-64
Δ65-144
Δ145-208
**
587 bp
*
434
- + - + - + - + - +
-/+ 37°C Pre-warm

generated only one repeat of elongation products with a pattern similar to the wild-type profile. Another P3 helix mutant containing a two base substitution found in a family with autosomal dominant dyskeratosis congenita (DKC) exhibited activity levels similar to hTR170 but was fully processive (Figure 3.5D, lane 5) (Vulliamy et al. 2001). Substitution of nucleotides 190-199 in the adjacent P1 helix reduced processivity but did not affect the efficiency of DNA synthesis (Figure 3.5D, lane 2). We recently found that the hTR190 and hTR180 mutants contain an additional small deletion of hTR nucleotides 200-201 (see Materials and Methods). The distinct catalytic phenotypes of the hTR180 and hTR190 mutants (Figure 3.5D) suggest that the 200/201 deletion is not a major determinant of these mutants' catalytic properties. Since nucleotides 170-189 were essential for repeat addition processivity, we investigated the mechanism underlying the hTR170 and hTR180 mutants' severe processivity defects.

3.5.11. Pseudoknot-mediated hTR dimerization is necessary but not sufficient for repeat addition processivity

Consistent with previous reports that the P3 helix is not essential for hTERT binding (Bachand and Autexier 2001; Ly et al. 2003b), we found that the hTR170 and hTR180 substitutions did not prevent hTR from binding to full-length hTERT, RID1 or RID2 (Appendix 1C). Since hTR interaction with the hTERT RID1 was not sufficient for repeat addition processivity, we investigated whether altered hTR-hTR interactions could account for the hTR170 and hTR180 mutants' processivity defects. Substitution of nucleotides 170-189 with complementary sequences is predicted to disrupt the pseudoknot P3 helix, which is formed by long-range base-pairing between nucleotides in the phylogenetically conserved regions CR2 and CR3 (Figure 3.5B) (Chen et al. 2000). Interestingly, disruption of long-range base-pairing potential in the *Tetrahymena* and *K. lactis* TR pseudoknots inhibits repeat addition processivity and affects template usage, respectively (Lai et al. 2003; Tzfati et al. 2003). hTR dimerization is mediated by *trans* base-pairing of the CR2 and CR3 sequences from separate hTR molecules (Figure 3.5B) (Ly et al. 2003b). To investigate the hypothesis that P3-mediated hTR-hTR interactions might regulate processivity, we analyzed the *in vitro* dimerization potential of the hTR170 and hTR180 mutants. We found that hTR170 exhibited dimerization defects as extensive as those observed for an hTR mutant lacking all of CR2 (Figure 3.5F-G: hTR170 and Δ65-

144). However, hTR180 dimerized as efficiently as wild-type hTR, implying that this mutant's impaired processivity was not attributable to dimerization defects detectable by this assay (Figure 3.5F-G). We also found that the fully-processive DKC mutant dimerized more efficiently than wild-type hTR (Figure 3.5F-G). These data imply that *trans* P3 base-pairing between full-length hTR molecules may not require the 3' end of the P3 helix (nucleotides 107-108 and 180-183), and may be regulated differently than the P3 interactions reconstituted in oligonucleotide model systems (Comolli et al. 2002; Ly et al. 2003b; Theimer et al. 2003). A recent study found that not all compensatory base-pairing substitutions in the *K. lactis* pseudoknot restored wild-type telomerase activity (Tzfati et al. 2003). Though it is not known if the *K. lactis* TR dimerizes, these observations suggest that the pseudoknot base-pairing structure alone is not sufficient for catalytic function. Similarly, we concluded that P3 helix-mediated hTR dimerization is not sufficient for repeat addition processivity.

Though the hTR180 mutant's processivity impairment could not be attributed to a loss of hTR-hTR interactions, we found that reductions in dimerization efficiency coincided with processivity defects in other mutants. The processivity-impaired hTR190 mutant dimerized as inefficiently as hTR170, indicating that P1 helix residues are important for hTR-hTR interactions (Figure 3.5F-G). In addition, deletion of CR2- or CR3-containing sequences inhibited dimerization and abolished processivity (Figure 3.5F-G; Figure 3.6A: Δ65-144, Δ145-208). Interestingly, the Δ145-208 mutant, which lacks both CR3 and P1 helix sequences, dimerized less efficiently than the Δ65-144 mutant, supporting the conclusion that the P1 helix participates in hTR-hTR interactions (Figure 3.5F-G). Since all of the dimerization-impaired hTR mutants we tested were also defective in processivity (Figure 3.5 and 3.6), we concluded that hTR dimerization may be necessary, though not sufficient for wild-type levels of repeat addition processivity.

3.5.12. *Trans*-acting hTRs restore dimerization and repeat addition processivity to a P3 helix mutant

To test the hypothesis that hTR dimerization is important for repeat addition processivity, we asked if *trans*-acting hTR fragments could restore processivity and dimerization to the hTR170 mutant. First, we mixed hTR170 with nucleotides 1-209 or 207-451, and found that only the pseudoknot/template domain could complement

hTR170's processivity defects (Figure 3.6A, lanes 4-6). An inactive CR4/CR5 mutant also reconstituted processivity when mixed with hTR170, confirming that *trans* complementation of processivity defects can occur in the context of overlapping hTR molecules, and is not dependent on the presence of a functional CR4/CR5 domain in the complementing molecule (Figure 3.6A, lane 7). Next, we asked which nucleotides in the pseudoknot/template domain were responsible for *trans* complementation of hTR170's processivity defects. We deleted nucleotides 1-64, 65-144 or 145-208 from hTRs containing an intact 3' region (nt 209-451). Nucleotides 1-64 contain the template, upstream sequences involved in P1 helix formation, and a downstream processivity-determining element (Figure 3.4A) (Chen et al. 2000; Chen and Greider 2003a). As expected, deletion of the hTR template region abolished telomerase activity (Figure 3.6A, lane 11). Nucleotides 65-144 include all of the sequences required to form the P2a.1, P2a and P2b helices, in addition to the CR2 residues involved in P3 base-pairing. Deletion of nucleotides 145-208 removes the entire upper strand of the pseudoknot region, including the CR3 sequences required for P3 base-pairing with CR2 (Figure 3.4A) (Chen et al. 2000). Deletion of nucleotides 145-208 or 65-144 disrupted hTR dimerization and abolished processivity (Figure 3.5F-G; Figure 3.6A, lanes 9-10). Mixing hTR170 with Δ65-144 or Δ1-64, which confers P3 base-pairing potential *in trans*, stimulated processivity by approximately 10-fold, and restored dimerization to wild-type levels (Figure 3.6A, lanes 13-14; Figure 3.6B and C). Similarly, mixing hTR180 with Δ65-144 or Δ1-64 reconstituted processivity, though less efficiently than hTR170, suggesting that *trans*-acting CR3 sequences also contribute to processivity (data not shown). However, mixing Δ145-208 with hTR170 restored neither dimerization nor processivity (Figure 3.6A, lane 12; Figure 3.6B and C). We concluded that reconstitution of *trans* P3 base-pairing potential and dimerization can confer processivity to the hTR170 mutant. Recent studies have identified a role for *trans*-acting *Tetrahymena* pseudoknot elements in repeat addition processivity (Lai et al. 2003; Mason et al. 2003). Though the mechanistic contribution of pseudoknot sequences and structures to repeat addition processivity remains unclear, our results establish a role for hTR-hTR interactions in the repeat addition processivity of human telomerase.

3.6. Discussion

3.6.1. Functional organization of the human telomerase complex

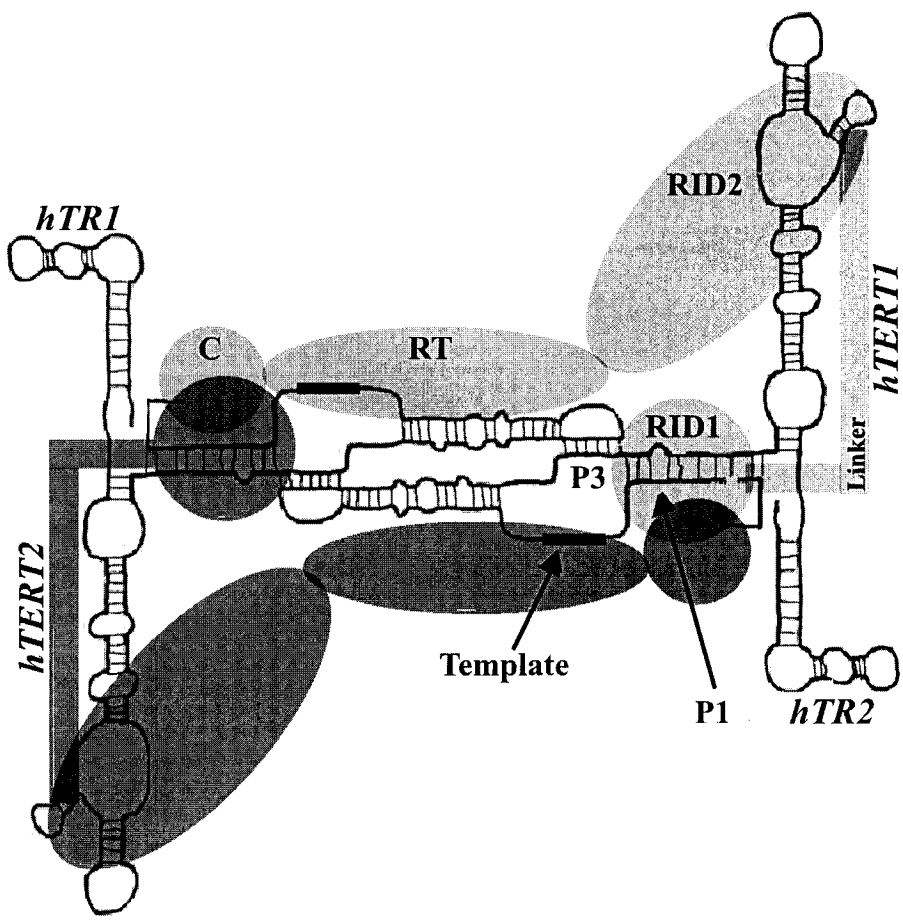
We found that processivity-mediating RID1 residues interact with the processivity-regulating hTR pseudoknot/template domain, and that hTR-interacting residues in RID2 and a RID2-interacting helix in CR4/CR5 are essential for DNA synthesis. These results imply that hTERT-hTR interactions contribute to both the DNA synthesis and processivity functions of human telomerase. In addition, we found that *trans*-acting hTERTs or hTRs confer repeat addition processivity to processivity-defective hTERT or hTR mutants, respectively, suggesting a role for telomerase multimerization in repeat addition processivity. RID1 interacted with processivity-determining hTERT C-terminal sequences, and hTR dimerization also contributed to repeat addition processivity. A schematic summary of the hTERT-hTR, hTERT-hTERT and hTR-hTR interactions reported in this study and others is presented in Figure 3.7 (Beattie et al. 2000; Lai et al. 2001; Chen et al. 2002; Ly et al. 2003b). The role of hTERT multimerization in repeat addition processivity and DNA synthesis may be analogous to the interdependent functioning of the HIV-1 RT's polymerase and RNase H accessory domains (see below). hTR-hTR interactions at the P3 helix interface could stabilize the hTR dimer structure, permitting one TR to allosterically influence the function or conformation of the other TR (Ly et al. 2003b). hTR interactions with RID1 and RID2 hTERT sequences essential for processivity and DNA synthesis, respectively, could facilitate the dynamic hTR rearrangements that are likely involved in telomerase catalysis. Such rearrangements could include the alternating use of telomerase's two potential active sites (Prescott and Blackburn 1997a; Wenz et al. 2001), though it is unknown if active site or template switching are an obligatory feature of telomerase catalytic function. An alternative model for the function of telomerase dimerization is that dimerization facilitates the simultaneous extension of two DNA substrates (Wenz et al. 2001). Such a hypothesis might account for the observation that the abundant *Tetrahymena* telomerase is active and processive as a monomer (Bryan et al. 2003).

3.6.2. RID1 as the major accessory domain of hTERT

Nucleic acid polymerases contain unique, structurally-unrelated major accessory domains that confer the specialized activities of each enzyme and interact with nucleic

Figure 3.7: Proposed functional domain organization of the human telomerase multimer.

Schematic of hTERT-hTR, hTERT-hTERT and hTR-hTR interactions in the human telomerase complex. RID1 is connected to the hTERT core polymerase domain (RID2, RT, C) by a flexible, catalytically inessential linker. The RID2-P6.1 interaction site is likely essential for DNA synthesis. RID1-pseudoknot/template and RID1-C terminus interactions may regulate repeat addition processivity. RID1 and the C-terminus can functionally interact *in trans* (see text), implying that RID1 and the polymerase domain of a single hTERT may interact with different hTR molecules. P3 helix-mediated hTR dimerization also contributes to repeat addition processivity and may facilitate the allosteric regulation of one hTR monomer by the other (see text). This schematic is adapted from the recently proposed hTR dimer structure (Ly et al. 2003b). RID2-C terminus/RT contacts (Arai et al. 2002) are not depicted in this model, but could form during the dynamic rearrangements likely entailed by telomerase catalysis.


hTR1
RID2
hTERT1
C
RT
RID1
Linker
P3
hTERT2
Template
P1
hTR2

acids upstream of the polymerase active site, but which are not required for basic polymerase function (reviewed in Sousa 1996). Previous work indicates that RID1 interacts with full-length hTR and is physically separable from the remainder of hTERT (Beattie et al. 2001). Also, deletion of RID1 reduces but does not abolish telomerase activity and affects the ability of human telomerase to extend non-telomeric primers (Beattie et al. 2001).We have identified the processivity-regulating hTR pseudoknot/template domain as a site in hTR that interacts with RID1. Furthermore, using a direct primer extension telomerase assay, we found that RID1 is not essential for DNA synthesis, and is physically and functionally separable from, though functionally interdependent with, the core polymerase domain of hTERT (RID2, RT, C). These data suggest that RID1 may be the major accessory domain of hTERT and that the unique catalytic property it confers is repeat addition processivity.

The HIV-1 RT is a well-characterized dimeric nucleic acid polymerase which has been used as a model for telomerase function in several previous structure-function studies (reviewed in Kelleher et al. 2002). However, the similarities we observed between hTERT and the HIV-1 RT's domain organization, and the pattern of hTERT and RT accessory domain interactions with nucleic acids and polymerase sequences are also true of many other nucleic acid polymerases (see Hostomsky et al. 1991; Sousa 1996; Wang et al. 1997; Hopfner et al. 1999, and citations therein). Like the HIV-1 RT's RNase H accessory domain, RID1's ability to interact with several catalytically-important hTERT regions could account for the effect of RID1 mutations on the DNA synthesis efficiency of telomerase (Hostomsky et al. 1991). The hTERT C terminus's association with RID1 could also influence repeat addition processivity by a mechanism analogous to the p51 thumb's regulation of the p66 subunit's RNase H function (Cameron et al. 1997). Interestingly, we found that processivity-defective C-terminal mutants functionally complemented a RID1 mutant's processivity defects, suggesting that RID1 and C-terminal processivity-determining sequences can be physically located on separate hTERT molecules (Figure 3.7; Table 3.1; data not shown). These observations imply that *trans* interactions in the TERT multimer can confer repeat addition processivity to human telomerase. Though protein fragments containing RID1 complemented overlapping RID1 mutants, our results do not indicate that RID1 always functions *in trans* in the human telomerase multimer.

Processivity-determining RID1 sequences also interacted with the hTR pseudoknot/template domain, which is a major determinant of repeat addition processivity (Figure 3.7) (this study) (Chen and Greider 2003a). Recent work demonstrates that the vertebrate pseudoknot/template domain mediates mouse- and human-specific differences in telomerase activity in a TERT-dependent fashion (Chen and Greider 2003a). Though this study reported that the mTR pseudoknot interacts more efficiently with hTERT than the hTR pseudoknot, TR-TERT interactions were evaluated using wild-type hTERT and an hTR pseudoknot/template domain missing the first 43 nucleotides of hTR (Chen and Greider 2003a). These hTR sequences are important for human telomerase activity and have been recently implicated in 5' template boundary definition and repeat addition processivity (Beattie et al. 2000; Chen and Greider 2003b; Ly et al. 2003a). Since hTR residues 1-39, which are absent in mTR, may be implicated in hTERT interactions (Beattie et al. 2000; Bachand and Autexier 2001), and as these nucleotides are protected *in vivo*, but not *in vitro* (Antal et al. 2002), it is possible that hTERT-hTR1-209 interactions could partially account for species-specific differences in repeat addition processivity. Interestingly, substitution of nucleotides 36-45 or 190-199, which alters P1 helix sequences, reduced processivity, suggesting that this region might be a candidate for RID1 interactions (Figure 3.5D; data not shown). The accessory domains of a number of polymerases such as the HIV-1 RT regulate the movement of nucleic acids during catalysis (Bahar et al. 1999), and repeat addition processivity is likely to entail dynamic rearrangements of the template RNA and/or DNA substrate within the catalytic active site. Our observations suggest a similar function for RID1.

The HIV-1 RNase H accessory domain also contributes to the stability of RT-primer-template complexes (reviewed in Götte et al. 1999). Repeat addition processivity may be partly regulated by telomerase-DNA interactions at a protein-dependent anchor site that is distinct from the catalytic core (Hammond et al. 1997; Wallweber et al. 2003, and references therein). Interestingly, *in vitro*-reconstituted human telomerase lacking RID1 cannot elongate non-telomeric primers, but this defect is rescued by coexpressing a second, inactive hTERT containing an intact RID1 (Beattie et al. 2001). Together with our data demonstrating that *trans* complementation restores processivity to RID1 mutants, these observations suggest that RID1 may interact with DNA substrates, and could constitute the TERT anchor site (Xia et al. 2000; Beattie et al. 2001).

3.6.3. RNA-dependent strand transfers as a model for telomerase repeat addition processivity

Our data indicate that the hTERT RID1 and dimerization-mediating hTR sequences are essential for repeat addition processivity. These observations, and the similarities we have found between RID1 and the HIV-1 RNase H accessory domain, suggest that repeat addition processivity may resemble the strand transfer events mediated by HIV-1 and other RTs (reviewed in Götte et al. 1999). During replication of HIV-1's double-stranded RNA genome, RNase H-dependent strand transfers at the 5' end of RNA templates facilitate the translocation of newly synthesized minus-strand DNA to the 3' end of the RNA acceptor-strand. By a mechanism that recalls the processivity-determining features of telomerase's 3' template-3' DNA substrate interactions (Chen and Greider 2003a), the 3' end of translocated donor-strand DNA then hybridizes to complementary sequences at the 3' end of either one of the homologous copies of genomic RNA, permitting a second round of DNA synthesis to begin. RNA-dependent strand transfers require the cleavage activity of the RNase H domain, suggesting that RID1 could mediate a similar function in hTERT. Endonuclease cleavage activity has been reported for yeast and ciliate telomerases (reviewed in Harrington 2003). However, the role of specific TERT sequences in endonucleolytic cleavage has not been characterized, and the similarities shared by the RNase H and RID1 accessory domains may not include cleavage function.

RNA-dependent strand transfers also require dimerization-initiating sequences (DIS) in the double-stranded RNA template, though the function of template dimerization in strand transfers is not fully understood (Balakrishnan et al. 2003). The primary RNase H-mediated invasion step of strand transfer can occur at sites that are physically distant from the DIS, implying that dimerization is not required for the initiation of this process (Balakrishnan et al. 2003). RNA-dependent strand transfers can proceed in either an intra- or inter-molecular fashion, though template-switching is not obligatory. A strand transfer model of repeat addition processivity could therefore explain the ability of telomerase dimers to use both their templates (Prescott and Blackburn 1997a; Wenz et al. 2001). Though RNA-dependent strand transfers and telomerase repeat addition processivity share some interesting features, it is unlikely that the mechanisms mediating these two processes

are identical. The extent of their potential similarities and the roles of RID1 and the TR pseudoknot in repeat addition processivity remain to be defined.

In this study, we identified elements in hTR and hTERT that regulate DNA synthesis and repeat addition processivity. Our data implicate telomerase multimerization in repeat addition processivity, and suggest that the mechanism mediating this telomerase-specific catalytic function may represent a variation on a catalytic mechanism shared by other reverse transcriptases.

3.7. Acknowledgements

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4. CHAPTER 4: An anchor site-type defect in human telomerase that disrupts telomere length maintenance and cellular immortalization

4.1. Preface

The preceding chapters described a role for hTERT RID1 in telomerase catalytic function, specifically repeat addition processivity. A report that was published shortly before the study described in Chapter 2 indicated that a subset of hTERT RID1 sequences were not required for catalytic function when telomerase activity was measured using the PCR-based TRAP assay, but were essential for telomere length maintenance when expressed *in vivo* (referred to as DAT sequences, for dissociates activities of telomerase). We examined the telomere length maintenance phenotype of a catalytically active hTERT RID1 mutant first described in Chapter 2, and determined that it exhibited an apparent DAT phenotype. We analyzed the activity of this mutant on different types of DNA substrates using the direct primer extension telomerase assay, and found that it exhibited catalytic impairment, including defects in repeat addition processivity, with a specific subset of DNA substrates. Examination of the DNA sequences that affected the telomerase activity of this mutant implied that its catalytic defect likely resulted from an impaired, anchor site-type affinity for DNA substrates. These observations constitute the first report of an anchor site-type mutant in any TERT, and implicate RID1 in interactions with the telomerase DNA substrate.

4.2. Abstract

Telomerase-mediated telomeric DNA synthesis is important for eukaryotic cell immortality. Telomerase adds tracts of short telomeric repeats to DNA substrates using a unique repeat addition form of processivity. It has been proposed that repeat addition processivity is partly regulated by a telomerase reverse transcriptase (TERT)-dependent anchor site; however, anchor site-mediating residues have not been identified in any TERT. We report the characterization of an N-terminal human TERT (hTERT) RNA interaction domain 1 (RID1) mutation that caused telomerase activity defects consistent with disruption of a template-proximal anchor site, including reduced processivity on short telomeric primers, and reduced activity on substrates with non-telomeric 5' sequences, but not on primers with non-telomeric G-rich 5' sequences. This mutation was located within

a subregion of RID1 previously implicated in biological telomerase functions unrelated to catalytic activity (N-DAT domain). Other N-DAT and C-terminal DAT (C-DAT) mutants and a C-terminally tagged hTERT-HA variant were defective in elongating short telomeric primers, and catalytic phenotypes of DAT variants were partially or completely rescued by increasing concentrations of DNA primers. These observations imply that RID1 and the hTERT C terminus contribute to telomerase's affinity for its substrate, and that RID1 may form part of the human telomerase anchor site.

4.3. Introduction

Telomerase is important for telomeric DNA replication in most eukaryotes. The minimal components of *in vitro*-reconstituted telomerase are the telomerase reverse transcriptase (TERT) and the telomerase RNA (TR) (reviewed in Harrington 2003). The TR bears a short template (11 nucleotides in human TR, hTR) that commonly encodes less than two telomeric DNA repeats (vertebrate telomeric repeat: TTAGGG) (Feng et al. 1995). Many telomerases add multiple telomeric repeats to a single DNA substrate by repetitive reverse transcription of the TR template (repeat addition processivity) (reviewed in Lue 2004). This telomerase-specific catalytic property may be important for telomere length maintenance *in vivo*, where telomeres in telomerase-negative cells from organisms such as the vertebrates are predicted to lose 50-100 nucleotides (nt) of DNA with each round of cell division (Harley et al. 1990).

It is proposed that telomerase-specific catalytic functions are mediated by interactions with its DNA substrate at two different sites: 1) an active site where the TR template and reverse transcriptase (RT) polymerase motifs interact with 3' sequences of the DNA substrate via base-pairing between the TR template and DNA; and 2) a template-independent anchor site that interacts with G-rich substrate 5' sequences (Morin 1989; Harrington and Greider 1991; Morin 1991; Collins and Greider 1993; Lee and Blackburn 1993; Melek et al. 1994; Melek et al. 1996; Hammond et al. 1997; Lue and Peng 1998; reviewed in Collins 1999; Harrington 2003; Lue 2004). Repeat addition processivity requires translocation of the TR template and DNA substrate such that the DNA substrate 3' end is realigned with template 3' sequences at the beginning of each round of template reverse transcription. The mechanism of this translocation and realignment likely entails conformational changes and movement of both the TR template

and DNA substrate (reviewed in Lue 2004). The anchor site is thought to prevent dissociation of telomerase from its DNA substrate during the translocation step of repeat addition processivity, and to facilitate alignment or positioning of primers in the active site (Morin 1989; Collins and Greider 1993; Lee and Blackburn 1993; Melek et al. 1994; Melek et al. 1996). Anchor site interactions also promote the elongation of partially telomeric and non-telomeric substrates, a property which could be important for telomerase-mediated chromosome healing *in vivo* (Harrington and Greider 1991; Morin 1991; Collins and Greider 1993; Melek et al. 1996; Wang and Blackburn 1997). In *Euplotes* and *Candida albicans* (*C. albicans*) telomerases, telomerase-associated factors such as p43 and Est1p stimulate repeat addition processivity and/or the mode and efficiency of telomerase-mediated elongation of suboptimal DNA substrates, suggesting the possibility that telomerase accessory proteins could contribute to the anchor site (Bednenko et al. 1997; Singh and Lue 2003; Aigner and Cech 2004).

Examination of the primer length and sequence preferences of endogenous yeast and ciliate telomerases suggests that the enzyme interacts in a template-independent fashion with telomeric DNA at two positions, the template-proximal and template-distal anchor sites (Lue and Peng 1998; Collins 1999). The template-proximal anchor site is predicted to interact with primers in a region immediately adjacent to the template-hybridizing nucleotides (up to ~ nt 12), whereas the template-distal anchor site associates with primer sequences further upstream; primer nucleotides in both of these regions contribute to repeat addition processivity and the affinity of yeast, ciliate and human telomerases for their DNA substrates, though template-distal anchor site sequences are not essential for repeat addition processivity (Morin 1989; Collins and Greider 1993; Lee and Blackburn 1993; Lue and Peng 1998; Collins 1999; Baran et al. 2002; Wallweber et al. 2003). Template-distal and template-proximal anchor site interactions with DNA primers do not require base-pairing with the TR template, and may be TERT-dependent. In *Euplotes*, a TERT-sized protein is cross-linked to single-stranded DNA (ssDNA) substrates 20-22 nt 5' of the 3' terminus, suggesting that the anchor site is TERT-dependent; in double-stranded DNA (dsDNA) substrates with a 3' overhang, an anchor site cross-link forms at the junction of ssDNA and dsDNA, 15-17 nt from the 3' end of the overhang, implying that TERT might also interact with dsDNA (Hammond et al.

1997). However, residues that mediate anchor site function have not been identified in any TERT.

Elongation of short telomeric substrates is likely regulated by interaction with the template-proximal anchor site, though it has been proposed that a TERT-interacting template recognition element (TRE) in the *Tetrahymena* TR (Lai et al. 2001; Miller and Collins 2002) might also contribute to telomerase activity on short primers (Lue and Peng 1998; Collins 1999; Baran et al. 2002). Previous studies have shown that endogenous human telomerase elongates 12 nt telomeric primers more efficiently than longer 18 nt telomeric substrates, and that the catalytic rate and activity of *Tetrahymena* and *Oxytricha* telomerases are enhanced on substrates containing two or fewer telomere repeats (Morin 1989; Zahler et al. 1991; Lee and Blackburn 1993). It has been proposed that DNA sequences predicted to interact with the template-proximal anchor site may regulate conformational changes in the enzyme-substrate complex; such conformational changes could facilitate alignment and/or positioning of the template/primer duplex in the catalytic active site, or unwinding of RNA/DNA duplexes during translocation (Lee and Blackburn 1993; Lue and Peng 1998; Baran et al. 2002).

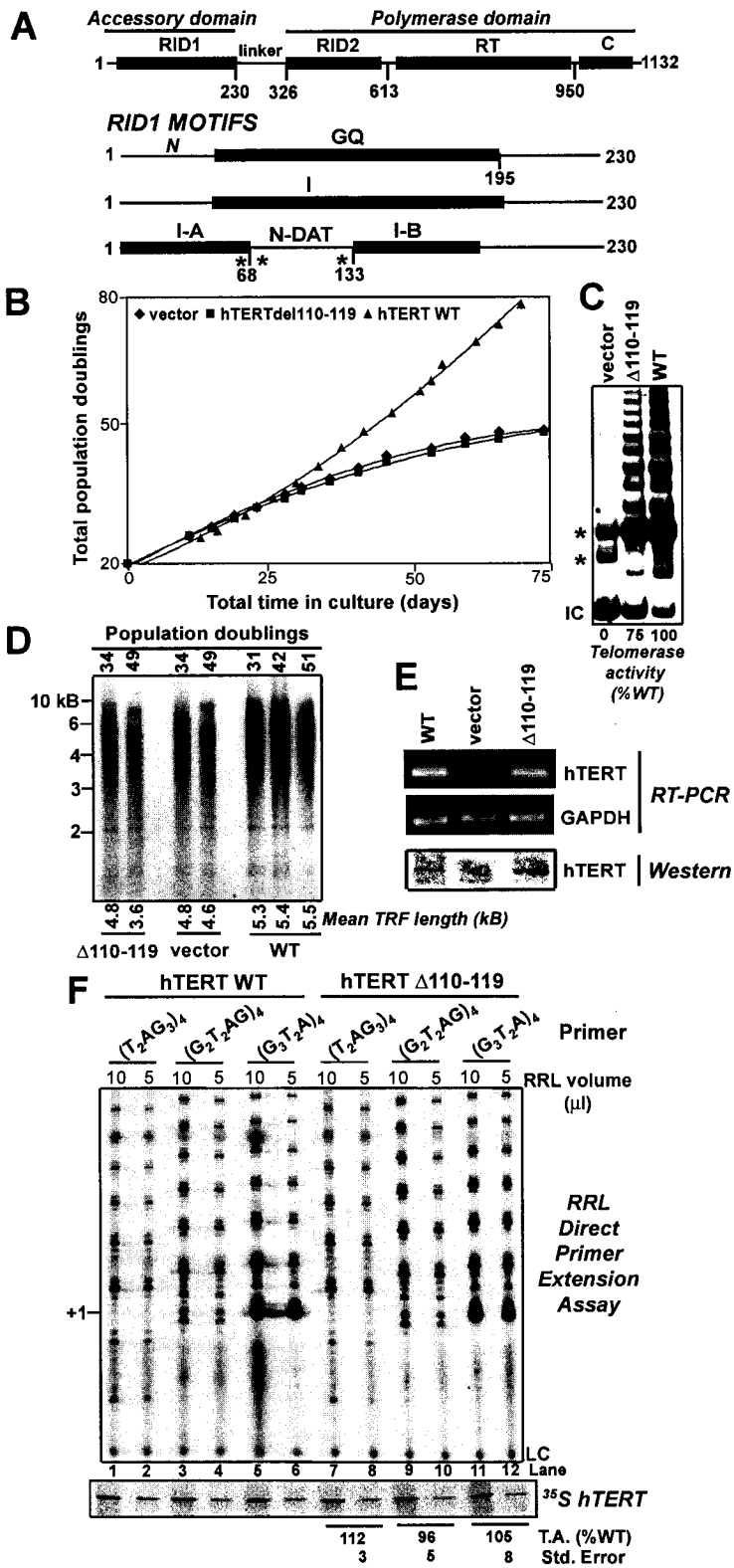
Mutations in telomerase-specific N- and C-terminal sequences of human and *Saccharomyces cerevisiae* (*S. cerevisiae*) TERTs (hTERT and Est2p, respectively) have been reported to dissociate the biological and catalytic activities of telomerase (N-DAT and C-DAT mutants) (Friedman and Cech 1999; Xia et al. 2000; Armbruster et al. 2001; Banik et al. 2002; Kim et al. 2003). Addition of a hemaglutinin (HA) tag or other extensions to the hTERT C terminus also confer a DAT-like phenotype (Counter et al. 1998; Kim et al. 2003). The *in vitro* telomerase activity of most N-DAT mutants is at least 60% of the activity observed for wild-type (WT) telomerase (Xia et al. 2000; Armbruster et al. 2001), and a C-DAT hTERT mutant exhibits 15-50% of WT telomerase activity (Banik et al. 2002). However, N-DAT, C-DAT and C-terminally modified TERTs do not immortalize telomerase-negative cells, and telomere shortening in cells expressing these mutants is as extensive as the telomere shortening in cells that express no telomerase (Counter et al. 1998; Friedman and Cech 1999; Xia et al. 2000; Armbruster et al. 2001; Banik et al. 2002; Kim et al. 2003).

The hTERT N-DAT domain is located between amino acids 68 and 133 (Armbruster et al. 2001) (Figure 4.1A). The first reports characterizing this hTERT region indicated that mutations in residues 70-85 impair but do not abolish *in vitro* telomerase activity detected using the PCR-based telomeric repeat amplification protocol (TRAP) assay and partially telomeric primers; a mutation in this sub-region of the yeast telomerase N-DAT sequences also affects telomerase activity (Xia et al. 2000; Armbruster et al. 2001; Moriarty et al. 2002b). However, hTERT amino acids 86-133 appeared to be catalytically inessential, though some mutations in the corresponding region of Est2p cause moderate to severe catalytic defects (Xia et al. 2000; Armbruster et al. 2001; Moriarty et al. 2002b). The limited proliferation phenotypes of cells expressing hTERT variants with mutations in N-DAT residues 125-136 or 128-134 is partially or fully rescued by fusion of these mutants to the telomere-binding proteins hTRF2 or hPOT1, respectively; these observations led to the proposal that some N-DAT sequences may be important for the recruitment and/or activation of telomerase at telomeres (Armbruster et al. 2003; Armbruster et al. 2004). However, N-DAT and C-DAT hTERT variants with mutations in amino acids 92-97 or 1127-1132, respectively, exhibit primer concentration-dependent catalytic defects when telomerase activity is measured using an entirely non-telomeric primer in the PCR-based TRAP assay (Lee et al. 2003). These mutants and a second variant with a mutation in N-DAT residues 122-127 also display catalytic defects in direct primer extension telomerase assays performed with a physiological telomeric DNA substrate, though catalytic impairment is not readily apparent when telomerase activity is measured using the TRAP assay and the standard, partially telomeric TRAP primer (Armbruster et al. 2001; Lee et al. 2003). These observations indicate that some hTERT DAT residues are catalytically important in the context of low primer concentrations, altered substrate sequences and more stringent assay conditions.

The most extensive DAT sequences in hTERT and Est2p are found within RNA interaction domain 1 (RID1), a putative TERT accessory domain that in human telomerase is essential for repeat addition processivity but not basic polymerase function, and which interacts with the processivity-regulating pseudoknot/template domain of hTR (see Figure 4.1A for alternative nomenclatures describing the RID1 region)

Figure 4.1: Effect of an N-DAT mutation on hTERT-mediated immortalization, telomere length maintenance and *in vitro* telomerase activity

(A) Upper panel: schematic depicting hTERT regions: RNA interaction domains 1 and 2 (RID1 and RID2), reverse transcriptase (RT) motifs and C terminus (C); hTERT accessory domain: RID1; hTERT polymerase domain: RID2 + RT + C (Moriarty et al. 2004). Lower panels: RID1 sequence motifs and functional regions previously identified in hTERT and Est2p, mapped onto hTERT. The GQ motif was identified by alignment of sequences conserved in Est2p and other TERTs, and overlaps with Est2p hypomutable Region I (Friedman and Cech 1999; Xia et al. 2000; Armbruster et al. 2001). I-A, I-B: hTERT RID1 sequences required for telomerase catalytic function (Armbruster et al. 2001). N-DAT: sequences previously implicated in telomere length maintenance, but not *in vitro* telomerase activity (Armbruster et al. 2001). Asterisks indicate the positions in hTERT of Est2p RID1 mutants that exhibit a DAT phenotype (Friedman and Cech 1999; Xia et al. 2000). **(B)** Elongation of 24 nt telomeric primers by rabbit reticulocyte lysate (RRL)-reconstituted wild-type (WT) and N-DAT mutant (Δ110-119) telomerases, as detected by a direct primer extension assay. Upper panel: telomerase-mediated elongation of DNA primers containing different permutations of the telomeric sequence. +1 indicates the position of products resulting from the addition of one nucleotide to a 24 nt substrate. Telomerase products lower than the +1 position arise from telomerase-associated nucleolytic primer cleavage (Huard and Autexier 2004; Oulton and Harrington 2004). LC: loading control. Middle panel: expression of ^{35}S-labeled hTERT in RRL samples. Bottom: hTERTΔ110-119 T.A. values were expressed relative to activity values for WT hTERT reactions performed using the same RRL volume and DNA substrate. Relative T.A. values were similar for reactions performed with 5 or 10 μl RRL-reconstituted telomerase. Average T.A. values were calculated from three independent experiments. **(C-F)** Proliferative lifespan (*C*), telomerase activity (*D*), telomere length (*E*), and expression of hTERT mRNA and protein (*F*) in polyclonal HA5 cell lines stably expressing vector alone, hTERTΔ110-119 or wild-type (WT) hTERT. Telomerase activity and hTERT protein and mRNA expression were examined in cell lines at population doubling (PD) 42-55. **(D)** Telomerase activity (T.A.), detected using the TRAP assay. The positions of PCR primer dimers (*) and internal controls (IC) are indicated.


A
Accessory domain
Polymerase domain
RID1
linker
RID2
RT
C
1
1132
230
326
613
950
RID1 MOTIFS
N
GQ
I
I-A
N-DAT
I-B
68
133
B
vector ■ hTERTdel110-119 ▲ hTERT WT
Total population doublings
Total time in culture (days)
C
vector
Δ110-119
WT
IC
0 75 100
Telomerase activity (%WT)
D
Population doublings
10 kB
6
4
3
2
Mean TRF length (kB)
Δ110-119 vector WT
E
WT
vector
Δ110-119
hTERT
GAPDH
RT-PCR
hTERT
Western
F
hTERT WT
hTERT Δ110-119
Primer
RRL volume (μl)
RRL Direct Primer Extension Assay
+1
LC
Lane
35S hTERT
T.A. (%WT)
Std. Error
112
3
96
5
105
8

(Moriarty et al. 2004). RID1 sequences are important for the catalytic activity of diverse telomerases (Figure 4.1A) (Friedman and Cech 1999; Beattie et al. 2000; Miller et al. 2000; Xia et al. 2000; Armbruster et al. 2001; Bachand and Autexier 2001; Lai et al. 2001; Moriarty et al. 2002b). Several groups have proposed that RID1 might constitute the anchor site, based on observations that this domain in Est2p interacts with DNA, and is essential for repeat addition processivity and elongation of entirely non-telomeric primers by human telomerase (Beattie et al. 2000; Xia et al. 2000; Lee et al. 2003; Moriarty et al. 2004). The RID1 domain may function cooperatively with the putative TERT C-terminal thumb, since the isolated Est2p C terminus interacts with DNA, and the hTERT C terminus has been implicated in repeat addition processivity, elongation of non-telomeric primers, and functional and physical interactions with RID1 (Beattie et al. 2001; Hossain et al. 2002; Huard et al. 2003; Lee et al. 2003; Moriarty et al. 2004).

We examined the catalytic phenotypes of a highly active hTERT RID1 DAT mutant and several less active N-DAT and C-DAT variants using a direct primer extension telomerase assay and DNA substrates that varied in length and 5' sequence. We investigated whether increasing concentrations of DNA substrate could rescue the catalytic defects of DAT mutants on these primers. Our results implicate RID1 and C-terminal hTERT sequences in telomerase's affinity for its DNA substrate, and indicate that residues in RID1 contribute to proximal anchor site-type functions in human telomerase.

4.4. Materials and methods

4.4.1. T7 promoter-driven RRL expression constructs

pET28b-hTERT WT, D868N (Bachand and Autexier 1999), Δ70-79, Δ110-119, Δ150-159, Δ508-517 (Moriarty et al. 2002b) and Δ1123-1132 (Huard et al. 2003) plasmids were previously described; pCR3.1-Flag-hTERT1-250 was previously described (Moriarty et al. 2004); pcDNA3.1-HA-hEST1A, pcDNA3.1-HA-hEST1B, pcDNA-N-MYC-TRF2 and hPOT1-expressing pPB320A constructs were provided by Drs. L. Harrington, T. De Lange and T. Cech, respectively (Baumann and Cech 2001; Snow et al. 2003). The T7 reporter-driven TRF2 construct was generated in the lab of T. De Lange, and has not been previously described. The pCI-Neo-hTERT-HA construct, provided by

Dr. R. Weinberg, was sub-cloned into the *EcoRI/SalI* sites of a pcDNA3 vector. Construct identity was confirmed by restriction digest and *in vitro* expression of ^{35}S-labeled hTERT-HA.

4.4.2. Retroviral constructs

Retroviral constructs expressing a GFP reporter gene and hTERT (WT or Δ110-119) were created by *EcoRI/NotI* digestion of pET28b-hTERT plasmids, and ligation of hTERT-encoding DNA into an *EcoRI/NotI* -digested pBMN-IRES-EGFP retroviral vector provided by Dr. G.P. Nolan. Infectious retrovirus-containing media collected from Phoenix packaging cells (G.P. Nolan) transiently transfected with pBMN-IRES-GFP-hTERT plasmids were passed through a 0.2 μM filter and stored at -80°C.

4.4.3. Stable cell lines

The hTERT-negative hTR-positive SV40-transformed human embryonic kidney cell line HA5 (provided by Dr. S. Bacchetti) was infected at population doubling 20 with packaging cell line media containing retroviruses expressing GFP reporter alone (vector), or GFP reporter gene and WT or Δ110-119 hTERT (WT and Δ110-119 hTERT, respectively). Five μg/ml hexadimethrine bromide (polybrene) (Sigma) was added to media to promote infection. GFP-expressing infected cells were selected by fluorescence-activated cell sorting with a Becton-Dickinson FACScan machine. Stable cell lines were grown in the presence of 5% CO_2, in MEM alpha medium (GIBCO) supplemented with 5% heat-inactivated FBS (GIBCO) and 1X antibiotic/antimycotic (GIBCO) (100 μg/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B). Population doubling number of polyclonal stable cell lines was calculated from cell numbers counted at each passage.

4.4.4. TRAP assays

TRAP assays were performed using 1 μg CHAPS cell extracts, as previously described (Moriarty et al. 2002a; Moriarty et al. 2002b).

4.4.5. Immunoblotting

Immunoblotting was performed with 50 μg CHAPS cell extracts and αhTERT antibody (Moriarty et al. 2002b). The hTERT peptide sequence used to generate αhTERT (Moriarty et al. 2002b) is the same as the sequence used to generate αTP2 hTERT antibody (Harrington et al. 1997b). A typographical error in the latter manuscript erroneously suggests that this peptide is different (L. Harrington, personal communication).

4.4.6. RT-PCR

Preparation of total cellular RNA and cDNA from stable cell lines using Trizol reagent, Superscript II reverse transcriptase (Invitrogen) and pd(N)$_6$ random hexamer (Amersham) were performed according to manufacturers' instructions. hTERT and GAPDH cDNAs were amplified using 1 ng/μl hTERT (5'-AAGTTCCTGCACTGGCTGATGAG-3', 5'-TCGTAGTTGAGCACGCTGAACAG-3') or GAPDH (5'-CGGAGTCAACGGATTTGGTCGTAT-3', 5'-TGCTAAGCAGTTGGTGGTGCAGGA-3') specific primers, 200 μM dNTPs and 0.05 U/ μl Taq DNA polymerase (Invitrogen). PCR conditions: 94°C 5 min, 31 cycles (94°C 45 sec, 57°C 45 sec, 72°C 1 min).

4.4.7. Telomere length measurement

Two μg *RsaI*/*HinfI*-digested genomic DNA were resolved by pulse field gel electrophoresis on 1% agarose gels (Bryan et al. 1995). In-gel hybridizations and calculations of mean telomere length from blots exposed to Phosphorimager cassettes were performed as described (Harley et al. 1990; Bryan et al. 1995).

4.4.8. *In vitro* telomerase reconstitution

^{35}S-labeled and unlabeled human telomerases were reconstituted in rabbit reticulocyte lysates (RRL), using equal amounts of WT hTR, as previously described (Moriarty et al. 2002b). In complementation assays (Supplemental Figure 4.6A and B), 0.75 μg hTERT-encoding DNA was mixed with 0.75 μg DNA encoding the specified proteins in a final RRL reaction volume of 20 μl.

4.4.9. Direct primer extension telomerase assay

The direct primer extension telomerase assay was performed as described (Huard et al. 2003; Moriarty et al. 2004). Reactions mixtures were supplemented with 1 mM dCTP as this nucleotide is present in the cellular context. All reactions were performed with 20 µl RRL-reconstituted telomerase and 1 µM primer, except where indicated. RRL alone did not mediate non-specific primer elongation under different assay conditions. This control was performed routinely, though not in every experimental replicate.

4.4.10. Quantification of repeat addition processivity and telomerase activity detected by direct primer extension assay

Telomerase activity was quantified by measuring the signals of the first six repeats of telomerase products, using gels exposed to Phosphorimager cassettes (Moriarty et al. 2004). The signals of products containing more than six telomeric repeats were not measured because non-specific "shadows" of variable intensities were frequently present on gels above this position, precluding accurate quantification of longer products. Mutant telomerase activity values were expressed relative to activity values for WT hTERT reactions from the same experiment performed with the same RRL volume and DNA substrate. Relative telomerase activity values were similar when reactions were performed with different volumes of RRL-reconstituted enzyme; therefore calculations of average telomerase activity values included data for all sample volumes. Repeat addition processivity values were calculated, as previously described, by measuring the signal intensity of the strongest product in each repeat (corresponding to the last G incorporated before enzyme translocation), normalizing this value to the number of incorporated radiolabelled dGTPs, and comparing the normalized values for different repeats to each other in the form of a ratio (Hardy et al. 2001). Repeat addition processivity values were measured between the first and second, and second and third telomere repeats, and both values were included in all statistical calculations. Processivity values were similar over the first three repeats when measured from primer extension assays performed with either 10 or 20 µl RRL-reconstituted telomerase (confirmed by two-way ANOVA tests). Therefore, calculations of average processivity included both of these sets of data. Signal quantification, non-linear regression analyses, and statistical calculations (average,

standard error, and Student's t-Test and two-way ANOVA calculations of statistical significance) were performed using ImageQuant, GraphPad Prism and Excel software.

4.4.11. Pulse-chase direct primer extension assay

Pulse chase telomerase assays were performed as previously described using 20 µl RRL-reconstituted telomerases in a final reaction volume of 40 µl (Moriarty et al. 2004).

4.5. Results

4.5.1. A true DAT mutant?

The direct primer extension telomerase assay reveals at least two classes of catalytically-impaired telomerases whose defects cannot be identified using the PCR-based TRAP assay: 1) highly non-processive enzymes whose extremely short products cannot be amplified in TRAP reactions; and 2) enzymes that exhibit nearly wild-type levels of telomerase activity in the TRAP assay, but are only weakly active in the direct primer extension assay (Huard et al. 2003; Moriarty et al. 2004). We and others have found that almost all mutations in conserved N- and C-terminal hTERT regions cause severe impairment of telomerase's ability to elongate telomeric primers in the direct primer extension assay (Huard et al. 2003; Lee et al. 2003; Moriarty et al. 2004). One exception was a mutation in the N-DAT region (Δ110-119), which did not impair the ability of *in vitro*-reconstituted mutant telomerase to elongate 18 or 24 nucleotide (nt) telomeric primers in the direct primer extension assay (Figures 4.1B and 4.3). A polyclonal SV40-transformed human embryonic kidney HA5 cell line stably expressing this mutant exhibited proliferation and telomere length maintenance defects similar to a cell line expressing vector alone (Figures 4.1C and E), though hTERT protein and mRNA levels were similar to those in cells stably expressing wild-type (WT) hTERT (Figure 4.1F). Telomerase activity in cells expressing hTERTΔ110-119 was slightly reduced compared to cells expressing WT hTERT, as measured using the TRAP assay and the standard TRAP TS primer (TS-GTT) (Figure 4.1D). The Δ110-119 mutant exhibited WT levels of telomerase activity when expressed in *S. cerevisiae*, rabbit reticulocyte lysates (RRL), or transiently in HA5 cells (Appendix 2) (Moriarty et al. 2002b). Therefore, we

initially concluded that hTERTΔ110-119 was likely a true DAT mutant that dissociates the *in vitro* and *in vivo* activities of telomerase (Armbruster et al. 2001).

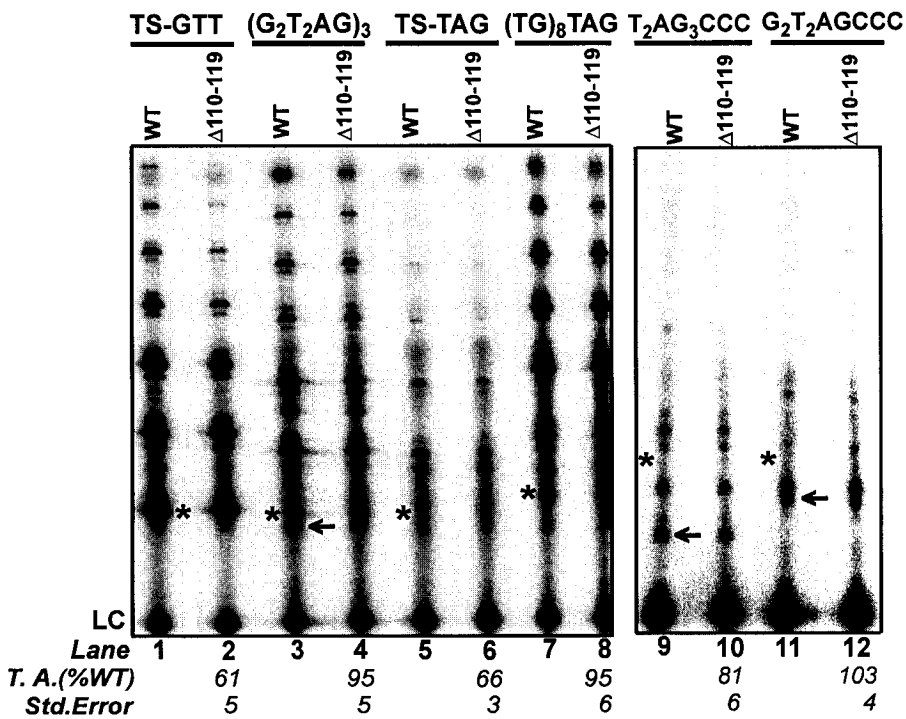
4.5.2. Catalytic defects of an N-DAT mutant on DNA substrates with non-telomeric 5' sequences

Altering the sequence of telomeric DNA substrates affects the catalytic activity of endogenous telomerases from diverse organisms, implying that telomerase has a sequence-specific affinity for its substrate (reviewed in Collins 1999; Harrington 2003; Lue 2004). We tested the activity of *in vitro*-reconstituted WT and Δ110-119 telomerases using primers containing non-telomeric sequences, and found that the N-DAT mutant exhibited catalytic defects on partially telomeric substrates, especially those with non-telomeric 5' sequences (Figure 4.2 and Supplemental Figure 4.5). Catalytic defects could be detected visually by comparing the abundance of longer products synthesized by WT and mutant telomerases; these observations were confirmed by measurement of telomerase activity values for different primers in multiple independent experiments (Figure 4.2 and Supplemental Figure 4.5). The expression levels of WT, Δ110-119 and other mutant hTERTs analyzed in this study varied by less than 10% in multiple independent experiments, indicating that the catalytic defects of hTERT mutants was not caused by differences in protein expression.

Primers with 5' 'backbones' consisting of vertebrate telomeric or G-rich sequences were efficiently elongated by the N-DAT mutant telomerase (Figure 4.2: $(G_2T_2AG)_3$ and $(TG)_8TAG$); however this mutant elongated TS-type primers containing entirely non-telomeric 5' sequences and a telomeric 3' end less efficiently than WT telomerase (Figure 4.2: TS-GTT, TS-TAG). The elongation defect of the Δ110-119 mutant on TS-type primers was similar for three distinct primers predicted to hybridize with the hTR template at different positions (TS-GTT, TS-TAG and TS-GGG) (Figure 4.2; data not shown), suggesting that this N-DAT mutation may not affect formation of the primer/template hybrid. A primer containing an entirely telomeric 5' backbone and three non-telomeric residues at the primer 3' end was elongated with similar efficiency by WT and Δ110-119 telomerases (Figure 4.2: G_2T_2AGCCC), supporting the hypothesis that the N-DAT mutant's catalytic defect was primarily dependent on 5' substrate sequences,

Figure 4.2: An N-DAT mutant exhibits elongation defects on DNA substrates with non-telomeric 5' sequences

Elongation of a telomeric substrate $(G_2T_2AG)_3$ and DNA primers containing: 1) a non-telomeric 'TS' 5' backbone and telomeric 3' sequences (TS-GTT and TS-TAG); and 2) a telomeric 5' backbone and non-telomeric 3' sequences (T_2AG_3CCC and G_2T_2AGCCC) by RRL-reconstituted wild-type (WT) and N-DAT mutant (Δ110-119) telomerases, as detected by a direct primer extension assay. The $(TG)_8TAG$ primer 5' backbone is composed of G-rich non-vertebrate telomeric sequences. All primers were 18-19 nt in length. Average relative telomerase activity (T.A.) and repeat addition processivity (R.A.P.) values calculated from four independent experiments are indicated at bottom. LC: loading control. Asterisks indicate the positions of products resulting from the addition of one nucleotide to each DNA substrate. Arrows indicate the shortest cleavage-derived elongation products visible on these gels.



Primer	Length	Telomeric backbone	5' sequence (5'-3')	3' sequence (5'-3')
TS-GTT	18 nt	no	AATCCGTCGAGCAGA	GTT
$(G_2T_2AG)_3$	18 nt	yes	GGTTAGGGTTAGGGT	TAG
TS-TAG	18 nt	no	AATCCGTCGAGCAGA	TAG
$(TG)_8TAG$	19 nt	non-vertebrate	TGTGTGTGTGTGTGTG	TAG
T_2AG_3CCC	18 nt	yes	TTAGGGTTAGGGTTA	CCC
G_2T_2AGCCC	18 nt	yes	GGTTAGGGTTAGGGT	CCC

and not on hybridization with the hTR template. DAT mutant-mediated elongation of a second primer consisting of a different permutation of the telomeric 5' backbone, and the same non-telomeric 3' residues, was 20% less efficient than that of WT enzyme (Figure 4.2: T_2AG_3CCC). Though both of these primers were the same length, the cleavage-derived elongation products generated with these substrates were shorter for T_2AG_3CCC than G_2T_2AGCCC, implying that the T_2AG_3CCC primer was more extensively cleaved prior to elongation (Figure 4.2). Similarly, when we examined the elongation of two 19 nt partially telomeric substrates containing a single internal telomeric repeat cassette at different positions, we found that the substrate that generated shorter cleavage-derived products was elongated less efficiently by Δ110-119 telomerase (Supplemental Figure 4.5, lanes 7-8 and 11-12). These observations suggested that differences in elongation efficiency might be due to primer length (see Figure 4.3). The DAT mutation did not appear to affect cleavage of substrates prior to elongation, since products shorter than the input primer were similar to those generated by WT enzyme (Figure 4.2 and Supplemental Figure 4.5). The DAT mutant's activity on a telomeric substrate containing a single nucleotide 3' mismatch insertion was also slightly reduced compared to WT enzyme, but this inhibition was less pronounced than the defects observed for primers with non-telomeric 5' backbones (compare Supplemental Figure 4.5, lanes 3 and 4 to Figure 4.2, lanes 2 and 6).

Fusion of hTRF2 or hPot1 to certain N-DAT mutants partially or fully rescues the limited proliferation phenotype of cells expressing these variants, and hPOT1 regulates telomerase activity on telomeric substrates (Armbruster et al. 2003; Armbruster et al. 2004; Kelleher et al. 2005). The telomerase-associated protein Est1p also stimulates the catalytic function of *C. albicans* telomerase on short substrates and primers containing non-telomeric 5' sequences, and can rescue the proliferation defects of *S. cerevisiae* cells expressing Est2p proteins with mutations in the RID1 region (Friedman et al. 2003; Singh and Lue 2003). Coexpression of telomere-binding proteins hTRF2 or hPOT1, or the hTERT-interacting human Est1p orthologues hEST1A or hEST1B, did not rescue the N-DAT mutant's TS-TAG substrate elongation defect (Supplemental Figure 4.6) (Snow et al. 2003). The Δ110-119 variant coimmunoprecipitated hEST1A and hEST1B as efficiently as WT hTERT in the presence of hTR (data not shown). Furthermore,

coexpression of a catalytically dead hTERT RT mutant (D868N) or the hTERT RID1 domain did not improve the N-DAT mutant's ability to elongate the TS-TAG primer, though we and others have shown that functionally complementing hTERT molecules can restore processive telomerase activity to other RID1 mutants (Supplemental Figure 4.6) (Beattie et al. 2001; Moriarty et al. 2004). This result suggests that more than one copy of the N-DAT region or RID1 domain may be required for elongation of substrates with non-telomeric 5' ends, or that elongation of partially telomeric substrates may require cis interactions between hTERT's polymerase and RID1 accessory domains (Moriarty et al. 2004).

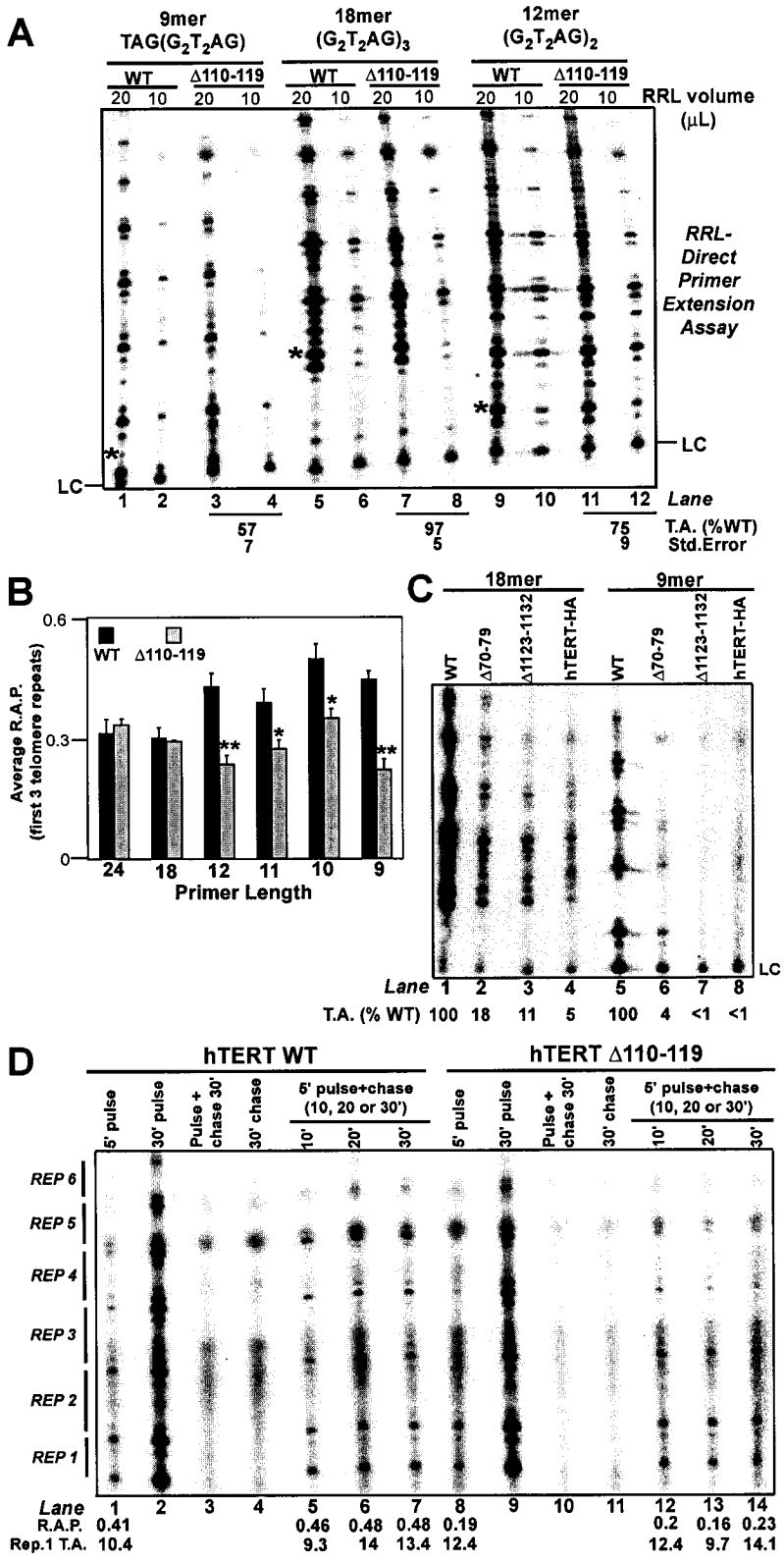
4.5.3. Substrate length-dependent repeat addition processivity and activity defects of an N-DAT mutant

Anchor site-type interactions with the 5' end of DNA substrates are thought to be facilitated by the presence of G-rich sequences. Since the telomerase activity of the Δ110-119 mutant appeared to be dependent on the presence of G-rich 5' sequences in DNA substrates, we investigated its ability to elongate entirely telomeric primers consisting of identical 3' sequences and 5' ends of varying lengths (shortened 5' ends) (Figure 4.3). Previous studies have shown that endogenous human telomerases elongates 12 nt telomeric primers more efficiently than longer 18 nt telomeric substrates, and that the catalytic rate and activity of *Tetrahymena* and *Oxytricha* telomerases are enhanced on substrates containing two or fewer telomere repeats (Morin 1989; Zahler et al. 1991; Lee and Blackburn 1993). It has been proposed that elongation of such short telomeric substrates is regulated by interaction with a template-proximal anchor site (Lue and Peng 1998; Collins 1999). Similar to these previous observations, quantitative analysis of repeat addition processivity within the first three telomere repeats synthesized by human telomerase indicated that WT telomerase was more processive on short (9-12 nt) primers than on longer (18-24 nt) substrates (Figure 4.3B). Repeat addition processivity values between the first and second, and second and third telomere repeats were similar to each other, and both values were included in all calculations shown in Figure 4.3.

WT and Δ110-119 telomerases exhibited similar levels of processivity on 24 and 18 nt primers (Figure 4.3B). However, the Δ110-119 variant did not exhibit the enhanced

Figure 4.3: N-DAT, C-DAT and hTERT-HA variants exhibit elongation defects on telomeric DNA substrates shortened at the 5' end.

WT, N-DAT (hTERTΔ70-79, Δ110-119), C-DAT (hTERTΔ1123-1132) and hTERT-HA mutant telomerase activities on short telomeric DNA substrates. Primers contained identical 3' sequences and progressively shortened 5' ends. Primer sequences: 24mer $(G_2T_2AG)_4$, 18mer $(G_2T_2AG)_3$, 12mer $(G_2T_2AG)_2$, 11mer $GT_2AG(G_2T_2AG)$, 10mer $T_2AG(G_2T_2AG)$, 9mer $TAG(G_2T_2AG)$. In (*A* and *C*), the telomerase activity (T.A.) values of hTERT mutants expressed relative to WT telomerase are indicated below each panel. LC: loading control. **(A)** Autoradiograph showing elongation of three representative telomeric primers of different lengths by WT and Δ110-119 telomerases. Asterisks indicate the positions of products resulting from the addition of one nucleotide to primer substrates. **(B)** Graphical summary of the repeat addition processivity (R.A.P.) of WT and Δ110-119 telomerases on short primers. Average R.A.P. values are expressed in arbitrary units, and standard error bars for each value are indicated; R.A.P. calculations are described in detail in Materials and Methods. Average R.A.P. values were calculated from four independent experiments, each of which included direct primer extension assays performed with 10 and 20 μl RRL-reconstituted telomerase; therefore, statistical calculations for each primer include R.A.P. data from 8 samples. Δ110-119 mean processivity values that differed significantly from WT mean processivity values are indicated by * ($0.05<p<0.01$) or ** ($p<0.01$). **(C)** Autoradiograph depicting elongation of 18 and 9 nt telomeric primers by Δ70-79, Δ1123-1132 and hTERT-HA telomerases. **(D)** Pulse-chase time course experiments. Pulse primer: 1 μM biotinylated 9mer $TAG(G_2T_2AG)$. Chase primer: 150 μM non-biotinylated $(TTAGGG)_3$. Control 1 (lanes 3 and 10): pulse and chase primers added simultaneously to demonstrate the efficiency of chase conditions. Control 2 (lanes 4 and 11): reactions performed with chase primer alone to demonstrate that non-biotinylated chase primer elongation products are not purified by streptavidin beads. Repeat addition processivity (R.A.P.) values and first repeat telomerase activity values (Rep. 1 T.A.) for individual samples (expressed in arbitrary units) are indicated at the bottom of each lane.


A
9mer
TAG(G2T2AG)
18mer
(G2T2AG)3
12mer
(G2T2AG)2
WT
Δ110-119
20 10 20 10 20 10 20 10 20 10 20 10
RRL volume (μL)
RRL-Direct Primer Extension Assay
LC
1 2 3 4 5 6 7 8 9 10 11 12 Lane
57 7
97 5
75 9
T.A. (%WT) Std.Error
B
Average R.A.P. (first 3 telomere repeats)
WT Δ110-119
0.6
0.3
0
24 18 12 11 10 9
Primer Length
C
18mer
9mer
WT
Δ70-79
Δ1123-1132
hTERT-HA
LC
Lane 1 2 3 4 5 6 7 8
T.A. (% WT) 100 18 11 5 100 4 <1 <1
D
hTERT WT
hTERT Δ110-119
5' pulse
30' pulse
Pulse + chase 30'
30' chase
5' pulse+chase (10, 20 or 30')
10' 20' 30'
REP 6
REP 5
REP 4
REP 3
REP 2
REP 1
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14
R.A.P. 0.41 0.46 0.48 0.48 0.19 0.2 0.16 0.23
Rep.1 T.A. 10.4 9.3 14 13.4 12.4 12.4 9.7 14.1

repeat addition processivity observed for WT telomerase on substrates 12 nt and shorter (Figure 4.3B), suggesting that this mutation disrupts the activity-stimulating function of the template-proximal anchor site. After the addition of three repeats to short primers, the DAT mutant became as processive as WT telomerase on most short primers (93, 89, 139, 137, 87 and 27% of WT repeat addition processivity on primers 24, 18, 12, 11, 10 and 9 nt in length, respectively), possibly because it elongated longer products more efficiently, or because telomerase entered a new mode of elongation following the addition of the first three repeats (Melek et al. 1994); therefore, we did not include processivity values for products longer than three repeats in the data summarized in Figure 4.3.

Pulse-chase telomerase assays indicated that the Δ110-119 mutant added the first repeat of telomeric DNA to a 9 nt substrate as efficiently as WT enzyme (Figure 4.3D, compare first repeat telomerase activity values), but synthesized fewer products containing multiple telomere repeats, implying a specific defect in repeat addition processivity on short primers (Figure 4.3D). As we have previously observed, WT telomerase exhibited quite limited repeat addition processivity over time in pulse-chase time course assays, as longer products were not detected after the first 5 minutes of chase conditions (Moriarty et al. 2004). This may indicate that recombinant human telomerase reconstituted in RRL is less processive or less stable than endogenous enzyme, or that human telomerase is less processive than previously assumed. Pulse-labeling of telomerase products for fewer than 5 minutes might have facilitated detection of chased longer products; however, we could not perform pulse-labeling for fewer than 5 minutes due to the reduced activity of WT and mutant telomerases on 9 nt primers.

It has previously been demonstrated that *Euplotes* telomerase forms an anchor site-type cross-link to both ssDNA and dsDNA substrates, implying that telomerase may also interact with double-stranded DNA (Hammond et al. 1997). Furthermore, the isolated RID1 region of Est2p can interact with both ssDNA and dsDNA (Xia et al. 2000). Since the DAT mutant was fully active and processive on telomeric and partially-telomeric G-rich primers 18 nt and longer (Figures 4.1 to 4.3), we hypothesized that extending the 5' length of short substrates by the addition of duplex sequences might also restore WT levels of activity. Non-telomeric double-stranded substrates with telomeric 3' overhangs of varying lengths were generated by adapting an oligonucleotide annealing

technique previously used to examine the activity of *Tetrahymena* telomerase on 3' overhang DNA; a variation of this method has also been used recently to examine the 3' overhang length requirements of recombinant and endogenous human telomerases in the PCR-based TRAP assay (Supplemental Figure 4.7) (Wang and Blackburn 1997; Rivera and Blackburn 2004). Using the direct primer extension assay, we found that WT telomerase could elongate dsDNA substrates containing 3' overhangs as short as 7 nt; interestingly, we also observed that the DAT mutant elongated substrates with telomeric overhangs 12 nt and shorter as efficiently as WT telomerase (Supplemental Figure 4.7). Similarly, extending the length of 5' sequences also stimulates the ability of *Tetrahymena* telomerase to elongate entirely non-telomeric substrates (Wang and Blackburn 1997). These observations suggest that extending the length of 5' sequences by adding either ssDNA or dsDNA sequences (Figures 4.1 to 4.3; Supplemental Figure 4.7) might engage a potential template-distal anchor site or dsDNA interaction site that could enhance the affinity or stabilize the association of telomerase with suboptimal DNA substrates.

4.5.4. Catalytic defects of other N-DAT, C-DAT and hTERT-HA variants on short DNA substrates

Since the N-DAT mutant characterized in these experiments clearly exhibited catalytic defects on short telomeric primers, we investigated the effect of primer length on the telomerase activities of other DAT-type mutants. A C-terminally HA-tagged hTERT variant (hTERT-HA) (Counter et al. 1998), a C-DAT mutant (Δ1123-1132) (Banik et al. 2002; Huard et al. 2003) and a second N-DAT mutant (Δ70-79) (Moriarty et al. 2002b) elongated 18 nt primers less efficiently than WT enzyme (Figure 4.3C). All of these variants were expressed at wild-type levels in RRL (data not shown). The Δ70-79 N-DAT mutant exhibited repeat addition processivity defects in addition to an overall impairment of telomerase activity on 18 nt primers (data not shown). These putative DAT variants were completely or almost completely inactive on 9 nt primers, indicating that they could not elongate a minimal substrate that was sufficient for WT telomerase function (Figure 4.3C). The reduced activity of these variants on both 18 nt and 9 nt telomeric primers prevented us from examining potential anchor site-type catalytic defects using the types of analysis performed for the Δ110-119 mutant (Figure 4.3B).

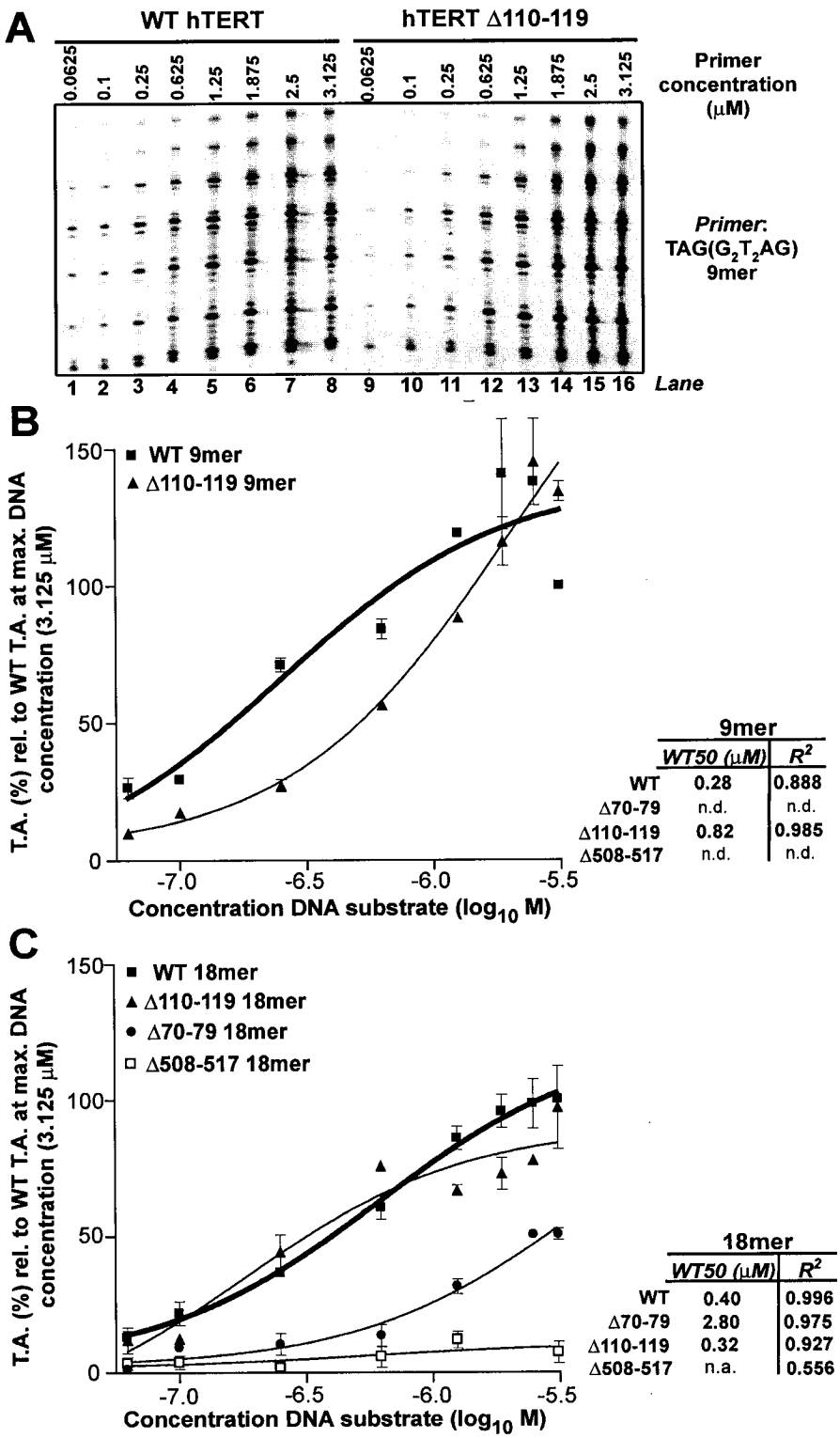
4.5.5. Catalytic defects of N-DAT and C-DAT mutants can be partially or fully rescued by increasing concentrations of DNA substrate

The presence of a template-independent anchor site in telomerase has been deduced not only from analysis of substrate sequences that influence telomerase activity, but also from the observations that 5' primer sequences regulate elongation in a concentration-dependent fashion (Harrington and Greider 1991; Collins and Greider 1993). We therefore examined whether inefficient elongation of telomeric primers by RID1 and C-terminal hTERT variants could be rescued in the presence of increasing concentrations of DNA substrate (Figure 4.4; data not shown; see text below). The telomerase activity of the Δ70-79 mutant improved with increasing concentrations of 18 nt substrate, and the elongation defects of the Δ110-119 variant were rescued at higher concentrations of 9 nt substrate (Figures 4.4B and C). The primer concentration-dependent catalytic phenotype of these mutants was specific, as increasing DNA concentrations did not restore telomerase activity to a completely inactive RNA interaction domain 2 (RID2) mutant (Δ508-517) defective in the ability to assemble with the telomerase RNA (Figure 4.4C) (Moriarty et al. 2002b; Moriarty et al. 2004).

We could not calculate K_m values for different telomerases and primers because the primer concentrations that would be required to reach saturation for more weakly active telomerases exceeded the binding capacity of the streptavidin magnetic beads used in the telomerase assay. Instead, dose response curves plotted from these data were used to estimate the primer concentrations required to achieve 50% of maximum WT telomerase activity. These values were similar for WT and Δ110-119 telomerases on 18 nt primers (Figure 4.4C). In contrast, the Δ110-119 and Δ70-79 mutants required approximately 3-fold and 7-fold greater concentrations of 9mer and 18mer DNA substrate, respectively, to reach 50% of the maximum telomerase activity of WT enzyme (Figures 4.4B and C). An approximately 8-fold greater concentration of 18 nt substrate was required for a C-DAT mutant (Δ1123-1132) to achieve 50% of the activity of WT telomerase on the same primer (data not shown). It has previously been reported that RID1 and the TERT C terminus can physically interact and regulate similar, though not identical catalytic properties, including repeat addition processivity and affinity for the DNA substrate

Figure 4.4: Increasing concentrations of DNA substrate rescue catalytic defects of N-DAT mutants

Elongation activities of WT, N-DAT (hTERTΔ110-119 and Δ70-79) and RID2 (hTERT Δ508-517) telomerases in the presence of different concentrations of telomeric DNA primers. **(A)** Representative autoradiograph showing elongation of a 9 nt telomeric substrate by WT and Δ110-119 telomerases at the indicated primer concentrations. **(B, C)** Graphical summary of the average telomerase activities (T.A.) and standard error (SE) values of WT and indicated mutant telomerases at increasing concentrations of 9 nt (*B*) or 18 nt (*C*) telomeric primers. The dose response curves for WT telomerase are indicated by bold lines. At least two independent repeats of each experiment were performed. In each experiment, the T.A. of WT and mutant telomerases at different concentrations of the same primer were expressed relative to the T.A. of WT enzyme at the maximum primer concentration (3.125 μM, -5.5 on the $\log_{10}$ M scale). Therefore, the WT value at 3.125 μM is 100% (SE is 0). R^2 values for dose response curves calculated by non-linear regression analysis are shown to the right of each graph, as are the primer concentrations at which WT and mutant telomerases were predicted by linear regression analysis to achieve 50% of the maximum measured activity of WT enzyme on the specified substrates (WT50). Note that in (B) WT T.A. at the maximum 9mer primer concentration is lower than the maximum observed T.A. because activity decreased at higher primer concentrations; a similar trend was observed for Δ110-119 telomerase on the same primer. Maximal WT T.A. (140% of T.A. at the maximum primer concentration) was observed at 1.875 μM primer (-5.7 on the $\log_{10}$ M scale); therefore, WT50 values for the 9mer primer were calculated as the primer concentrations at which T.A. was predicted to achieve 70% of WT T.A.. The SE values for Δ110-119 T.A. were very small at lower concentrations of 9mer primer, and are not readily discernible on this graph. In (*C*), the SE bar for Δ110-119 T.A. at the maximum primer concentration overlaps with the WT datapoint at the same primer concentration; average WT T.A. at this concentration had a SE of 0. The Δ508-517 RID2 mutant exhibited activity levels only slightly above background at all primer concentrations, and the WT50 concentration was predicted to be infinite (n.a.). n.d: not determined.


A
WT hTERT
hTERT Δ110-119
0.0625
0.1
0.25
0.625
1.25
1.875
2.5
3.125
Primer concentration (μM)
Primer: TAG(G2T2AG) 9mer
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 Lane
B
WT 9mer
Δ110-119 9mer
T.A. (%) rel. to WT T.A. at max. DNA concentration (3.125 μM)
150
100
50
0
-7.0
-6.5
-6.0
-5.5
Concentration DNA substrate (log10 M)
9mer
WT50 (μM)
R2
WT 0.28 0.888
Δ70-79 n.d. n.d.
Δ110-119 0.82 0.985
Δ508-517 n.d. n.d.
C
WT 18mer
Δ110-119 18mer
Δ70-79 18mer
Δ508-517 18mer
T.A. (%) rel. to WT T.A. at max. DNA concentration (3.125 μM)
150
100
50
0
-7.0
-6.5
-6.0
-5.5
Concentration DNA substrate (log10 M)
18mer
WT50 (μM)
R2
WT 0.40 0.996
Δ70-79 2.80 0.975
Δ110-119 0.32 0.927
Δ508-517 n.a. 0.556

(Beattie et al. 2000; Xia et al. 2000; Peng et al. 2001; Hossain et al. 2002; Huard et al. 2003; Lee et al. 2003; Moriarty et al. 2004). Our observations that hTERT C-terminal variants are profoundly impaired in the ability to elongate short telomeric substrates and exhibit a reduced affinity for DNA substrates suggests that the TERT C-terminus might also contribute to anchor site-type functions, perhaps by cooperative interaction with or regulation of the RID1 domain.

Interestingly, the 3-fold and 7-fold reductions in WT50 telomerase activity values we observed for deletion 110-119 and deletion 70-79 mutant-mediated elongation of 9-mer and 18-mer primers respectively, were comparable to the template-independent reductions in affinity recently measured for human telomerase substrates containing substituted 5' sequences; this previous study indicates that substitution of telomeric sequences 4-8 nt upstream of the 3' end with non-telomeric sequences reduces the affinity of endogenous human telomerase for primer DNA by 2.6-fold, and that substitution of nt 4-18 reduces affinity by 8.3-fold (Wallweber et al. 2003). Increasing substrate concentrations also improved, but did not fully rescue the activity defects caused by a RID1 mutation outside the N-DAT region (Δ150-159); however, this mutant is entirely non-processive, and its predicted WT50 value on 18 nt primers was exceedingly large (data not shown; Moriarty et al. 2004). Collectively, these observations suggested that the RID1 and C-DAT hTERT regions are important for telomerase's affinity for its DNA substrate, and that RID1 contributes to proximal anchor site-type functions in human telomerase.

4.6. Discussion

4.6.1. Is RID1 part of the human telomerase anchor site?

RID1 may contribute to telomerase anchor site interactions with DNA substrates, since deletion of hTERT RID1 generates a completely non-processive enzyme with catalytic defects on telomeric and non-telomeric primers; furthermore, the isolated Est2p RID1 interacts with DNA (Beattie et al. 2000; Xia et al. 2000; Moriarty et al. 2004). This hypothesis is substantiated by our observations that the catalytic activity of an N-DAT RID1 mutant was specifically dependent on the presence of G-rich sequences in the 5' region of DNA primers, and on the length of substrate 5' sequences. Furthermore, this

mutant exhibited specific repeat addition processivity defects on short telomeric primers, and its impaired catalytic phenotype on a short telomeric primer was rescued by increasing DNA substrate concentration. The presence of a template-independent anchor site in telomerase was originally deduced based on the observations that altering the length or G-rich composition of 5' substrate sequences affected the processive elongation of primers in a concentration-dependent fashion (Harrington and Greider 1991; Morin 1991; Collins and Greider 1993). Therefore, we concluded that the catalytic phenotype of the RID1 mutant characterized most extensively in this study is consistent with an anchor site-type defect.

We have been unsuccessful in our attempts to analyze hTERT-DNA interactions by more direct, telomerase activity-independent methods that would permit the examination of anchor site function in other N-DAT and RID1 mutants that exhibit catalytic defects on both long and short primers. However, the sensitivity of these mutants to altered substrate concentration suggests that RID1 sequences in addition to residues 110-119 likely contribute to telomerase's affinity for its DNA substrate. This conclusion is supported by a recent report that DNA concentration affects the ability of a number of other N-DAT mutants to elongate non-telomeric primers (Lee et al. 2003). Collectively, these observations suggest that the entire hTERT RID1 domain may contribute directly or indirectly to interactions with much of the telomerase substrate.

4.6.2. Evidence of a role for a template-proximal anchor site in human telomerase repeat addition processivity

We found that recombinant WT human telomerase, like endogenous human and *Oxytricha* telomerases, was more processive on telomeric substrates containing two or fewer telomere repeats than on primers consisting of 3 or 4 telomere repeats (Morin 1989; Zahler et al. 1991). In *Tetrahymena* telomerase, DNA sequences predicted to interact with the putative template-proximal anchor site also contribute to repeat addition processivity, and the use of short telomeric primers (<12 nt) increases enzyme catalytic rate (Collins and Greider 1993; Lee and Blackburn 1993; Baran et al. 2002). These observations indicate that the putative proximal anchor site-interacting residues of telomeric substrates can stimulate the activities of processive ciliate and human telomerases. In contrast, DNA

sequences predicted to interact with the template-proximal anchor site of the predominantly non-processive *S. cerevisiae* telomerase negatively regulate activity, perhaps as a result of extensive base-pairing interactions between the TR template and DNA substrate that partly constrain repeat addition processivity (Lue and Peng 1998; Bosoy and Lue 2004b).

Oxytricha telomerase activity is reduced on certain long primers as a result of conformational changes in the substrates themselves; however, Lee et al. have demonstrated that the enhanced catalytic rate of *Tetrahymena* telomerase on short substrates is unlikely to be due to such conformational changes (Zahler et al. 1991; Lee and Blackburn 1993). This implies that stimulation of activity may be attributable to telomerase interactions with template-proximal DNA sequences. The hypothesis that upregulation of telomerase activity on short telomeric substrates is dependent on the enzyme itself is supported by our observation that mutation of hTERT RID1 significantly reduced the enhanced repeat addition processivity of human telomerase on short telomeric substrates but did not affect processive elongation of longer primers. These data imply that RID1 residues 110-119 may regulate template-proximal anchor site-type functions in human telomerase, a conclusion that is supported by the reduced affinity of this mutant for short DNA substrates, and by its sensitivity to the presence of G-rich 5' sequences in primers. Interestingly, our observation that human telomerase preferentially elongates short telomeric substrates complements recent data indicating that hPOT1 binding to internal sites in telomeric primers also stimulates telomerase processivity, likely by restricting the length of primer sequences available for interaction with telomerase (Lei et al. 2005). Thus, hPOT1 might promote telomere elongation *in vivo* by regulating the exposure of substrate sequences that can interact with the template-proximal anchor site of telomerase.

hTERT RID1 mutations, including deletion of residues 110-119, impair both hTR interactions and affinity for telomeric substrates, and RID1 associates with the hTR pseudoknot/template domain (this study; Moriarty et al. 2002b; Moriarty et al. 2004). At present, we cannot determine if hTERT RID1's affinity for the DNA substrate requires the presence of hTR. However, the observations that cross-linking of DNA substrates to the anchor site of *Euplotes* TERT is dependent on TR, and that Est2p RID1 interacts with

both TR and DNA, suggest that the telomerase anchor site may not be entirely TERT-dependent (Hammond et al. 1997; Xia et al. 2000). DNA sequences predicted to interact with the template-proximal anchor site could regulate conformational changes in the enzyme-substrate complex that facilitate alignment and/or positioning of the template/primer duplex in the catalytic active site, or unwinding of RNA/DNA duplexes during translocation (Lee and Blackburn 1993; Lue and Peng 1998; Baran et al. 2002). In ciliates, 5' substrate sequences regulate default positioning of the template in the active site (Yu and Blackburn 1991; Melek et al. 1994; Melek et al. 1996; Wang and Blackburn 1997; Wang et al. 1998). A repeat addition processivity- and activity-stimulating template recognition element (TRE) in the *Tetrahymena* TR that interacts with TERT also regulates default template positioning, and is proposed to form sequence-specific contacts with the DNA substrate and/or other regions of the telomerase ribonucleoprotein (Licht and Collins 1999; Lai et al. 2001; Miller and Collins 2002). Thus, hTERT RID1 might mediate repeat addition processivity by bridging or regulating interactions between the TR template and DNA 3' end.

4.6.3. Catalytic phenotypes of DAT mutants

Data reported here and previously collectively indicate that the C-DAT region and most N-DAT sequences are important for human telomerase catalytic function, especially when telomerase activity is measured using the direct primer extension assay or modified TRAP assays performed with entirely non-telomeric primers (this study; Armbruster et al. 2001; Huard et al. 2003; Lee et al. 2003). We found that the telomerase activity of most DAT-type variants was profoundly reduced on short telomeric primers. Since catalytic impairment on short primers might account for the inability of these mutants to maintain telomere length, it will be important to determine the length of the telomeric substrate accessible to human telomerase *in vivo*. The effects of N-DAT mutations spanning amino acids 86-91, 98-109 and 128-133 on telomerase activity have not been examined by these more stringent activity assays; however, since these sequences are short and interspersed among regions known to be catalytically important, we propose that all N-DAT sequences likely contribute to telomerase catalytic function. This conclusion does not preclude the possibility that N- and C-DAT regions have biological functions in addition

to their role in catalysis, as yeast and human N-DAT and C-DAT TERT sequences may interact directly or indirectly with proteins that could recruit or activate telomerase at the telomere, including Est1p, Est3p, and hnRNPs A1 and C1/C2 (Ford et al. 2000; Friedman et al. 2003; Lee et al. 2003; Singh and Lue 2003).

4.6.4. Conclusions

RID1 is important for repeat addition processivity, anchor site-type sequence- and length-specific affinity for the DNA substrate, and interactions with the TR pseudoknot/template domain, ssDNA and dsDNA (this study) (Xia et al. 2000; Beattie et al. 2001; Lee et al. 2003; Moriarty et al. 2004). Further characterization of this fascinating, telomerase-specific domain will be essential for our understanding of telomerase's unique catalytic properties.

4.7. Acknowledgements

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4.8. Supplemental Material

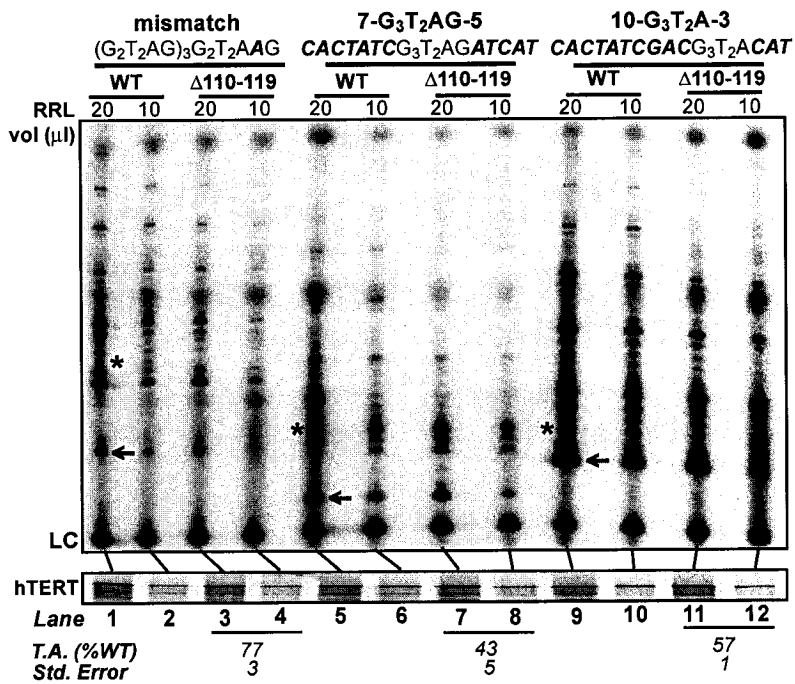
4.8.1. Materials and methods

Direct primer extension assays shown in Supplemental Figures 4.5-4.7 were performed as described in the primary text. Assays described in Figure 4.7 were performed as follows. 40 pmoles TAATcBio primer, with ^{32}P-5'-end-labeled TAATcBio primer added as a trace primer, were incubated with 40 pmoles unlabeled TAAT18mer, TAAT12mer, TAAT11mer, TAAT10mer, TAAT9mer, TAAT8mer or TAAT7mer in a

10 μl final volume of Oligo Anneal Buffer (10 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM $MgCl_2$). Primer sequences are described in Supplemental Table 4.1. Primer mixtures were denatured at 95°C for 5 minutes, allowed to cool in the temperature block for 30 minutes after turning off the heat, then stored on ice before the direct primer extension assay. To test the efficiency of double-stranded product formation, electrophoretic mobility shift assays were performed with 0.5 μl aliquots of this mixture before each direct primer extension assay. Mixtures were electrophoresed on 12% non-denaturing acrylamide gels containing 50 mM Tris pH 8.3, 50 mM boric acid and 1 mM $MgCl_2$, in a running buffer of similar composition for 3.5 h at 10 mA. Direct primer extension assays were performed with the remainder of each annealing reaction, as described in the main text, using 20 μl of RRL sample in a final reaction volume of 40 μl. The 3' terminus of the TAATcBio primer used to form the double-stranded portion of overhang substrates was coupled to a biotin residue, preventing telomerase-mediated elongation of this primer. TAAT primers containing telomeric sequences that formed the 3' overhang did not contain a biotin residue, and could only be purified from telomerase reaction mixtures in complex with the biotinylated TAATcBio primer.

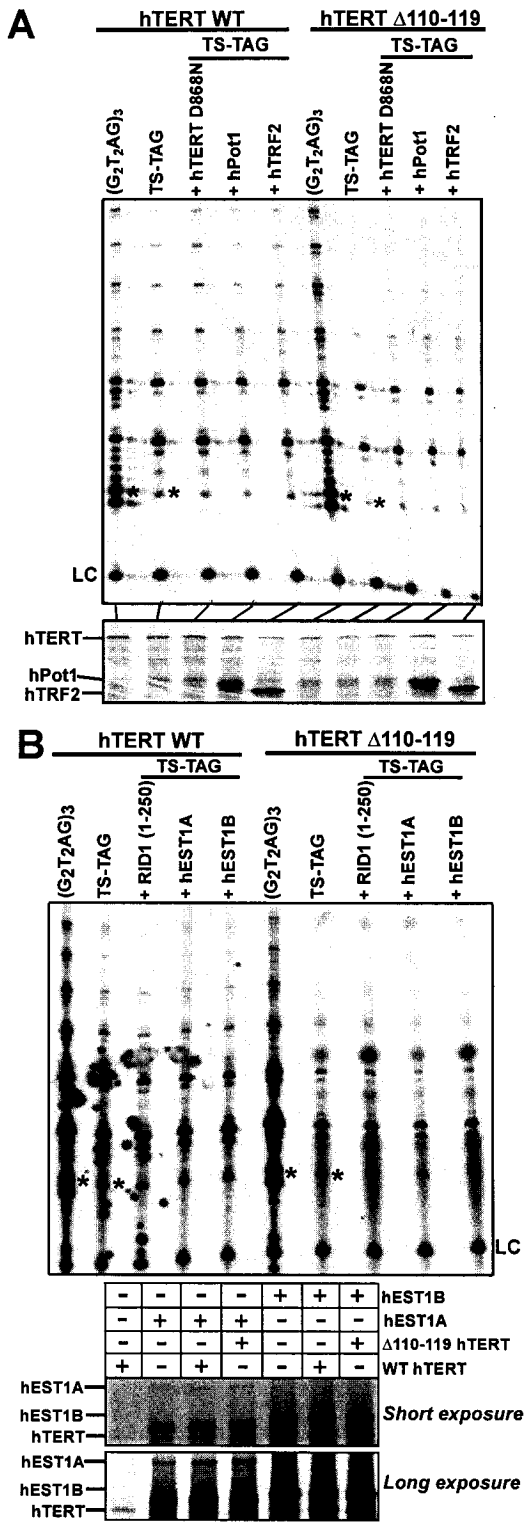
Supplemental Figure 4.5: Elongation of additional partially telomeric primers by Δ110-119 and WT telomerases

Elongation of various primers by RRL-reconstituted wild-type (WT) and N-DAT mutant (Δ110-119) telomerases, as detected by a direct primer extension assay. Primer sequences are shown above the top panel, with non-telomeric sequences indicated in italics. LC: loading control. Asterisks indicate the positions of products resulting from the addition of one nucleotide to each DNA substrate. Arrows indicate the shortest cleavage-derived elongation products visible on these gels. Top panel: direct primer extension assays. Middle panel: expression of ^{35}S-labeled hTERT in RRL samples. Bottom: hTERTΔ110-119 average T.A. values expressed relative to activity values for WT hTERT reactions performed using the same RRL volume. Relative T.A. values were similar for reactions performed with 10 or 20 μl RRL-reconstituted telomerase. Average values were calculated from three independent experiments.


mismatch
(G2T2AG)3G2T2AAG
7-G3T2AG-5
CACTATCG3T2AGATCAT
10-G3T2A-3
CACTATCGACG3T2ACAT
WT
Δ110-119
WT
Δ110-119
WT
Δ110-119
RRL
vol (μl)
20 10 20 10 20 10 20 10 20 10 20 10
LC
hTERT
Lane 1 2 3 4 5 6 7 8 9 10 11 12
T.A. (%WT)
77
43
57
Std. Error
3
5
1

Supplemental Figure 4.6: Inefficient elongation of non-telomeric primers by Δ110-119 telomerase is not rescued by co-expression with hEST1A, hEST1B, hPOT1, hTRF2, hTERT D868N or hTERT RID1.

(A, B) hTERT D868N, hPOT1 or hTRF2 (*A*) and hTERT RID1, hEST1A or hEST1B (*B*) were co-expressed with WT or hTERT Δ110-119 telomerase in RRL, and elongation of the partially telomeric primer TS-TAG in these mixtures was examined. Expression levels of ^{35}S-labeled proteins cosynthesized in RRL are shown in lower panels.

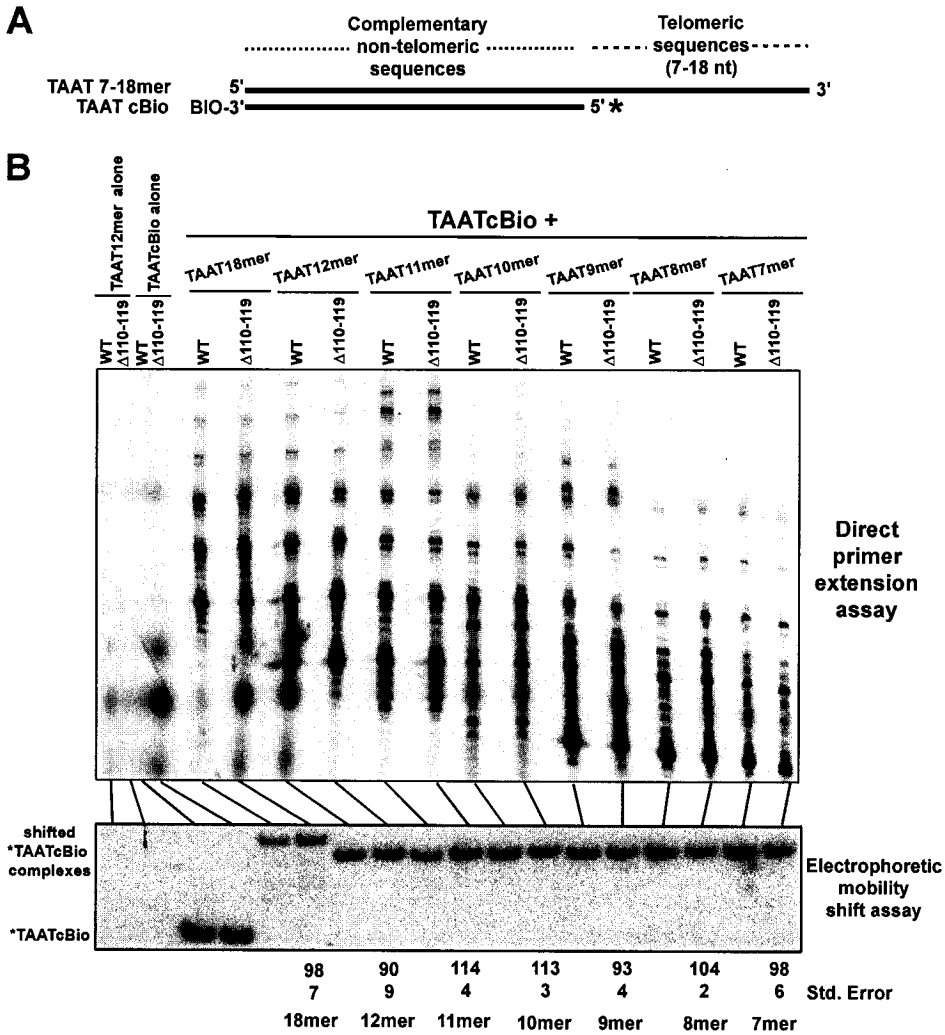

A
hTERT WT
hTERT Δ110-119
TS-TAG
TS-TAG
(G2T2AG)3
TS-TAG
+ hTERT D868N
+ hPot1
+ hTRF2
(G2T2AG)3
TS-TAG
+ hTERT D868N
+ hPot1
+ hTRF2
LC
hTERT
hPot1
hTRF2
B
hTERT WT
hTERT Δ110-119
TS-TAG
TS-TAG
(G2T2AG)3
TS-TAG
+ RID1 (1-250)
+ hEST1A
+ hEST1B
(G2T2AG)3
TS-TAG
+ RID1 (1-250)
+ hEST1A
+ hEST1B
LC
hEST1B
hEST1A
Δ110-119 hTERT
WT hTERT
hEST1A
hEST1B
hTERT
Short exposure
hEST1A
hEST1B
hTERT
Long exposure

Supplemental Figure 4.7: Elongation of double-stranded substrates with single-stranded 3' overhangs of varying lengths by Δ110-119 and WT telomerases.

(A) Schematic depicting the strategy used to generate double-stranded substrates with single-stranded 3' overhangs. The 5' end of the TAATcBio oligo is ^{32}P-end-labeled, as indicated by the asterisk. Primer sequences are described in Supplemental Table 4.1. **(B)** Direct primer extension telomerase assays performed with WT and Δ110-119 telomerases and double-stranded substrates containing telomeric 3' overhangs of the indicated lengths (upper panel). The final concentration of overhang-containing substrate used in these assays was the same as the final concentration of single-stranded substrates used in experiments described in Figures 4.1-4.4 and Supplemental Figures 4.5 and 4.6 (1 μM). Controls: TAAT12mer alone (demonstrating that non-biotinylated primers elongated by telomerase in the absence of annealed TAATcBio sequences were not purified by streptavidin beads), TAATcBio alone (demonstrating that this primer is not elongated by telomerase). The efficiency of double-stranded substrate formation in each sample was monitored by examining the electrophoretic mobility of trace-labeled TAATcBio primer in the presence or absence of TAAT7-18mers (electrophoretic mobility shift assay: lower panel). Average relative telomerase activity (T.A.) values for the Δ110-119 mutant calculated from 2 independent experiments are indicated at bottom.

Supplemental Table 4.1: Sequences of primers used to generate double-stranded substrates with single-stranded 3' overhangs

Primer	*Sequence*
TAATcBio	5'-ATTATTTTCTTAAATTAAACAAACT-3'-Bio
TAAT18mer	5'-AGTTTGTTTAATTTAAGAAAATAATTTAGGGTTAGGGTTAGGG-3'
TAAT12mer	5'-AGTTTGTTTAATTTAAGAAAATAATTTAGGGTTAGGG-3'
TAAT11mer	5'-GTTTGTTTAATTTAAGAAAATAATTTAGGGTTAGG-3'
TAAT10mer	5'-GTTTGTTTAATTTAAGAAAATAATTTAGGGTTAG-3'
TAAT9mer	5'-GTTTGTTTAATTTAAGAAAATAATTTAGGGTTA-3'
TAAT8mer	5'-GTTTGTTTAATTTAAGAAAATAATTTAGGGTT-3'
TAAT7mer	5'-GTTTGTTTAATTTAAGAAAATAATTTAGGGT-3'


A
Complementary non-telomeric sequences
Telomeric sequences (7-18 nt)
TAAT 7-18mer 5' 3'
TAAT cBio BIO-3' 5'*
B
TAAT12mer alone
TAATcBio alone
TAATcBio +
TAAT18mer
TAAT12mer
TAAT11mer
TAAT10mer
TAAT9mer
TAAT8mer
TAAT7mer
WT
Δ110-119
Direct primer extension assay
shifted *TAATcBio complexes
*TAATcBio
Electrophoretic mobility shift assay
98 7 18mer
90 9 12mer
114 4 11mer
113 3 10mer
93 4 9mer
104 2 8mer
98 6 7mer
Std. Error

5. CHAPTER 5: Summary and general discussion

The specific aim of this doctoral work was to characterize the roles of the telomerase-specific TERT N terminus in the human telomerase catalytic mechanism and in nucleic acid interactions. In the course of this work, we identified two RNA interaction domains in the hTERT N terminus (RID1 and RID2) that mediated distinct catalytic functions and interacted with different regions of hTR (Chapters 2 and 3). We found that RID2 was functionally inseparable from the hTERT RT motifs and C-terminal sequences, and that in concert these hTERT regions likely constitute a core polymerase domain of hTERT (Chapter 3). In contrast, the physically and functionally separable RID1 domain was not required for basic polymerase function, but was instead essential for the telomerase-specific property of repeat addition processivity (Chapter 3). Our data suggest that RID1 regulates repeat addition processivity at least in part through its interactions with a processivity-determining region of hTR (Chapter 3). We found that RID1 is also important for the affinity of human telomerase for its DNA substrate, likely because it contributes to template-proximal anchor site-type interactions with the 5' end of substrates (Chapter 4). Finally, we found that *trans*-acting RID1 sequences and *trans*-acting hTR molecules could restore repeat addition processivity to entirely non-processive hTERT and hTR mutants, respectively, suggesting that repeat addition processivity may be one of the catalytic properties conferred by human telomerase multimerization (Chapter 3).

5.1. The RID2-P6.1 interaction site and telomerase assembly

In the study described in Chapter 2, we identified TERT-conserved sequences in the hTERT N terminus that reduced hTR interactions by 50% or more when mutated or deleted. We called this region RID2 to distinguish it from a second, more N-terminal RNA interaction domain which caused a smaller reduction in hTR association when mutated (RID1). During the course of our study, a report that defined the boundaries of the high affinity hTR interaction domain of hTERT using truncated hTERT fragments was published; the region identified by this method corresponds well to the sequences we identified as RID2 (Lai et al. 2001). Corresponding sequences in *Tetrahymena* and *S.*

cerevisiae TERTs are also required for high affinity TR interactions, suggesting that RID2 may be essential for telomerase RNP assembly in diverse telomerases (Friedman and Cech 1999; Bryan et al. 2000b; Miller et al. 2000; Lai et al. 2001; Bosoy et al. 2003). Our observation that the hTERT RID2 includes a vertebrate-specific sequence motif (Chapter 2: VSR motif) concurs with previous reports that the *Tetrahymena* TERT high affinity TR interaction domain also includes a ciliate-specific motif (CP2) (Miller et al. 2000), though these motifs are located at different positions in the human and *Tetrahymena* TERT N termini, respectively. This indicates that some sequences in the high affinity TR interaction domains of different TERTs may have co-evolved with their cognate TRs, and may account at least partly for the ability of the otherwise relatively well-conserved TERT RID2 region to assemble with TRs whose distinct structures vary widely in different groups of organisms.

It is now clear that RID2-mediated telomerase assembly in diverse eukaryotes may be an important target for telomerase regulation. Recent reports indicate that the yeast and human PINX1 telomerase inhibitors interact with the TERT RID2; in yeast, PinX1p and TR associations with RID2 are mutually exclusive, whereas in human cells hTR is present in complexes containing hTERT and PINX1 (Banik and Counter 2004; Lin and Blackburn 2004). PINX1 is a nucleolar protein, though human PINX1 also interacts with the negative regulator of telomere length TRF1 at telomeres (Zhou and Lu 2001; Banik and Counter 2004; Lin and Blackburn 2004 and references therein). Since hTERT is sequestered in the nucleolus in normal but not transformed or cancerous human cells, it has been proposed that PINX1 might negatively regulate telomerase assembly by competing with the TR for binding to RID2 (Wong et al. 2002; Lin and Blackburn 2004). Alternatively, PINX1 might also disrupt the RNP structure of assembled telomerase complexes, which could account for its interaction with TRF1 at telomeres, and for its ability to inhibit human telomerase catalytic activity *in vitro* (Zhou and Lu 2001; Banik and Counter 2004).

As has been observed for *Tetrahymena* and yeast TERTs, we found that deletion of small groups of contiguous RID2 residues abolished telomerase catalytic function (Chapter 3) (Friedman and Cech 1999; Bryan et al. 2000b). Substitutions in the hTR P6.1 helix, which has previously been implicated in high affinity vertebrate TERT interactions,

caused an identical catalytic phenotype and prevented hTR association with RID2, implying that RID2-P6.1 interactions may mediate human telomerase assembly (Chapter 3) (Mitchell and Collins 2000; Chen et al. 2002). The isolated *Tetrahymena* TERT high affinity TR interaction domain also interacts with a catalytically important *Tetrahymena*-specific TR stem structure (Helix II) (Lai et al. 2002; O'Connor et al. 2005), indicating that the mode of RID2 association with TR may be conserved, even if the specific elements that it interacts with are not. This hypothesis is supported by recent evidence that a yeast-specific helix formed by long-range base-pairing in the *S. cerevisiae* TR (Stem 2) is required for TERT interactions and telomere length maintenance *in vivo*, though its role in catalysis has not yet been investigated (Livengood et al. 2002; Chappell and Lundblad 2004; Dandjinou et al. 2004; Lin et al. 2004). The minimal high affinity TR interaction domain of yeast TERT has not yet been mapped in isolation from the remainder of TERT. It will be interesting to know if, like *Tetrahymena* and human TERTs, this domain will be sufficient for association with Stem II of the yeast TR, and if it will include yeast-specific sequences that contribute to TR interactions.

The high affinity TR interaction domain of TERT, and Helix II and flanking sequences of the TR regulate 5' template boundary definition in *Tetrahymena* telomerase (Autexier and Greider 1995; Miller et al. 2000; Lai et al. 2002). Furthermore, the 5' boundary-defining element from yeast TRs has been implicated in interactions with an undefined region of *S. cerevisiae* TERT, though this element does not appear to be as important for TERT interactions as Stem 2 (Seto et al. 2003; Lin et al. 2004). These observations suggest the possibility that 5' template boundary definition may be regulated by TERT-TR interactions in all telomerases. We have been unable to determine if the hTR P6.1 helix or hTERT RID2 contribute to 5' template boundary definition, as mutations in these regions abolish human telomerase activity (Chapter 3; Moriarty et al. 2005). The P1b helix regulates 5' template boundary definition in human telomerase, though this structure is not required for high affinity interactions with hTERT (Chen and Greider 2003b; Ly et al. 2004). We have found that disruption of P1b affects template usage and interaction with an hTERT RID1 mutant, suggesting that the association of P1b and RID1 might mediate 5' template boundary definition in human telomerase (Moriarty et al. 2005). Furthermore, this mutant incorporates a non-cognate nucleotide at a product

positions specified by hTR sequences 5' of the template boundary, suggesting that RID1-P1b interactions could regulate 5' template boundary definition (Moriarty et al. 2005). hTERT C-terminal mutants also incorporate elevated levels of the same non-cognate nucleotide; however, C-terminal variants do not exhibit defects in association with P1b, implying that the C terminus prevents incorporation of non-cognate nucleotides by other mechanisms (Moriarty et al. 2005). Further characterization of hTERT sequences that potentially contribute to 5' template boundary definition will likely require partial purification of recombinant telomerase complexes, or possibly the use of recombinant telomerase reconstituted in a system other than rabbit reticulocyte lysates.

All previously identified conserved sequence motifs in RID2 (CP, QFP and T) contribute to high affinity TR association in yeast, ciliate and human TERTs, implying that RID2 sequences may interact with their respective TR stem structures in a cooperative fashion as a single domain. This hypothesis is supported by our own and others' observations that isolated RID2 sequences are sufficient for specific association with the high affinity TERT-interacting TR stem structures in human and *Tetrahymena* telomerases (Chapter 3) (Lai et al. 2002; O'Connor et al. 2005). We found that RID2 was not functionally separable from the hTERT RT motifs and C terminus with respect to catalysis (Chapter 3). However, this does not necessarily imply that RID2 cannot function as an independent domain with respect to TR interactions. To date, TERT-TR interactions in all telomerases have been primarily studied using immunoprecipitation-based assays because it has proved difficult to generate sufficient quantities of soluble, purified protein to examine interactions by more direct methods. An important goal for telomerase structure-function studies in the next few years will be to obtain an extensively purified recombinant form of RID2 that can be used to directly characterize the binding properties of this region in the presence and absence of other TERT sequences. The generation of soluble, purified protein would be useful for structural studies of RID2 in complex with the interacting stem structure of TR, and could also be used to screen small molecule libraries for compounds that could disrupt RID2 interactions with its associated TR helix. Identification of compounds that inhibit RID2-dependent assembly with hTR might be useful for the development of telomerase-specific therapeutic agents for cancer treatment, since disrupting such interactions could mimic the effect of the endogenous telomerase

inhibitor PINX1. Though methods for obtaining extensively purified recombinant hTERT have not yet been developed, two very recent studies have reported the recombinant expression and partial purification of the *Tetrahymena* TERT N-terminus, RID1 and RID2 from *E. coli*, suggesting that the goal of obtaining purifying hTERT N-terminal fragments might indeed be possible (O'Connor et al. 2005; Prathapam et al. 2005).

5.2. The RID1-pseudoknot/template domain interaction site

In Chapter 2, we described a low affinity hTERT N-terminal hTR interaction domain (RID1) identified using a quantitative RNA binding assay and hTERT variants bearing small internal sequence deletions. During the course of our study, another group that mapped TR interaction sites in *Tetrahymena* and human TERTs using N-terminally truncated TERT fragments, found that progressive deletion of RID1 sequences causes a small but visible reduction in the association of TR; substitutions in the *S. cerevisiae* RID1 region also reduce TR interactions, suggesting that RID1 is a conserved site of TR interaction in TERT (Friedman and Cech 1999; Lai et al. 2001) The interaction defects observed for truncated or internally mutated RID1 variants are small compared to the inhibition of TR association that results from deleting RID2 sequences; therefore RID2 has been identified as the major TR interaction site in TERT (Chapter 2) (Lai et al. 2001).

Previous studies also indicated that, like RID2, isolated yeast and human RID1 sequences can interact independently with the TR; in yeast, RID1 association with the TR appears to be relatively non-specific, as antisense TR can interact with RID1, and since non-specific ssDNAs and dsDNA competitively inhibit TR association (Xia et al. 2000; Beattie et al. 2001). Recently, it has been reported that the *Tetrahymena* TERT RID1 region can also interact independently in a low affinity fashion with TR (O'Connor et al. 2005). In Chapter 3, we further investigated the interaction of isolated hTERT RID1 sequences with hTR, and found that it associated with the processivity-regulating hTR pseudoknot/template domain, which had previously been shown to interact with hTERT independently of the P6.1 helix-containing CR4/CR5 domain (Beattie et al. 2000; Mitchell and Collins 2000; Bachand and Autexier 2001). This interaction was likely functionally-specific, as RID1 did not associate with an unrelated *Drosophila* dsRNA, and since the minimal RID1 sequences that were required for interaction with the

pseudoknot/template domain were sufficient to restore repeat addition processivity to a non-processive RID1 mutant. Interestingly, the *Tetrahymena* TERT RID1 region has recently been shown to associate independently and directly with processivity-regulating TR sequences and structures that are distinct from TR structures involved in RID2 interactions (O'Connor et al. 2005). This observation implies that RID1 interactions with processivity-regulating regions of TRs may be functionally conserved.

Curiously, we found that the isolated RID1 domain interacted more strongly with WT hTR than either the isolated RID2 domain or full-length hTERT (Chapter 3). This may indicate that sequences such as RID2 in the intact hTERT molecule compete with RID1 for association with hTR. Alternatively, or additionally, RID1-interacting hTERT sequences such as the putative C-terminal thumb (Chapter 3) might regulate RID1-hTR interactions. The latter hypothesis is especially interesting since our own and others' observations implicate RID1 and the C terminus in the regulation of similar catalytic functions, including repeat addition processivity, affinity for DNA substrates and suppression of incorporation of non-cognate nucleotides (Chapters 3 and 4) (Xia et al. 2000; Hossain et al. 2002; Huard et al. 2003; Lee et al. 2003; Moriarty et al. 2005). Analysis of C-terminally truncated proteins indicates that C-terminal TERT sequences are not required for high affinity TR association in human and *Tetrahymena* TERTs (Beattie et al. 2000; Bachand and Autexier 2001; Lai et al. 2001). However, the isolated *S. cerevisiae* TERT C terminus can interact with RNA/DNA hybrids composed of telomeric and telomere-complementary sequences, suggesting that this region of TERT might associate with the RNA/DNA hybrid formed during reverse transcription of the TR template (Hossain et al. 2002). Furthermore, using competitive quantitative RNA binding assays we have recently found that a small internal C-terminal hTERT sequence deletion significantly enhances the association of hTR with hTERT, suggesting that the C terminus may influence TR interactions indirectly, perhaps by impairing the hTR-interacting functions of RID1 or RID2 (Moriarty et al. 2005). One plausible method for investigating the possible role of hTERT sequences such as the C terminus and RID2 in modulating RID1-hTR interactions would be to perform quantitative RNA binding assays with RID1 and increasing concentrations of competitor hTERT variants. As for RID2, the

generation of soluble, purified recombinant RID1 (and other hTERT domains or subdomains) would be immensely useful for such analyses.

Though we and others have demonstrated that the pseudoknot/template domain of vertebrate TR associates independently with TERT, the hTR sequences or structures mediating this interaction have not yet been defined (Chapter 3) (Beattie et al. 2000; Mitchell and Collins 2000; Bachand and Autexier 2001; Chen and Greider 2003a). As described in greater detail in the introductory literature review of this thesis (section 1.5.3.3), the most likely candidates for this association are the pseudoknot region itself, and structures flanking the template, including helices P1 and P2a.1. The pseudoknot structure and adjacent sequences have been implicated in TERT interactions in yeast and ciliate telomerases, supporting the prediction that the hTR pseudoknot may associate with hTERT (Gilley and Blackburn 1999; Chappell and Lundblad 2004; Lin et al. 2004). Other TERT-interacting regions in the yeast and ciliate TR pseudoknot/template domains include helices that are important for 5' template boundary definition (Lai et al. 2002; Seto et al. 2003; Lin et al. 2004). The observation that the P1b helix regulates 5' template boundary definition in human telomerase (Chen and Greider 2003b) suggests that, as for the 5' boundary-regulating stems of other TRs, P1b may associate with hTERT. We are currently mapping the RID1-interacting sites in the hTR pseudoknot/template domain, and have found that mutation of P1b disrupts hTR interactions with a RID1 mutant (Moriarty et al. 2005). It remains possible, however, that this strategy will not successfully identify all hTERT association sites in hTR, especially if these sites mediate transient interactions. The use of purified recombinant protein in electrophoretic mobility shift or UV cross-linking assays might facilitate such mapping. Alternatively, substitution of uracil residues with 4-thiouracil in target hTR regions followed by UV-crosslinking and immunoprecipitation of telomerase complexes might permit identification of hTR nucleotides that mediate transient hTERT interactions. This strategy has been used successfully to determine that a ciliate-specific loop in the *Tetrahymena* TR associates with TERT (Lai et al. 2003).

5.3. RID1 interactions with the DNA substrate

In Chapter 4, we described catalytic evidence suggesting that hTERT RID1 contributes to telomerase's affinity for telomeric substrates and to template-proximal anchor site-type functions. We have unsuccessfully tried to directly examine DNA interactions with RID1 and full-length hTERT by multiple methods, including electrophoretic mobility shift and supershift assays performed with unpurified and partially purified telomerases, immunoprecipitation-based and non-immunoprecipitation-based UV-crosslinking assays, and a streptavidin pull-down assay designed to detect ^{35}S-labeled proteins that interact with biotinylated DNA substrates (data not shown). Therefore, we cannot positively conclude that hTERT RID1, like the yeast RID1 region, interacts directly with DNA substrates (Xia et al. 2000). We have performed all of these assays with recombinant telomerases and hTERT fragments expressed in RRL because hTERT protein expression levels in human cells are very low, even following transient transfection with plasmids that direct hTERT expression from strong viral promoters (data not shown). The only reports to date that have characterized telomerase-DNA interactions by non-catalytic methods have used TERT fragments expressed and partially purified from *E. coli* (*S. cerevisiae* RID1 and C terminus), or endogenous telomerase partially purified from ciliates in which telomerase is robustly expressed (*Euplotes*) (Hammond et al. 1997; Xia et al. 2000; Hossain et al. 2002). This may suggest that the source and purity of enzyme used in more direct assays might affect detection of DNA interactions. Data from our lab indicate that hTERT RID1 is expressed extremely poorly in *E. coli*. Therefore, an important future goal will be to explore methods for reconstituting and purifying RID1 from other systems such as insect cells that permit greater expression of functional (though predominantly insoluble) hTERT. Alternatively, RID1-mediated interactions could be examined *in vivo*, using a chromatin immunoprecipitation assay recently developed for hTERT, though hTERT-DNA complexes detected by this method might not reflect direct interactions between the enzyme and its DNA substrate (Sharma et al. 2003). Finally, if telomerase-DNA interactions are transient, then telomerase-DNA complexes might be effectively detected by UV cross-linking assays performed with DNA primers substituted with photoreactive

5-iododeoxypyrimidines, or by laser cross-linking methods more suitable for labile nucleoprotein complexes (Hockensmith et al. 1991; Hammond et al. 1997).

It will also be important to examine the role of RID1, and other hTERT sequences, in telomerase function at the telomere itself. Both the yeast and human RID1 regions contain sequences that are essential for telomere length maintenance *in vivo*, but not catalytic function *in vitro* (N-DAT region in hTERT) (Xia et al. 2000; Armbruster et al. 2001). We and others have shown that most N-DAT sequences are in fact catalytically important, especially for elongation of short telomeric primers (Chapter 4; Lee et al. 2003). However, the biological relevance of these observations is difficult to interpret in the absence of crucial information about the length of the 3' overhang substrate that is actually accessible to human telomerase *in vivo*. Furthermore, the mechanism by which the 3' overhang substrate is "revealed" to telomerase is not yet understood. For example, hPOT1 has recently been shown to either inhibit or stimulate human telomerase-mediated elongation of telomeric substrates *in vitro*, in a manner that is dependent on the telomeric repeat permutation at the 3' end of the substrate and the position of hPOT1 binding (Kelleher et al. 2005; Lei et al. 2005). Our data (Chapter 4) suggest that hPOT1 might stimulate telomerase activity under certain conditions by presenting a short substrate that is preferentially elongated by telomerase. However, the mechanisms regulating hPOT1 binding to internal and terminal 3' overhang sequences (and hence presentation of the telomerase substrate) are unknown. We observed that an N-DAT mutant caused only a 50% reduction in repeat addition processivity and 3-fold reduction in affinity for short DNA substrates, though telomere length was as short in cells expressing this mutant as in those expressing vector alone (Chapter 4). If the normal substrate for telomerase is a short 3' overhang presented by hPOT1, then the specific defect of this N-DAT mutant on short substrates might account for its *in vivo* phenotype. Furthermore, it is likely that the lowest concentration of DNA primer used in telomerase activity assays vastly exceeds the concentration of physiological substrates accessible to telomerase in cells. Therefore, even a 3-fold reduction in telomerase's affinity for its DNA substrate or a 50% reduction in processivity might abolish telomerase function *in vivo*. Finally, mice heterozygous for either *mTERT* or *mTR* display haploinsufficiency phenotypes with respect to telomere length, and a substantial body of evidence implicates telomerase haploinsufficiency in

human bone marrow failure syndromes, including dyskeratosis congenita (Hathcock et al. 2002; Erdmann et al. 2004; Ly et al. 2004; Marrone et al. 2004; Yamaguchi et al. 2005, and references therein), suggesting that inhibition of telomerase catalytic activity by as little as 50% might have serious consequences in mammalian cells. Therefore, understanding the physiological context of telomerase activity, including the length of the telomerase substrate and the mechanism by which it is exposed for telomerase-mediated elongation, will be important for interpretation of *in vitro* catalytic defects such as those described in Chapter 4. Another, related goal would be to develop assays that permit direct detection and measurement of the telomeric sequences incorporated into telomeres by WT and mutant human telomerases. This might be achieved by adapting techniques that have been used to clone and sequence individual yeast telomeres, or by examining the incorporation of 'marked' telomeric repeats by telomerases containing a mutated template (Marusic et al. 1997; Forstemann et al. 2000).

5.4. RID1 catalytic functions

In Chapter 3 we found that removal of most hTERT RID1 sequences abolishes the repeat addition processivity of human telomerase without eliminating basic polymerase function. In contrast, deletion of *Tetrahymena* TERT RID1 sequences abolishes telomerase activity (Lai et al. 2001). It is not yet known if, as in *Tetrahymena*, the yeast RID1 is essential for telomerase activity, since the effect of large-scale deletion of RID1 sequences on catalytic function has not been examined in yeast TERT. Only two conserved residues in the *Tetrahymena* RID1 region have been mutagenized; of these, one is essential for telomerase activity, whereas substitution of the second reduces but does not eliminate catalytic function (Miller et al. 2000). Substitution of TERT-conserved residues in the *S. cerevisiae* RID1 region impairs but does not completely abolish telomerase activity, but the mechanistic basis for this catalytic impairment has not yet been reported (Friedman and Cech 1999; Xia et al. 2000). Since the contributions of the RID1 region to the activity of diverse telomerases have not been systematically investigated, it therefore remains difficult to form general conclusions about the catalytic function of this telomerase-specific domain. It is possible that RID1 may confer distinct

functions to different telomerases, since the N-terminal half of this domain is only moderately conserved among TERTs (Bosoy et al. 2003).

In Chapter 3, we proposed that RID1 may be a TERT accessory domain because the hTERT RID1 is functionally and physically separable from the remainder of hTERT, is not required for basic polymerase function, is essential for the telomerase-specific property of repeat addition processivity, and interacts with the putative hTERT C-terminal thumb. Polymerase accessory domains interact with nucleic acids outside the polymerase active site, and are frequently responsible for nuclease and/or proofreading activities and sequence-specific nucleic acid interactions (Sousa 1996; Hubscher et al. 2002). Our data suggesting that hTERT RID1 may interact with telomerase substrate nucleotides outside the active site support the hypothesis that RID1 may constitute an accessory domain, as does our observation that RID1 interacts with the hTR pseudoknot/template domain (Chapters 3 and 4).

The functional similarities we found between RID1 and the accessory domains of other nucleic acid polymerases suggest that RID1 might confer nuclease activity, which is a tightly-associated property of human and other telomerases (Huard and Autexier 2004; Oulton and Harrington 2004, and references therein). Nuclease activity may be important for revealing preferred substrates for elongation, proofreading, or reinitiation of stalled DNA synthesis, as is observed for the nuclease activity of RNA polymerases (Harrington and Greider 1991; Collins and Greider 1993; Melek et al. 1996; Huard and Autexier 2004). To date, telomerase-associated nuclease activity has been monitored by comparing the length of the shortest products synthesized by telomerase to the size of labeled input primers migrated on the same gel; these products are thought to reflect the elongation of substrates that are cleaved prior to elongation. We have not observed obvious differences in the lengths of cleavage-dependent elongation products generated by WT and RID1 mutant telomerases (Chapters 3 and 4); however, this does not preclude the possibility that other RID1 sequences may contribute to nuclease function, or that RID1-dependent nuclease activity could be important at later phases of the telomerase catalytic cycle such as reinitiation of a new round of template reverse transcription following stalling at the 5' boundary of the template (Melek et al. 1996). To date, telomerase-dependent primer cleavage has been detected only as products that migrate more rapidly than input primers

in telomerase activity-based assays, and has not been visualized directly. Since primer cleavage in these cases may be coupled to initiation events, the development of a direct nuclease assay would be useful for examining the possible contribution of nuclease activity to catalytic functions such as proofreading and reinitiation of stalled DNA synthesis.

We found that the hTERT RID1 is essential for the repeat addition processivity of human telomerase, and that a RID1 mutation caused template-proximal anchor site-type catalytic defects; furthermore, several RID1 mutations reduced the affinity of telomerase for its DNA substrates and impaired interactions with hTR (Chapters 2 to 4). It has been proposed that substrate sequences predicted to interact with the template-proximal anchor site of *Tetrahymena* telomerase might be important for alignment or positioning of the template/substrate duplex in the polymerase active site, or for unwinding of RNA/DNA duplexes formed during reverse transcription of the template (Lee and Blackburn 1993). Either or both of these proposed functions could make essential contributions to repeat addition processivity. An alignment or positioning function could facilitate the realignment of the primer and template 3' ends following Type II translocation. A potential helicase function might also permit Type II translocation by disrupting the RNA/DNA hybrid formed during template reverse transcription. In Chapter 3, we proposed that telomerase repeat addition processivity might resemble the strand transfer events required for complete replication of retroviral genomes. Interestingly, recent evidence indicates that a newly identified primer grip in the RNase H accessory domain of the murine leukemia virus RT regulates strand transfer efficiency and accuracy, and positioning of the template-primer in the polymerase active site (Mbisa et al. 2005). Together, these observations suggest the intriguing possibility that RID1 might confer repeat addition processivity by regulating the movement and positioning of DNA substrates with respect to the TR template. This hypothesis may be supported by our recent observations that RID1 mutations affect template usage (Moriarty et al. 2005). Furthermore, a recent review of telomerase repeat addition processivity reports that *S. cerevisiae* TERT RID1 mutations cause catalytic defects consistent with impaired positioning of template/primer duplexes in the active site, though a full description of these data has not yet been published (Lue 2004). Evidently, numerous questions

concerning the catalytic function of RID1 have not yet been addressed. Some of the most important experiments that must be performed include precise mapping of all of the potential RID1 interaction sites in the hTR pseudoknot/template domain and telomeric DNA substrates, perhaps by footprinting techniques performed with purified protein, and investigation of the possibility that RID1 interacts with RNA/DNA duplexes. It will also be important to determine if hTR and DNA substrates can associate simultaneously with RID1, or if they compete for interaction with RID1. Finally, careful analysis of the template usage phenotypes of RID1 mutants will be required to characterize the potential role of this domain in substrate positioning and alignment.

5.5. The human telomerase multimer and catalytic function

In 2001, it was reported that human telomerase contains two functionally cooperating hTR molecules, and is likely to contain two functionally interacting hTERTs (Beattie et al. 2001; Wenz et al. 2001). The conclusions of these studies were supported by our own and others' work demonstrating that hTERT molecules can interact with each other *in vitro* in an hTR-independent fashion, and that hTR can dimerize *in vitro* in the absence of hTERT (Chapters 2 and 3) (Arai et al. 2002; Ly et al. 2003b).Gel filtration of endogenous and recombinant human telomerase complexes suggests that the minimal functional human telomerase likely contains two hTRs and two hTERTs (Wenz et al. 2001). The hypothesis that telomerase is a dimer is supported by reports that functional and physical TR-TR and TERT-TERT interactions also occur in *Euplotes* and *S. cerevisiae* telomerases; however, *Tetrahymena* telomerase is functional as a monomer, and the *Tetrahymena* TERT and TR components do not dimerize *in vitro* (Prescott and Blackburn 1997a; Wang et al. 2002; Bryan et al. 2003). Thus, while there is growing evidence that a number of telomerases may function as dimers, it is clear that dimerization is not a conserved feature of all telomerases. Future work in this field must investigate the molecular basis for differences in the stoichiometry of diverse telomerases, and the possible catalytic or biological functions that are regulated by dimerization.

Previous studies indicate that separately inactive hTERT or hTR variants can functionally complement each others' catalytic defects to restore human telomerase activity (Tesmer et al. 1999; Beattie et al. 2001). In the experiments described in Chapters

2 to 4 of this thesis, we used a functional complementation approach to map hTR and hTERT sequences that can function *in trans*, and to investigate the molecular basis for these interactions and their catalytic function. Our major findings were that RID2, the RT motifs and C terminus function together in the same hTERT molecule, whereas RID1 can function *in trans*, and that *trans*-acting hTR or hTERT molecules can restore repeat addition processivity to completely non-processive enzymes (Chapters 2 and 3). These observations suggested that one of the functions of human telomerase dimerization may be to confer repeat addition processivity. However, all of these experiments were performed using unfractionated recombinant telomerase complexes, and do not therefore constitute proof that telomerase dimerization is essential for human telomerase repeat addition processivity. It will be especially important to repeat a subset of these experiments using enzymes fractionated by gel filtration or other methods, since it has been reported that the monomeric *Tetrahymena* telomerase exhibits moderate repeat addition processivity (Bryan et al. 2003). Furthermore, conditions in crude RRL lysates expressing telomerase components could facilitate *trans* interactions that are maintained only weakly or not at all in isolated enzyme complexes. Therefore, the work described in this thesis constitutes only preliminary steps in investigating the potential catalytic function(s) of human telomerase multimerization.

Dimerization or oligomerization is a feature of many, but not all nucleic acid polymerases, and is especially common among the RTs encoded by retroviruses and retroelements. The function of reverse transcriptase dimerization remains unclear, but in the HIV-1 RT and some retroelement RTs, dimerization may create an extended surface that can interact with regions of the template and primer that are implicated in enzyme-specific functions such as splicing and RNase H activity (Ding et al. 1998; Huang et al. 1998; Blocker et al. 2005). This may also be true of human telomerase, since our data and others' indicate that hTERT N-terminal and C-terminal regions involved in hTERT-hTERT interactions *in vitro* are also those that are important for functional and/or physical association with the TR and DNA substrates (Chapters 2 to 4; Arai et al. 2002). In Chapters 3 and 4, we proposed that an association between RID1 and the hTERT C terminus might cooperatively regulate interactions with hTR and DNA substrates. The association of RID1 and C-terminal sequences has not been examined in intact hTERT

molecules, and it would be useful to investigate such potential interactions in functional RNPs reconstituted in the presence and absence of DNA substrates; such experiments could be performed using fluorescent resonant energy transfer (FRET) or β-lactamase protein fragment complementation techniques that have been successfully used to examine the interaction and orientation of subunits in multimeric protein complexes (Galarneau et al. 2002). The hypothesis that RID1-C-terminal interactions cooperatively regulate nucleic acid interactions might be tested by measuring the association of hTR and DNA substrates with telomerases reconstituted from equimolar mixtures of RID1 and C-terminal hTERT mutants, though analyzing DNA interactions by this method would require the development of direct DNA binding assays. However, none of these approaches can provide a comprehensive, three-dimensional picture of hTERT-nucleic acid interactions, which may not be fully understood until the crystal structures of human telomerase in complex with the TR and DNA substrate have been solved.

Enzymatic probing, chemical modification and nuclear magnetic resonance (NMR) studies of naked ciliate and human TRs *in vitro* suggest that the TR pseudoknot is not stable, and may exist in equilibrium with a hairpin structure; it has been proposed that such conformational changes could be important for the telomerase catalytic mechanism, perhaps by functioning as a molecular switch (Bhattacharyya and Blackburn 1994; Lingner et al. 1994; Zaug and Cech 1995; Antal et al. 2002; Theimer et al. 2003; Theimer et al. 2005). This hypothesis may be especially relevant for hTR, which has been shown to dimerize via CR2 and CR3 sequences that form the vertebrate pseudoknot structure (Ly et al. 2003b). hTR dimerization is predicted to disrupt the pseudoknot, thus revealing or stabilizing the alternate hairpin structure. Data we described in Chapter 4 suggest that dimerization-mediated disruption of the hTR pseudoknot might play an important role in human telomerase repeat addition processivity. Interestingly, RNA pseudoknots are potent inhibitors of the HIV-1 RT; evidence from HIV-1 RT structural and kinetic studies suggests that RNA pseudoknots cause a shift in polymerase domain equilibrium from an open to a closed conformation, and are also likely to strongly compete with template-primer duplexes for interaction with the polymerase nucleic acid binding cleft (Jaeger et al. 1998; Kensch et al. 2000, and references therein). These observations suggest the intriguing possibility that the conserved TR pseudoknot might similarly affect the nucleic

acid interactions and catalytic function of telomerase, especially as the TR pseudoknot interacts or is predicted to interact with the TERT components of diverse telomerases (section 1.5.3.2). If the TR pseudoknot does in fact negatively regulate some aspect of telomerase catalytic function, then TR dimerization interactions or other mechanisms that disrupt the pseudoknot might be expected to stimulate telomerase activity under some conditions, as we found in Chapter 3. It would therefore be very interesting to determine if adding the isolated hTR pseudoknot or non-specific RNA pseudoknot aptamers to human telomerase reconstitution mixtures affects nucleic acid interactions and telomerase catalytic function. However, recent evidence suggests that a stable pseudoknot conformation is also important for human telomerase activity (Theimer et al. 2005). The hypothesis that a pseudoknot formed *in cis* contributes to telomerase catalytic function is supported by the conservation of this structure in all TRs studied to date.

Though dimerization potentially provides two catalytic active sites capable of dNTP addition, some RTs such as HIV-1 contain only one active site (reviewed in Götte et al. 1999). The number of active sites in human and other telomerases is unknown, but might be determined by examining the telomerase activity of size-fractionated telomerase heterodimers containing a functional catalytic aspartate residue in only one hTERT subunit. The partially purified multimeric *S. cerevisiae* telomerase interacts functionally with at least two DNA substrates, and size-fractionated dimeric *Euplotes crassus* telomerase complexes contain more than one DNA substrate (Prescott and Blackburn 1997a; Wang et al. 2002). These observations have led to the proposal that telomerase dimerization might facilitate alternating reverse transcription of the two TR templates (template switching), or simultaneous elongation of sister chromatids *in vivo*; template switching, which is commonly observed in retroviral RTs, might also promote telomerase repeat addition processivity (Prescott and Blackburn 1997a; Wenz et al. 2001; Wang et al. 2002). Recent data indicates that human telomerase does not employ template-switching after the first ten repeats of telomeric DNA have been added to substrates, though it is unknown if template switching is important during the early stages of telomerase-mediated DNA synthesis (Rivera and Blackburn 2004). Template-switching has not been examined in other telomerases. However, the ability of certain multimeric telomerases to interact functionally with more than one DNA substrate suggests that repeat addition

processivity might not be dependent on template switching, but could instead be mediated by alternating addition of telomeric repeats to different substrates using the same template. We found that *trans*-acting RID1 sequences could not restore WT levels of telomerase activity or repeat addition processivity to a RID1 mutant that exhibited anchor site-type catalytic defects and reduced affinity for short DNA substrates; furthermore, the repeat addition processivity of this mutant was reduced by 50% compared to WT enzyme (Chapter 4). It would be interesting to determine if human telomerase, like *Euplotes* and yeast telomerases, can interact with more than one DNA substrate, and to examine whether disruption of RID1 sequences prevents or impairs the interaction with multiple DNA primers.

5.6. Concluding remarks

Telomerase is a fascinating, ancient enzyme that confers replicative immortality to most eukaryotic cells. Many unique features of the telomerase catalytic mechanism, including repeat addition processivity, anchor site-type interactions with G-rich DNA substrates and the requirement for a specialized, structurally complex integral RNA molecule containing a short template were identified in the twelve years that preceded the discovery of the TERT catalytic subunit in 1997. However, the cloning of TERT homologues from multiple eukaryotes finally permitted detailed examination of the mechanistic basis for many of telomerase's catalytic properties. The development in the same year of an RRL-based system for reconstituting active ciliate and vertebrate telomerases was also instrumental to this endeavor. In 2000, the first successful alignment of N-terminal sequences from multiple TERTs indicated that the N-terminus contains the most extensive region of conserved, telomerase-specific sequences in the TERT proteins. Since then, we and other research groups have determined that the TERT N terminus is a major site of telomerase-specific catalytic functions and nucleic acid interactions in diverse telomerases. Despite these advances, our understanding of most aspects of the telomerase catalytic mechanism is still rudimentary. Detailed biochemical investigation of many of telomerase's catalytic features will require substantial quantities of soluble, extensively purified, active recombinant telomerase, a goal which has not yet been achieved for human or other telomerases. For the same reason, structural studies of the

telomerase enzyme may not be possible within the next few years. In the meantime, numerous questions concerning telomerase function can and must be addressed using currently available techniques and materials. Investigation of the telomerase catalytic mechanism is important for understanding the functional relationship of this unique and fascinating enzyme to other nucleic acid polymerases. Intensive research is also justified because telomerase is a major target for the design of anticancer therapeutics. Our work and others' suggest that the TERT N terminus may prove to be one of the most clinically and catalytically interesting sites in the telomerase enzyme.

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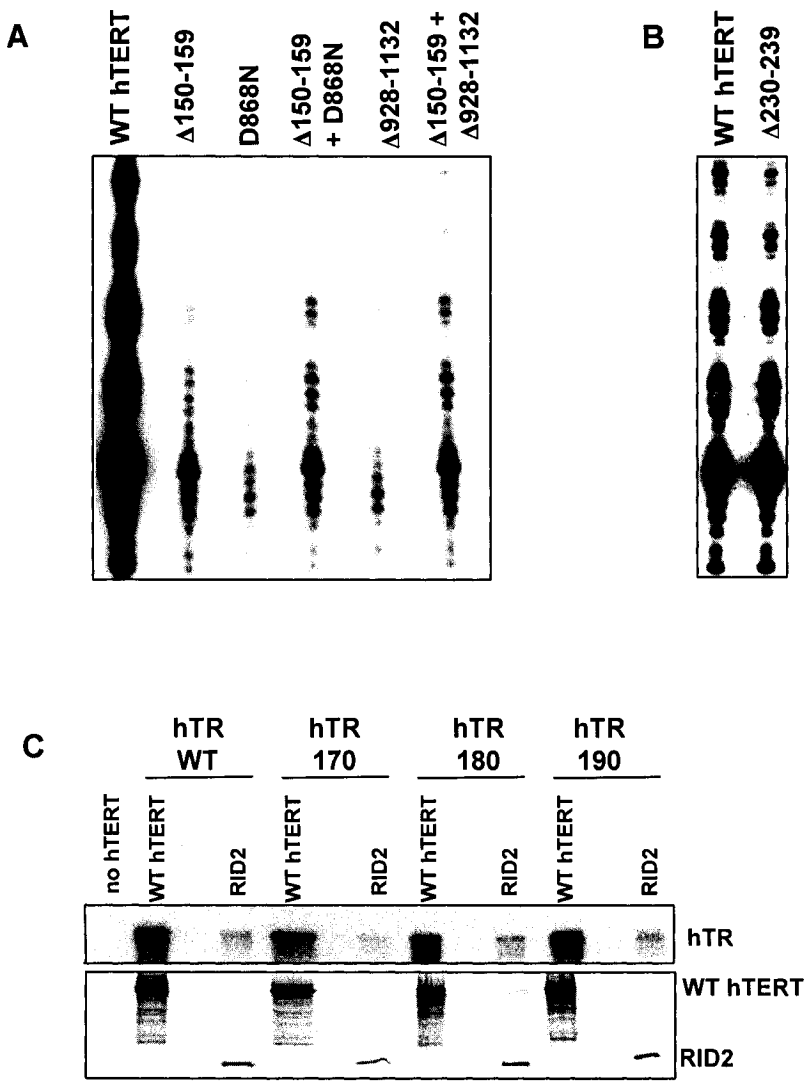
APPENDICES

1. Data accompanying Chapter 3
2. Data accompanying Chapter 4
3. Letters providing permission to reprint Figures 1.1-1.5, 1.7, 1.10, 1.11 and 1.13
4. Copyright permission letters for Chapters 2 to 4
5. Research compliance certificate for use of biohazard materials
6. Research compliance certificates for use of radioactive materials

APPENDIX 1

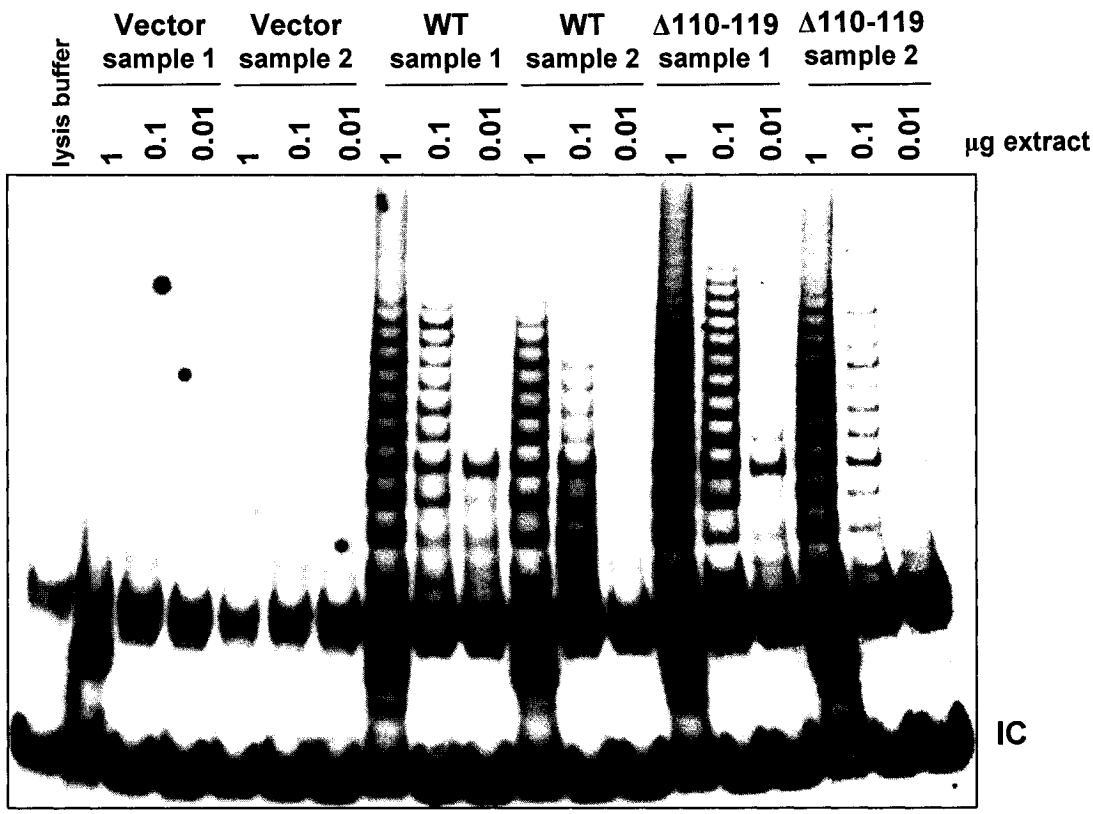
Appendix 1: Data accompanying Chapter 3



(A) and **(B)** Telomerase activity of indicated hTERT mutants expressed in RRL in the presence of WT hTR. Telomerase activity was detected by direct primer extension assay, using 1 μM biotinylated $(TTAGGG)_3$ primer (as described in the Materials and Methods of Chapter 3). **(C)** Interaction of ^{32}P-labeled WT hTR and indicated variants with ^{35}S-labeled Flag-tagged WT hTERT and Flag-RID2 following immunoprecipitation with anti-Flag antibody.

APPENDIX 2

Appendix 2: Data accompanying Chapter 4



Telomerase activity of HA5 cells transiently transfected with vector alone, WT hTERT or Δ110-119 hTERT. Telomerase activity was detected by TRAP assay using 1.0, 0.1 or 0.01 μg cell extract. The telomerase activities of samples from two independent transfections are shown. IC: PCR internal control.

APPENDIX 3

APPENDIX 4

APPENDIX 5

APPENDIX 6

Canadian Nuclear Safety Commission | Commission canadienne de sûreté nucléaire

04094-7-07.0

NUCLEAR SUBSTANCES AND RADIATION DEVICES LICENCE | PERMIS PORTANT SUR LES SUBSTANCES NUCLÉAIRES ET LES APPAREILS À RAYONNEMENT | Licence Number / Numéro de permis

I) TITULAIRE DE PERMIS

Conformément à l'article 24 (2) de la Loi sur la sûreté et la réglementation nucléaires, le présent permis est délivré à:

Hôpital Général Juif/
Jewish General Hospital
Sir Mortimer B. Davis
3755, chemin de la
Côte Ste-Catherine
Montréal (Québec)
H3T 1E2
Canada

Ci-après désigné sous le nom de «titulaire de permis»

II) PÉRIODE

Ce permis est valide du 1er août 2002 au 31 juillet 2007.

III) ACTIVITÉS AUTORISÉES

Le présent permis autorise le titulaire à avoir en sa possession, transférer, importer, exporter, utiliser et stocker les substances nucléaires et les équipements autorisés qui sont énumérés dans la section IV) du présent permis.

Le présent permis est délivré pour le type d'utilisation: études de laboratoire - 10 laboratoires ou plus où des radio-isotopes sont utilisés ou manutentionnés (836)

IV) SUBSTANCES NUCLÉAIRES ET ÉQUIPEMENT AUTORISÉ

ARTICLE	SUBSTANCE NUCLÉAIRE	SOURCE NON SCELLÉE QUANTITÉ MAXIMALE	ASSEMBLAGE DE LA SOURCE SCELLÉE QUANTITÉ MAXIMALE	ÉQUIPEMENT - FABRICANT ET MODÈLE
1	Carbone 14	400 MBq	s/o	s/o
2	Calcium 45	40 MBq	s/o	s/o
3	Cérium 141	100 MBq	s/o	s/o
4	Chrome 51	100 MBq	s/o	s/o
5	Fer 55	100 MBq	s/o	s/o
6	Fer 59	1 GBq	s/o	s/o
7	Hydrogène 3	2 GBq	s/o	s/o
8	Iode 125	1 GBq	s/o	s/o
9	Phosphore 32	5 GBq	s/o	s/o
10	Phosphore 33	100 MBq	s/o	s/o
11	Soufre 35	3 GBq	s/o	s/o
12	Scandium 46	100 MBq	s/o	s/o
13	Strontium 85	20 MBq	s/o	s/o
14	Césium 137	s/o	40 kBq	s/o
15	Césium 137	s/o	1480 kBq	Beckman LS (series)
16	Radium 226	s/o	370 kBq	PerkinElmer Wallac 1200 series LS Counters
17	Europium 152	s/o	740 kBq	PerkinElmer Wallac 1400 series LS Counters

La quantité totale d'une substance nucléaire non scellée possédée ne doit pas excéder la quantité maximale qui est indiquée pour une source non scellée correspondante. La quantité de substance nucléaire par source scellée ne doit pas excéder la quantité maximale indiquée par source scellée correspondante. Les sources scellées doivent être utilisées seulement dans l'équipement indiqué corrrespondant.

V) ENDROIT(S) OÙ LES ACTIVITÉS AUTORISÉES PEUVENT ÊTRE EXERCÉES

utilisées ou entreposées à (aux)

endroit(s) suivant(s):

Institut Lady Davis de Recherches
Médicales
3755 chemin Côte Ste-Catherine
Montréal (Québec)

VI) CONDITIONS

1. Interdiction visant l'utilisation chez les humains
Le permis n'autorise pas l'utilisation des substances nucléaires dans ou sur le corps d'une personne.
(2696-0)

2. Classification des zones, pièces et enceintes
Le titulaire de permis désigne chaque zone, pièce ou enceinte où on utilise plus d'une quantité d'exemption d'une substance nucléaire non scellée à un moment donné selon la classification suivante :

(a) de niveau élementaire si la quantité ne dépasse pas 5 LAI,
(b) de niveau intermédiaire si la quantité utilisée ne dépasse pas 50 LAI,
(c) de niveau supérieur si la quantité ne dépasse pas 500 LAI,
(d) de confinement si la quantité dépasse 500 LAI;
(e) à vocation spéciale, avec l'autorisation écrite de la Commission ou d'une personne autorisée par celle-ci.

À l'exception du niveau élementaire, le titulaire de permis n'utilise pas de substances nucléaires non scellées dans ces zones, pièces ou enceintes sans l'autorisation écrite de la Commission ou d'une personne autorisée par celle-ci.
(2108-1)

3. Liste des laboratoires
Le titulaire de permis tient à jour une liste de toutes les zones, salles et enceintes dans lesquelles plus d'une quantité d'exemption d'une substance nucléaire est utilisée ou stockée.
(2569-1)

4. Procédures de laboratoire
Le titulaire affiche en tout temps et bien en évidence dans les zones, les salles ou les enceintes où des substances nucléaires sont manipulées une affiche sur la radioprotection qui a été approuvée par la Commission ou une personne autorisée par la Commission et qui correspond à la classification de la zone, de la salle ou de l'enceinte.
(2570-1)

5. Surveillance thyroïdienne
La personne
a) qui utilise à un moment donné une quantité d'iode 125 ou d'iode 131 volatiles dépassant :
(i) 5 MBq dans une pièce ouverte,
(ii) 50 MBq dans une hotte,
(iii) 500 MBq dans une boîte à gants,
(iv) toute autre quantité dans une enceinte de confinement approuvée par écrit par la Commission ou une personne autorisée par celle-ci;
(b) qui est impliqué dans un déversement mettant en cause plus de 5 MBq d'iode 125 ou d'iode 131 volatiles;
(c) chez laquelle on détecte une contamination externe à l'iode 125 ou l'iode 131;
doit se prêter à un dépistage thyroïdien dans les cinq jours suivant l'exposition.
(2046-7)

6. Dépistage thyroïdien
Le dépistage de l'iode 125 et de l'iode 131 internes se fait :
(a) par mesure directe à l'aide d'un instrument capable de détecter 1 kBq d'iode 125 ou d'iode 131;
(b) par essai biologique approuvé par la Commission ou une personne autorisée par celle-ci.
(2600-1)

7. Essai biologique thyroïdien
Si la charge thyroïdienne dans une personne dépasse 10 kBq d'iode 125 ou d'iode 131, le titulaire de permis doit présenter immédiatement un rapport préliminaire à la Commission ou à une personne autorisée par celle-ci. Dans un délai de 24 heures, la personne en question doit subir des essaies biologiques par une personne autorisée par la Commission à offrir un service de dosimétrie interne.
(2601-4)

8. Dosimétrie des extrémités
Le titulaire de permis veille à ce que toute personne qui manipule un contenant renfermant plus de 50 MBq de phosphore 32, de strontium 89, d'yttrium 90, de samarium 153 ou de rhénium 186 porte une bague dosimètre. Le dosimètre est fourni et lu par un service de dosimétrie autorisé par la Commission.
(2578-0)

9. Critères de contamination
En ce qui a trait aux substances nucléaires figurant au tableau «Classification des radionucléides» du guide sur les demandes de permis, le titulaire de permis veille à ce que :
(a) la contamination non fixée dans toutes les zones, pièces ou enceintes où on utilise ou stocke des substances nucléaires non scellées ne dépasse pas :
(i) 3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 30 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 300 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2;
(b) la contamination non fixée pour toutes les autres zones ne dépasse pas :
(i) 0,3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 3 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 30 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2.
(2642-2)

10. Déclassement
Avant le déclassement d'une zone, d'une pièce ou d'une enceinte où s'est déroulée l'activité autorisée, le titulaire de permis veille à ce que :
(a) la contamination non fixée pour les substances nucléaires figurant au tableau «Classification des radionucléides» du guide sur les demandes de permis ne dépasse pas :
(i) 0,3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 3 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 30 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2;
(b) la mise en disponibilité de toute zone, pièce ou enceinte contenant une contamination fixée soit approuvée par la Commission ou une personne autorisée par celle-ci;
(c) toutes les substances nucléaires et tous les appareils à rayonnement ont été transférés conformément aux conditions du permis;
(d) tous les panneaux de mise en garde contre les rayonnements ont été retirés ou ont été rendus illisible.
(2571-2)

11. Stockage
Le titulaire :
a) veille à ce que seules les personnes autorisées par lui aient accès aux substances nucléaires radioactives ou aux appareils à rayonnement stockés;
b) veille à ce qu'à tout endroit occupé à l'extérieur de la zone, de la salle ou de l'enceinte de stockage le débit de dose provenant des substances ou appareils stockés ne dépasse pas 2,5 microSv/h;
c) a des mesures en place pour assurer que les limites de dose indiquées dans le Règlement sur la radioprotection ne sont pas dépassées en raison du stockage de ces substances ou appareils.
(2575-0)

12. Évacuation (laboratoires)
Lorsqu'il évacue des substances nucléaires non scellées dans une décharge municipale ou un réseau d'égouts, le titulaire de permis