Exploring the Regulation of the Telomerase Reaction Cycle through Unique Protein, DNA, and RNA Interactions

by

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ABSTRACT

Telomerase is a unique reverse transcriptase that has evolved specifically to extend the single stranded DNA at the 3' ends of chromosomes. To achieve this, telomerase uses a small section of its integral RNA subunit (TR) to reiteratively copy a short, canonically 6-nt, sequence repeatedly in a processive manner using a complex and currently poorly understood mechanism of template translocation to stop nucleotide addition, regenerate its template, and then synthesize a new repeat. In this study, several novel interactions between the telomerase protein and RNA components along with the DNA substrate are identified and characterized which come together to allow active telomerase repeat addition. First, this study shows that the sequence of the RNA/DNA duplex holds a unique, single nucleotide signal which pauses DNA synthesis at the end of the canonical template sequence. Further characterization of this sequence dependent pause signal reveals that the template sequence alone can produce telomerase products with the characteristic 6-nt pattern, but also works cooperatively with another RNA structural element for proper template boundary definition. Finally, mutational analysis is used on several regions of the protein and RNA components of telomerase to identify crucial determinates of telomerase assembly and processive repeat synthesis. Together, these results shed new light on how telomerase coordinates its complex catalytic cycle.

DEDICATION

Dedicated to my loving parents, brother, and sister. Without their support, encouragement, and love I would not be where I am today.

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TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION: AN OVERVIEW OF THE TELOMERASE RIBONUCLEOPROTEIN ENZYME

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Introduction

1.1 History and significance of the telomerase enzyme

More than three-quarters of a century ago, Muller and McClintock found that the linear chromosomes of eukaryotic organisms required an essential terminal capping structure necessary for genomic stability (McClintock, 1941; Muller, 1938). While chromosomes lacking these capping structures, termed 'telomeres', were unstable and deleteriously affected cell survival, chromosomes with intact telomere structures were stable and mainatained genomic integrity (McClintock, 1941). Over time the function of telomeres for chromosome stability, partioning, masking from double-stranded DNA repair, and cellular proliferation was elucidated (Blackburn, 2001; Lange, 2002). The function of telomeres for cellular proliferation was of particular interest due to the apparent inherent limitation of conventional DNA polymerases for the replication of the chromosome termini (Lingner et al, 1995; Olovnikov, 1973; Watson, 1972). This end-replication problem, if left unabated results in chromosome uncapping, exposure of chromosome ends to double-stranded DNA repair machinery, genomic instability, and related deleterious effects upon cellular survival [reviewed in (de Lange, 2009)].

The cellular solution to the progressive loss of telomeric DNA and eventually genetic material is the unique DNA polymerase called telomerase (Greider & Blackburn, 1985; Greider & Blackburn, 1987). Telomerase is a reverse transcriptase that has become specialized for the addition of telomeric DNA repeats to the ends of chromosomes to offset the loss after each replication. Unlike traditional RTs, the telomerase enzyme is made up of two components required for activity: the telomerase reverse transcriptase

(TERT, also known as TRT, Est2) protein and the integral telomerase RNA (TR, also known as TER, TERC, TLC1). The TERT protein component is the catalytic unit responsible for DNA polymerization, while the TR provides the template for repeat addition. Together these two components make up the minimal catalytic core of telomerase, though *in vivo* a variety of accessory proteins are known to associate with one or both components for proper telomerase biogenesis, localization, and regulation (Egan & Collins, 2010; Fu & Collins, 2003; Kiss et al, 2010; Venteicher & Artandi, 2009).

Control of telomerase activity to maintain telomere length is very important, with mis-regulation leading to a number of diseases and disorders in humans and other species. In humans, the expression of telomerase is tightly regulated, being turned off in most somatic cells and only expressed in germ-line and stem cells (Hiyama & Hiyama, 2007). Without telomerase, somatic cells are effectively limited in their replicative capability and repeated replications will eventually lead to entering a stage of senescence and ultimately cell death (Hayflick & Moorhead, 1961). Overcoming this limit, most often through the reactivation of telomerase, is an important step to the development of cancer. As a result, nearly 90% of human cancers show an up-regulation of telomerase (Kim et al, 1994). In the opposite extreme, mutations in telomerase or accessory proteins that reduce telomerase activity in cells that normally express it can lead to a number of human diseases such as Aplastic Anemia, Idiopathic Pulmonary Fibrosis, and Dyskeratosis Congenita (Podlevsky & Chen, 2012).

Despite its short RNA template, telomerase is known to add long tracts of DNA to the ends of telomeres with a single binding event (Greider & Blackburn, 1987; Shippen-

Lentz & Blackburn, 1990). This processive addition is achieved through two distinct steps: the enzymatic addition of multiple nucleotides composing a single repeat to the 3' end of the DNA (nucleotide addition processivity) followed by a regeneration of the template without full dissociation for multiple additions of the template sequence (repeat addition processivity). This two-step processes is unique among polymerases and requires a novel mechanism. First, the telomeric DNA must anneal to the 5' end of the RNA template in the TERT active site (Figure 1.1). With the RNA/DNA properly positioned, DNA polymerization occurs, reverse transcribing the short RNA template onto the 3' end of the DNA. At the end of the template, telomerase undergoes a unique template translocation event to regenerate the template for addition repeat synthesis (Figure 1.1).

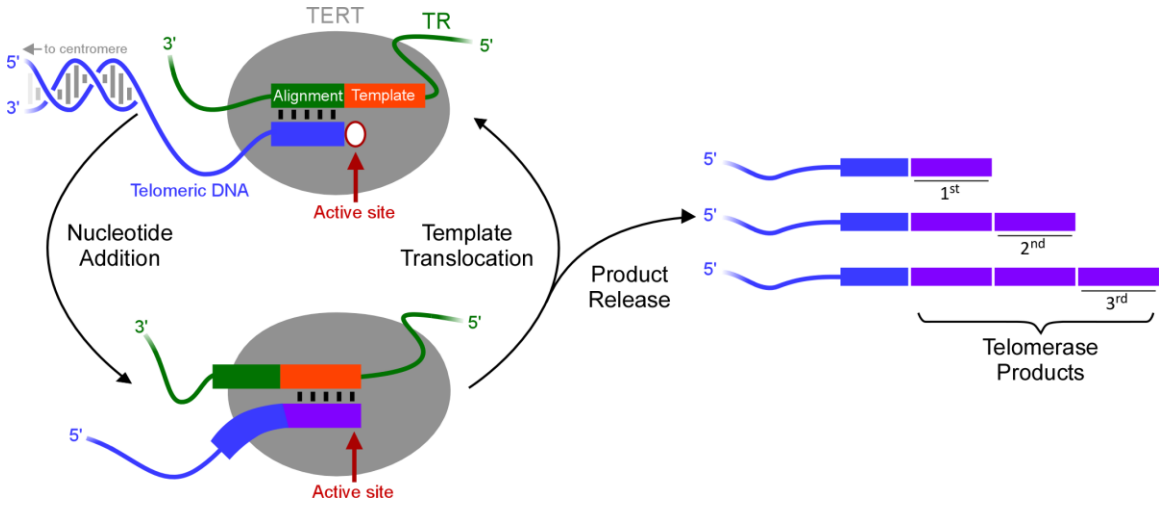

Figure 1.1: The unique telomerase catalytic cycle. Telomerase functions as a conventional reverse transcriptase by synthesizing single-stranded DNA onto the ends of a telomeric DNA primer (*blue*) from an intrinsic RNA template (*orange*), *nucleotide addition*. Apart from conventional polymerases, upon reaching the end of the template, telomerase has the capacity to regenerate the template by a complex mechanism of *template translocation*. Following this, an additional round of *nucleotide addition* then proceeds generating longer telomerase products (*violet*). Unsuccessful template translocation terminates the synthesis of additional telomeric repeats by *product release*.

1.2 Telomerase Reverse Transcriptase

The protein component of telomerase, TERT, has retained several structural features of prototypical reverse transcriptases as well as evolved its own specialized structural features. These conserved structural features of telomerase are commonly divided into four domains: the telomerase essential N-terminal (TEN) domain, the telomerase RNA binding domain (TRBD), the reverse transcriptase (RT) domain, and the C-terminal extension (CTE).

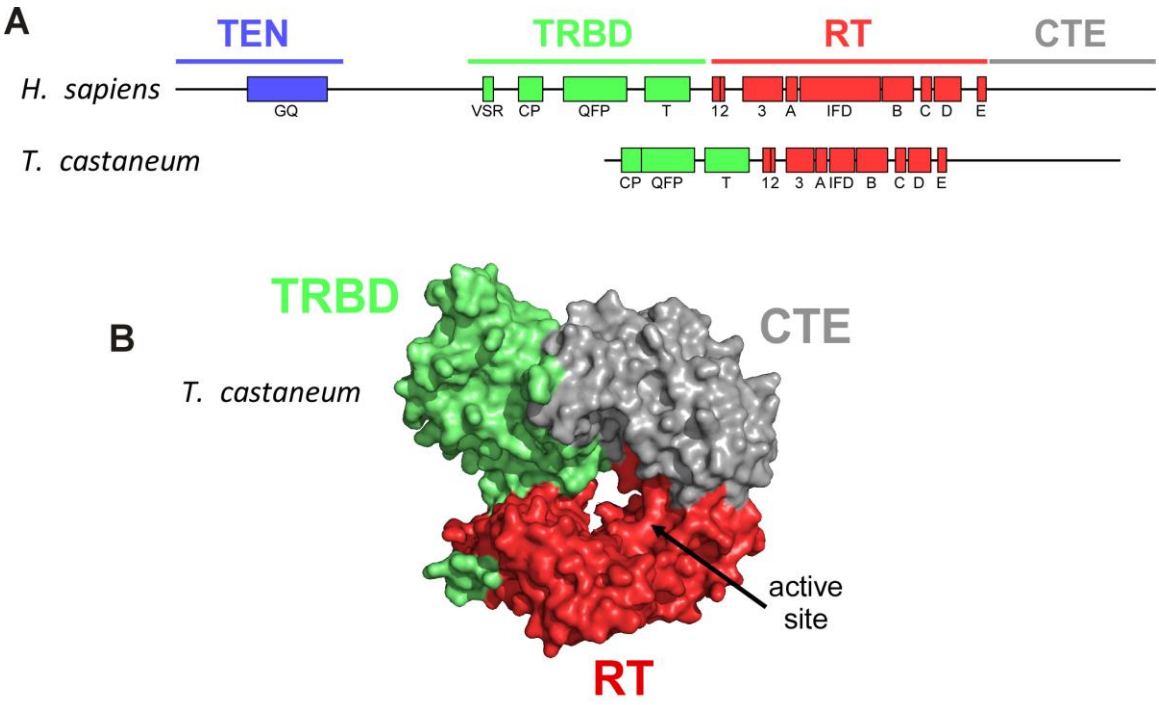

Figure 1.2: Structural organization of the catalytic TERT protein. (a) TERT is composed of four structural domains: telomerase essential N-terminal (TEN, *blue*) domain, telomerase RNA-binding domain (TRBD, *green*), reverse transcriptase (RT, *red*), and the C-terminal extension (CTE, *grey*). The TEN and TRBD are telomerase-specific, essential for template translocation, and not found among conventional polymerases. Important motifs are colored similarly in the encompassing domains. (b) The crystallized *Tribolium castaneum* TERT showing major protein domains. The *Tribolium castaneum* TERT protein forms a ring structure unseen in other polymerases.

The enzymatic core of TERT has retained many evolutionarily conserved structural motifs with conventional reverse transcriptases. This RT domain makes up a structural and functional homolog of the "fingers" and "palm" domains of traditional RTs and is divided into seven conserved motifs: 1, 2 and A, B, C, D, and E (Figure 1.2A). Like other RTs, the fingers domain (motifs 1 and 2) of TERT has been shown through biochemical methods to be involved in binding incoming nucleotides and positioning the RNA template (Bosoy & Lue, 2001; Gillis et al, 2008; Wyatt et al, 2010). Meanwhile, the palm domain (motifs A, B, C, D, and E) contains the catalytic active site for polymerization as well as a primer grip region for holding of the telomeric DNA. Similar to conventional RTs, the TERT active site contains three absolutely conserved aspartic acids (one in motif A and two in motif C) that catalyze nucleotide addition by a two-metal ion mechanism (Gillis et al, 2008; Lingner et al, 1997; Nakamura et al, 1997). Mutation of any of these three conserved aspartic acids leads to completely inactive telomerase *in vitro* and telomere shortening *in vivo* (Bryan et al, 2000; Counter et al, 1997; Harrington et al, 1997; Nakayama et al, 1998; Weinrich et al, 1997; Wyatt et al, 2010). Additionally, an invariant lysine residue in motif D is thought to act as a general acid to activate the pyrophosphate leaving group of the incorporated nucleotide triphosphate (Sekaran et al, 2010). Mutation of this lysine in telomerase leads to severely reduced telomerase activity *in vitro* (Bryan et al, 2000; Miller et al, 2000; Sekaran et al, 2010). The primer grip region of motif E has been shown through biochemical assays to be important for single stranded DNA binding and telomere maintenance (Peng et al, 2001; Wyatt et al, 2007). The crystal structure of *T. castaneum* TERT shows an interaction between the primer grip loop and the 3' end of the DNA strand of the

RNA/DNA duplex bound in the active site, presumably facilitating proper alignment of the duplex for nucleotide addition (Mitchell et al, 2010).

Diverging from traditional RTs, the TERT RT domain also contains several telomerase specific motifs to perform functions unique to telomerase. One such conserved feature is a large insertion in the traditional fingers domain of reverse transcriptases, dubbed the insertion in fingers domain (IFD). Mutation studies in IFD have identified this domain as essential for telomerase repeat addition processivity and telomere maintenance (Lue et al, 2003). Structurally, this domain appears to be important for proper arrangement of the protein and does not make direct contact with the DNA or RNA (Gillis et al, 2008; Mitchell et al, 2010). Another telomerase specific motif, named motif 3 due to its location between motifs 2 and A, has been shown to be more directly involved in repeat addition. Mutational analysis of this conserved motif showed independent effects on both the repeat addition processivity and rate of repeat addition of the telomerase enzyme (Xie et al, 2010). Composed of a long α-helix and a flexible linker region, motif 3 is positioned to make direct contact with the RNA/DNA duplex bound to the active site. Taken together, the structural and biochemical data support a role of motif 3 in facilitating the strand separation and template realignment of the RNA/DNA duplex during template translocation (Xie et al, 2010).

Also characteristic of TERT proteins is a long N-terminus that contains two telomerase specific domains; the telomerase N-terminal domain (TEN) and the telomerase RNA binding domain (TRBD). The TEN domain contains a high affinity DNA “anchor” site which specifically binds single-stranded telomeric DNA (Finger & Bryan, 2008; Jacobs et al, 2006; Lue & Li, 2007; Romi et al, 2007; Sealey et al, 2010).

This binding of the DNA upstream of the 3' end in the active site is known to prevent complete disassociation of the DNA from the enzyme during processive repeat addition and thus increases repeat addition processivity (Wyatt et al, 2010). Separate from its known DNA binding capability, several residues inside the TEN domain have been found to be important for telomerase function. A conserved leucine residue (L14 in *T. thermophilia*) has been shown to have an effect on the rate of repeat addition in ciliates even when the primer is tethered to the enzyme, showing an additional role of the TEN domain in repeat addition outside of primer retention (Eckert & Collins, 2012; Qiao et al, 2010; Zaug et al, 2008). In human TERT, mutation of the L14 or L13 residues resulted in a drastic decrease in activity without altered RAP, though a double mutation of L14 and L13 did show reduced RAP, reinforcing the importance of a leucine residue for processivity. The TEN domain also contains a low-affinity RNA interacting domain (RID1) which shows interactions with the TR template/pseudoknot domain, though the significance of this interaction is currently unknown (Lai et al, 2001; Moriarty et al, 2004; Podlevsky & Chen, 2012). The high-affinity RNA interacting domain (RID2) is contained in the TRBD and is responsible for the major interaction between TERT and the TR (Moriarty et al, 2004). As a result, the TRBD is a universally conserved domain of TERT proteins. The TRBD can be broken down into three separate conserved regions: CP, QFP, and T motifs. Together, these three motifs form a highly helical structure in both *T. thermophila* and *T. castaneum* crystal structures and make up the major RNA binding pocket (Figure 1.2B) (Gillis et al, 2008; Rouda & Skordalakes, 2007). In addition to its importance for RNA binding, the T motif has been shown to play a role in repeat addition processivity of the telomerase reaction, likely due to its interactions with the

RNA backbone of the RNA/DNA duplex when bound to the active site (Drosopoulos & Prasad, 2010; Mitchell et al, 2010). While not universally conserved, different lineages appear to contain an additional motif in the TRBD, the VSR in humans and CP2 in ciliates, which appear to be important for RNA binding in these species (Lai et al, 2002; Moriarty et al, 2002).

While the C-terminus of the TERT protein, called the C-terminal extension (CTE), contains very little sequence homology to traditional RTs, the structure and function of the domain remains similar to the "thumb" domain of retroviral RTs (Figure 1.2B). Functionally, the CTE has been shown to have an effect on telomeric DNA binding and telomerase activity and processivity (Hossain et al, 2002). Structurally, the CTE undergoes long range protein-protein interactions with the TRBD domain to form the characteristic ring-like shape of the TERT protein (Gillis et al, 2008). It has also been proposed that the CTE might have interactions with telomerase RNA, bound to the TRBD, to strengthen the interface between the two domains and promote proper protein folding (Bley et al, 2011).

1.3 Telomerase RNA

Unlike conventional RTs, the telomerase enzyme includes an integral RNA component necessary for enzyme activity. In addition to providing the RNA template for telomere extension, the TR also contains various other motifs necessary for telomerase activity. Unlike the well conserved TERT protein, the TR is remarkably divergent among different phylogenetic lineages (Figure 1.3). While different species contain different TR motifs, two in particular are universally conserved: the template pseudoknot domain and

three-way junction or stem-terminal element (Figure 1.3) (Blackburn & Collins, 2010; Brown et al, 2007; Chen & Greider, 2004; Chen et al, 2002; Chen et al, 2000; Lin et al, 2004). Together, these two domains make up the necessary components for telomerase enzymatic activity *in vitro*, even when added *in trans* with the TERT protein (Mitchell & Collins, 2000; Tesmer et al, 1999). In humans, the TR contains a third major domain known as the H/ACA domain (Figure 1.3B) (Chen et al, 2000; Mitchell et al, 1999a; Mitchell et al, 1999b). This domain is homologous to that of small nucleolar (sno) and small cajal body-specific (sca) RNAs, containing two stem-loops separated by box H and box ACA moieties (Jády et al, 2004). In the TR, a complex of four proteins (dyskerin, NOP10, NHP2, and GAR1) bind to the H/ACA domain and are required for proper biogenesis and localization (Egan & Collins, 2010; Mitchell et al, 1999a; Vulliamy et al, 2001).

The secondary and tertiary structure of the TR has provided many useful insights into telomerase function. The template/pseudoknot domain functions to properly position the RNA template into the active site and define the template boundary. Biochemical and structural studies have shown that the pseudoknot forms into a short triple helix which is important for proper telomerase activity (Chen & Greider, 2005; Ly et al, 2003; Qiao & Cech, 2008; Shefer et al, 2007; Theimer et al, 2005). The exact mechanism of the triple helix towards telomerase activity remains poorly understood, though the template and pseudoknot are always very close to each other in the secondary structure (Chen et al, 2000). NMR structural determination has revealed that the P2a and P2b stems between the triple helix and the template adopt a sharp kink, due to a conserved five nucleotide bulge junction, which might facilitate proper positioning of the template (Zhang et al,

2011; Zhang et al, 2010). In the templating region, the 5' end is used as template for telomeric repeat synthesis, while a smaller non-templating section at the 3' end is used as a realignment region where the extended DNA anneals at the start of each repeat synthesis. Upstream of the template is a structural template boundary element (TBE) which defines the 5' end of the template sequence. In humans, the TBE is composed of helix P1b with the single stranded RNA between the 5' end of the template and the stem determining the template length (Chen & Greider, 2003b). Mutations that disrupt P1b and lengthen or shorten the linker region has been shown to alter the normal repeat sequence (Chen & Greider, 2003b; Theimer & Feigon, 2006). Also upstream of the template pseudoknot domain in human TR is a guanosine-rich 5' end which is believed to form a G-quadruplex structure. The formation of this G-quadruplex structure and association with the HEXH box RNA helicase RHAU has been shown increase TR accumulation within cells (Lattmann et al, 2011; Sexton & Collins, 2011).

Another conserved feature of telomerase RNA important for proper enzymatic activity is a template distal stem-loop moiety (Figure 1.3). In vertebrate this moiety is the CR4/5 domain composed of a three way junction of helices made from conserved stems P5, P6, and P6.1 (Blackburn & Collins, 2010; Brown et al, 2007; Chen et al, 2002; Chen et al, 2000). The presence of this three way junction structure is necessary for telomerase activity both *in vitro* and *in vivo*, with disruptions of the stems or even the mutation of only two conserved residues in L6.1 enough to eliminate telomerase activity (Bley et al, 2011; Chen et al, 2002). Cross-linking studies have suggested that interactions between the CR4/5 and both the TRBD and CTE of TERT are necessary for proper protein folding (Bley et al, 2011). A three-way junction structure similar to the CR4/5 domain of

vertebrates has also been found as a conserved feature of budding yeasts through phylogenetic analysis and is also important for telomerase activity *in vitro* and *in vivo* (Brown et al, 2007). Ciliates, while not possessing a three-way junction structure, have a conserved stem-loop IV which is important for TR-TERT interactions and proper telomerase function (Figure 1.3A) (Blackburn & Collins, 2010).

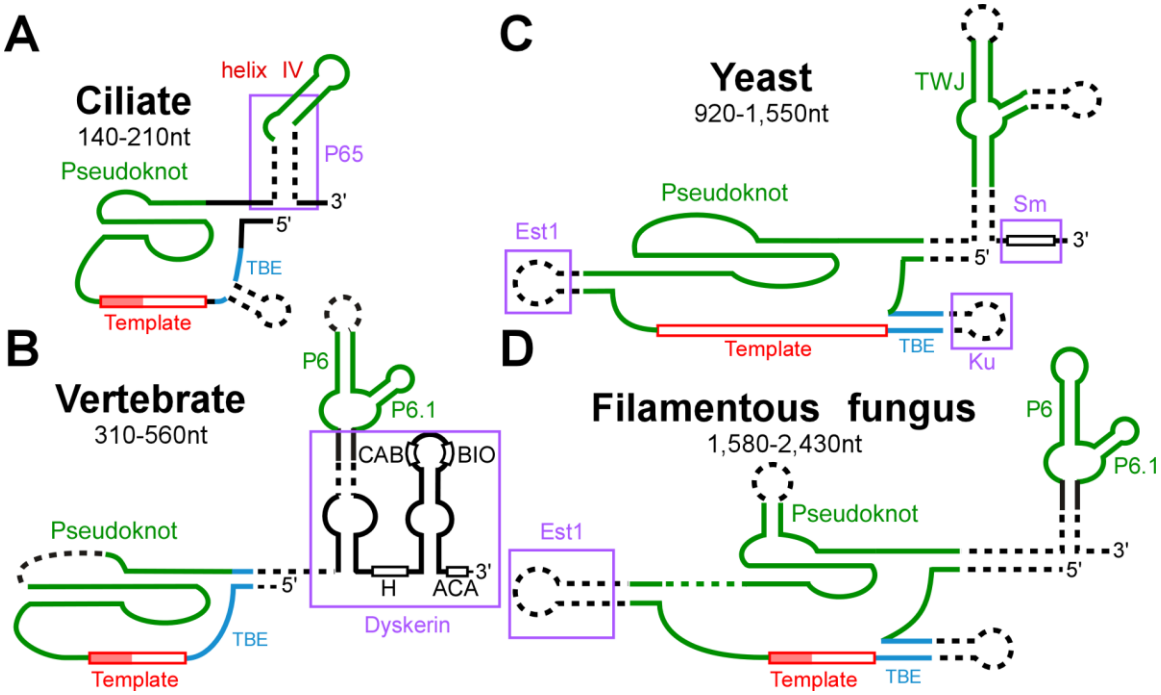

Figure 1.3: The conserved core of the highly divergent TR. Known secondary structure diagrams for ciliate (A), vertebrate (B), yeast (C), and filamentous fungus (D). Conserved pseudoknot domain and distal stem loop moieties (*green)* are highlighted in each group. Template (*red*) regions along with the template boundary elements (TBE, *blue*) are also shown. Additionally, known accessory protein binding sites denoted by boxed regions (*purple*).

Outside of the telomerase elements required for enzymatic activity, telomerase RNA also contains a variety of structural motifs for proper biogenesis, processing, and localization of the RNA. In vertebrate TR, the 3' end contains a conserved H/ACA domain. Two copies of a protein complex composed of dyskerin, NOP10, NHP2, and

GAR1 bind to this domain and facilitate proper RNA maturation, 3' processing, and RNP biogenesis in cells (Kiss et al, 2010; Li, 2008; Mitchell et al, 1999a; Vulliamy et al, 2006). Two additional conserved moieties are found in a stem-loop of the H/ACA domain, a Cajal body localization (CAB box) important for proper RNA localization and biogenesis promoting (BIO box) moiety (Egan & Collins, 2012; Mitchell et al, 1999a; Reichow et al, 2007; Venteicher & Artandi, 2009).

Mutations in the TR gene that affect active telomerase levels in the cell have been linked to a number of human telomere-mediated diseases such as Aplastic Anemia, Idiopathic Pulmonary Fibrosis, and Dyskeratosis Congenita (Alder et al, 2008; Alder et al, 2011; Armanios et al, 2007; Dokal, 2003; Du et al, 2009; Fogarty et al, 2003; Marrone et al, 2004; Tsakiri et al, 2007; Vulliamy, 2002; Vulliamy et al, 2006; Yamaguchi et al, 2003). These disorders appear to be caused by haploinsufficiency and the lone functional TR allele does not allow for sufficient TR accumulation, leading to reduced telomerase levels and telomere shortening (Armanios et al, 2005). Disease mutations tend to locate within the three functional domains: template-pseudoknot, CR4/5, and H/ACA domains (Podlevsky et al, 2008). Mutations in these regions can disrupt RNA base-pairing and/or RNA structure that are important for enzymatic activity or RNA processing and accumulation. Such a decrease in telomerase activity or RNA accumulation levels has been experimentally observed for many of the noted disease mutations (Alder et al, 2008; Alder et al, 2011; Armanios et al, 2007; Cristofari et al, 2007; Fu & Collins, 2003; Ly et al, 2005; Theimer et al, 2003; Theimer et al, 2007).

1.4 Telomerase Mechanism

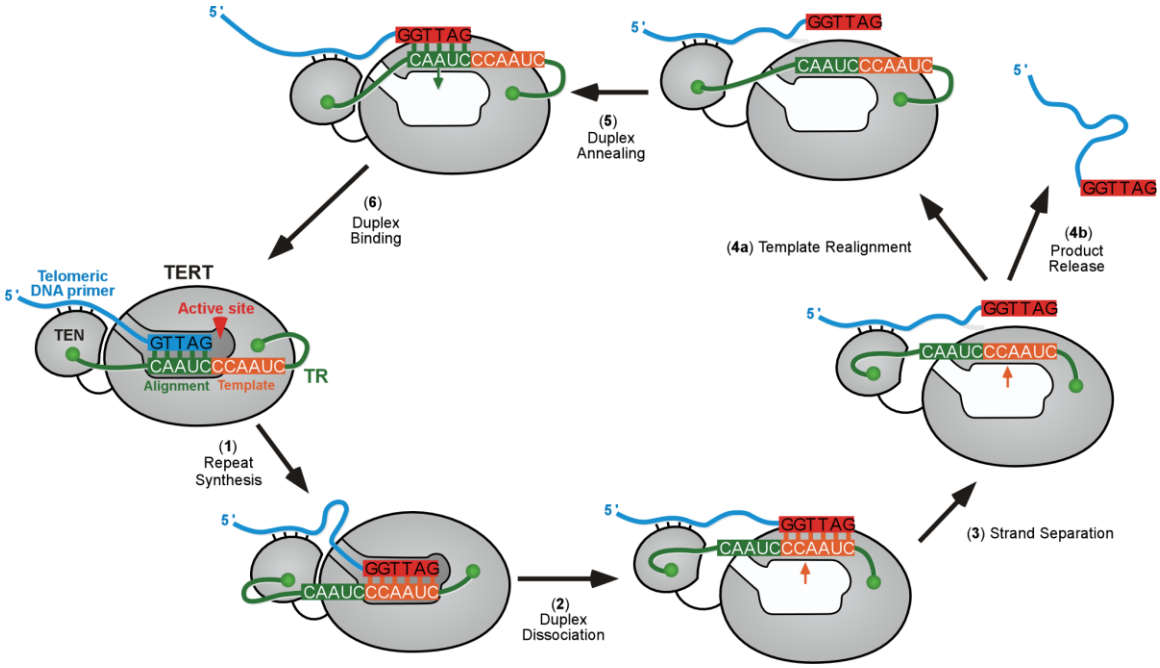

Figure 1.4: A working model for the telomerase template translocation mechanism. (a) Schematic of human telomerase bound to a telomeric DNA primer (blue). The TR alignment region (green) is base-paired with the 3'-end of the telomeric DNA primer (blue) to form 5 base pairs adjacent to the active site (red arrow). The TR template (orange) is constrained by flanking sequences bound to the TERT protein, while the 5' region of the telomeric DNA is bound to the TEN domain (gray). TERT (gray) catalyzes the addition of six deoxyribonucleotides to the 3' end of the DNA primer by reverse transcribing the TR template sequence (step 1). After nucleotide addition, a new repeat is generated. After reaching the end of the template, the duplex dissociates from the active site (step 2). Outside the active site, the RNA/DNA duplex undergoes template translocation involving strand separation (step 3) and template realignment (step 4a) to reform 5 base pairs (step 5). This reannealed duplex can then bind back to the active site for further nucleotide addition. Unsuccessful realignment of the DNA primer to the RNA template eventually results in complete dissociation of the DNA product from the enzyme (step 4b). Strand separation and template realignment (steps 3 and 4a) are postulated as reversible, with multiple binding/separation steps possible.

Due to the unique role of telomerase compared to other polymerases, a novel mechanism for telomeric repeat synthesis has evolved. While other RTs bind pre-

annealed RNA/DNA duplex and use a long RNA template to synthesize DNA in a processive manner, telomerase uses single stranded DNA as substrate and uses an internal, short RNA template to add multiple repeats of a repetitive sequence in a processive manner. The repetitive nature of telomerase extension lends itself to be represented as a repeat cycle with 5 distinct steps; nucleotide addition, duplex disassociation, strand separation, template realignment, and duplex binding (Figure 1.4).

Nucleotide Addition

The mechanism for polymerization of incoming nucleotides is similar to other RTs and employs a two metal ion mechanism. The three invariant aspartic acid residues in the palm domain of TERT coordinate two magnesium ions in the active site (Gillis et al, 2008). Free nucleotides are bound by the nucleotide binding pocket between the fingers and palm domain of the TERT protein and positioned in the active site (Gillis et al, 2008). The RNA/DNA duplex is positioned in the active site by cooperation of many regions of TERT with motif T, motif 3, the fingers domain, and the CTE implicated in proper duplex alignment (Gillis et al, 2008; Mitchell et al, 2010; Xie et al, 2010). When a proper base pairing of the bound nucleotide and the first free templating residue of the RNA/DNA duplex is made, the polymerization reaction occurs and a pyrophosphate is released. Once polymerization is complete, the RNA/DNA duplex must be moved through the active site to place the 3' end of newly extended DNA into the active site. This translocation of the duplex during nucleotide addition has been proposed to be facilitated by both the flanking single stranded DNA and RNA through possible secondary structure formation in the DNA and stretching or compaction of the bound

RNA (Berman et al, 2011; Jarstfer & Cech, 2002). Furthermore, there is evidence in both human and yeast that the length of the extended RNA/DNA duplex is maintained at a constant, short length of five to seven base pairs by unpairing at the 5' end of the DNA for every new base pair made at the 3' end (Förstemann & Lingner, 2005; Qi et al, 2012). This is supported by the *T. castaneum* crystal structure where the central cavity of the protein is large enough to accommodate a duplex of seven to eight base pairs (Gillis et al, 2008). The unpairing of base pairs at the 5' end of the DNA also appears to have functional significance in duplex translocation through the active site during processive nucleotide addition. When the template region of the TR is deleted and telomerase is supplied a preannealed RNA/DNA duplex as substrate, telomerase could only extend a short 5 base pair duplex processively if a short 5' single stranded DNA overhand was present and showed a higher affinity to the duplex with the overhang (Qi et al, 2012). Together, this data suggests that the TERT protein binds unpaired single stranded DNA at the 5' end to pull the duplex through the active site after nucleotide addition to prepare for the next addition.

Duplex Dissociation

Once telomerase reaches the end of its short template, the duplex must dissociate from the active site, the two strands of the duplex must be separated, and then the two strands must be realigned at the beginning of the template for subsequence repeat additions. To achieve this, there are two major models that telomerase could follow; the DNA could remain bound to the active site while the RNA is separated and repositioned relative to the DNA or the RNA/DNA duplex could dissociate together and reposition outside the active site. Recently, biochemical data has supported the latter model by

showing that the active site of telomerase becomes unoccupied and accessible to a short external RNA/DNA duplex during repeat synthesis (Qi et al, 2012). This suggests that full dissociation of the RNA/DNA duplex from the active site is the first step of template translocation. This is also supported by a comparison between the crystal structures of *T. castaneum* TERT with and without an RNA/DNA duplex bound in the active site. It is predicted that the separation of the duplex in the active site would require a significant conformation change to break not only the hydrogen bonds of the duplex, but also the protein-nucleic acid contacts holding the duplex in the active site. However, breaking only the protein-nucleic acid contacts would require far less energy and is more consistent with the relatively minor changes in the protein seen between the two crystal structures (Gillis et al, 2008; Mitchell et al, 2010). Furthermore, correlation between repeat addition rate in mutated telomerases and duplex turnover rates with duplex substrates hints that duplex dissociation may be the rate-limiting step of template translocation (Qi et al, 2012). In support of full duplex dissociation occurring before strand separation, it has been observed in several species that the stability of the telomerase with a bound primer is dependent on the sequence of the primer, but did not correlate with base pairing between the DNA primer and RNA template (Finger & Bryan, 2008; Hammond & Cech, 1998; Wallweber et al, 2003). This suggests that protein nucleic acid contacts play an important role in stabilizing the RNA/DNA duplex.

Strand Separation

Once the RNA/DNA duplex dissociates from the active site, the RNA and DNA strands must separate from each other and then realign so that the DNA 3' end is back at the beginning of the template region through base pairing with the template alignment

region. The method the enzyme employs to separate the two strands in a rapid manner remains elusive, though it has been noted that if the duplex is maintained at a short length of five to six base pairs, the duplex would separate spontaneously at 37°C in a reasonable time scale compared to known repeat addition rates (Qi et al, 2012). Using the nearest neighbor thermodynamic parameters, the off rate (k_{off}) of the 6 bp RNA/DNA duplex (CUAACC/GGTTAG), can be approximated near $3ms^{-1}$ at 37°C, which gives a half-life ($T_{1/2}$) of 0.2ms (Sugimoto et al, 1995; Turner, 2000)(Qi et al, 2012).

Template Realignment

Once the two strands are separated, the DNA could completely dissociate from the protein, leading to a termination of repeat addition, or it could re-anneal back to the template alignment region of the RNA to prepare for another addition. The ratio of DNA dissociated to template realignment is called the translocation efficiency and is the determinant of telomerase repeat addition processivity. To limit DNA dissociation in telomerase with high repeat addition processivity, the telomeric DNA sequence remains bound the TEN domain of TERT even while not bound to the central cavity near the active site (Finger & Bryan, 2008; Jacobs et al, 2006; Wyatt et al, 2007; Zaug et al, 2008). This interaction tethers the telomeric DNA to the protein during translocation, where the DNA must dissociate from the active site for repeat synthesis. In addition to, or perhaps in concert with, the TEN domain anchor site, two telomerase accessory proteins, POT1 and TPP1, have been shown to greatly decrease the probability of DNA dissociation through direct interactions with both the single stranded DNA and TERT protein (Latrick & Cech, 2010; Wang et al, 2007). The POT1-TPP1 complex has also been implemented in telomerase recruitment to telomeric DNA which may be related to

its processivity promoting function (Latrick & Cech, 2010; Zhong et al, 2012). While the DNA is held onto the protein, the free 3' end can once again find the free RNA template and base pair with the template realignment region. Proper repositioning of the template and DNA to prevent reannealing to the templating region is aided by steric forces of the TR scaffold which stretch and compress in the regions flanking the template (Berman et al, 2011). Once positioned, the efficiency of realignment is determined in part by the length of commentary sequence in the realignment region with longer sequences producing more processive enzymes (Chen & Greider, 2003a).

Duplex Binding

Once the DNA realigns with the RNA template, the duplex is then free to rebind to the active site for another round of repeat addition. Since the newly formed duplex is very short (five nucleotides in humans), it would naturally be very unstable at 37°C. Rapid binding of the duplex to stabilize it with protein-nucleic acid contacts before it dissociates would then be important for efficient template translocation. In support of this, experiments using both duplex substrates and short eight nucleotide primers lacking upstream protein interactions to hold them to TERT during translocation showed a strong correlation between binding affinity of the substrates and the repeat addition processivity of various TERT mutants (Qi et al, 2012; Xie et al, 2010). A strong binding affinity was shown to correlate with high translocation efficiency, decreasing the number of products which dissociate after repeat addition without going through another cycle (Qi et al, 2012). This suggests that the duplex binding affinity of TERT is a major determinant of repeat addition processivity. It has also been noted that repeat addition processivity can be stimulated by the concentration of dGTP, whether by enhancing the rate of addition as

in human or the translocation efficiency in Chinese hamster telomerase (Maine et al, 1999). Experiments in *T. thermophila* show that this dGTP dependent processivity is not due to its role as the first nucleotide incorporated at the beginning of nucleotide addition and is unlikely to affect DNA primer retention, leaving the exact mechanism of processivity stimulation unknown (Hardy et al, 2001). With the newly realigned duplex bound back the TERT active site, it is then in proper position to undergo nucleotide addition again to add another telomeric repeat.

1.5 Conclusion

As more becomes known about telomerase, its unique role in Eukaryote biology is becoming clearer. In the transition from circular to linear chromosomes, life likely used an ancestral single-copy RT gene to extend each chromosome 3' end to counter act progressive DNA loss upon replication. Over time, that ancestral RT evolved to become highly specialized in its role of maintaining chromosome ends that would eventually form the unique telomere complex. The most notable adaptation is the internalization of the RNA template for DNA polymerization in the form of an integral telomerase RNA component. While little is known about this ancestral TR due to its highly divergent nature through evolution, discovery of new TRs from different phylogenetic groups is slowly increasing our understanding of TR evolution. In all TRs discovered to date, a core structure has been found composed of a RNA pseudoknot secondary structure in close special proximity to the templating region as well as a structural feature used to define the 5' end of the template (Figure 1.3). While the exact function of the pseudoknot structure remains elusive, its presence and importance in telomerases from such diverse

groups such as ciliate, yeasts, and vertebrates strongly suggests that this structure evolved early in the ancestral TR and plays such a vital role in telomerase that it has been universally conserved since. Outside the template pseudoknot domain, another important, yet less conserved, feature of telomerase is a template distal stem-loop moiety important for RNP assembly and telomerase activity. In vertebrates and both budding and fission yeasts, this takes the form of a three-way junction structure (Figure 1.3). While sequence, structural, and even mutational studies have shown strong homology between the three-way junction structure of these groups as well as its importance to telomerase activity and telomere maintenance, this structure appears far less universal in TR structures and functions. While the TR from both budding yeasts *K. lactis* and *S. cerevisiae* contains the conserved TWJ structure, it appears to be dispensable for telomere maintenance in *S. cerevisiae* while essential in *K. lactis* like with vertebrates (Brown et al, 2007; Zappulla et al, 2005). Despite this, the wide conservation of this structure and the functional similarities between yeasts and vertebrates suggests that a TWJ structure was an important feature of an ancestral TR before the branching of yeast and vertebrate lineages. In the earlier branching ciliates, no TWJ junction structure is found, but a conserved and functionally important stem-loop IV shows some similarity. Like the TWJ in vertebrates, stem-loop IV in ciliates provides a high affinity binding location to the TERT protein and is required for RNP assembly (Robart et al, 2010). However, unlike vertebrate TWJ, stem-loop IV appears to have little to no effect on the catalytic function of telomerase in the holoenzyme (Robart et al, 2010). In an evolutionary context, it is unclear if the TWJ structure in yeasts and vertebrates evolved from a structure more similar to the ciliate stem-loop IV after ciliate branched off, or if a loss-of-function

removed the TWJ structure of the ancestral TR from ciliates. While the pool of identified and characterized TRs is continually growing, ultimately there is a great need in the field to expand the reach of this pool into more diverse phylogenetic groups. With more TRs from a larger variety of specie groups, we will be able to expand our knowledge of TR evolution.

Unlike the TR, the TERT protein has remained much more conserved, though many adaptations have been made to become specialized for its unique biological role. Classically, reverse transcriptases are associated with viruses which use them to replicate their RNA genomes into a DNA intermediate during infection. Structural and functional studies of viral RTs over the decades since their first discovery in 1970 have created a solid understanding of a "traditional" reverse transcriptase. More recently, reverse transcriptases have been found in not only viruses, but also in bacterial and eukaryotic genomes. In eukaryotes, a large and diverse group of RT's are associated with retrotransposons which are self-replicating genetic elements found all throughout eukaryotic genomes. In humans, retrotransposon derived sequences make up about 50% of the entire genome and are thought to play vital roles in gene regulation (Belfort et al, 2011). While a diverse group, telomerase shares quite a few similarities with other eukaryotic RTs which could shed clues on its evolutionary origin. While most retrotransposons use an endonuclease activity to insert its genomic material in the middle of the genome, several RTs such as the Penelope-like element (PLE) and long interspersed element-1 (LINE-1) have been shown to act in a endonuclease-independent mechanism at free DNA 3' ends in a manner similar to telomerase and even have been shown to associate with and act on telomere ends (Kopera et al, 2011). In Drosophila, due

to a loss of telomerase at some point in its evolution, several non-telomerase retrotransposons have come together to compensate for telomerase loss and created a novel method to maintain telomere ends (Pardue & DeBaryshe, 2011). Together, these observation suggest a common ancestral RT in early eukaryotes that diverged and specialized into retrotransposons and telomerase independently to fulfill distinct functional roles.

1.6 Projects

While our knowledge of the telomerase enzyme grows each year, there are still many gaps in our understanding that need to be filled. As a ribonucleoprotein complex which acts on single stranded DNA, there are many independent parts of telomerase that must come together to perform its important function. This study aims to identify and characterize several novel interactions and pieces of telomerase important for activity, processivity, and assembly of the active enzyme.

In chapter 2, the interaction between telomerase and the duplex formed from the RNA template and telomeric DNA are characterized. Particularly, we examine how the sequence of the RNA/DNA duplex affects duplex usage and nucleotide addition. Using a template free telomerase system, duplexes with different permutations of the telomeric sequence are examined. Several duplex modifications are also used to find the factors in the duplex sequence which affect its usage by the telomerase enzyme.

In chapter 3, the role of the template sequence in the native template containing telomerase is explored. Template sequence permutations and mutations are also introduced into the native *in vivo* reconstituted telomerase enzyme to examine how duplex sequence recognition affects the native enzyme and what role it plays in the

telomerase reaction. We also explore how other known RNA interactions work in relation to the novel sequence dependence found in this study.

In chapter 4, mutational analysis is employed to characterize several novel residues in the TERT protein and RNA components. A well conserved adenosine residue found in the junction of the TWJ region of many TRs was determined through structural studies to likely be important for binding of the TWJ to the TRBD region of the TERT protein. Mutations of this residue in multiple species showed that this conserved residue is indeed important for telomerase activity in all species tested and appears to be a conserved mechanism for TWJ binding. Several mutations found in the TERT protein and shown to be linked to disease in humans were also studied. Specifically, two motif T mutations were discovered to be linked to Hoyeraal Hreidarsson syndrome due to decreases in telomerase processivity. We investigated the motif T domain using alanine screening to determine the motif's role in processivity. We then characterized one of the disease mutant residues, K570, to determine a possible mechanism for it defects in processivity.

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CHAPTER 2

TELOMERASE RECOGNIZES THE SEQUENCE OF THE RNA/DNA DUPLEX TO REGULATE NUCLEOTIDE ADDITION

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and

Brown, A. F., Qi, X., Chen, Y., Podlevsky, J. D., Mingyi, X., & Chen, J. J.-L. (2014). A Self-Regulating Template in Human Telomerase. *PNAS*. (In review)

2.1 Introduction

The ends of human chromosomes are composed of precise repetitions of a 6-nucleotide (nt) sequence synthesized by the specialized reverse transcriptase (RT), telomerase (Meyne et al, 1989; Moyzis et al, 1988). The telomerase core enzyme is minimally composed of the catalytic telomerase reverse transcriptase (TERT) and the integral telomerase RNA (TR) components. Human TR (hTR) is a 451-nt RNA containing an exceedingly short 11-nt template which encodes for the telomeric DNA repeat GGTTAG (Figure 2.1, left). The resulting highly repetitive tract of DNA is bound in a sequence-specific manner by the shelterin complex which protects natural chromosome termini from end-to-end fusions and other DNA damage responses (Palm & de Lange, 2008; Sfeir & de Lange, 2012). High fidelity synthesis of telomeric DNA repeats by telomerase is crucial for maintaining telomere function and chromosome stability. Appending the termini of telomeres with even single-nucleotide variations in the telomeric DNA repeat sequence is sufficient for compromising the protective function of the shelterin complex, culminating in deleterious genome instability and cell death (Guiducci et al, 2001; Kim et al, 2001; Li et al, 2004; Marusic et al, 1997; Stohr & Blackburn, 2008; Stohr et al, 2010; Vulliamy et al, 2006).

While TR sequences are highly divergent across taxa, the TR template itself is highly conserved (Chen et al, 2000; Xie et al, 2008). Within vertebrates, the template sequence is conserved as a specific register with the 5' boundary defined physically by a long-ranged based-paired region known as helix P1 (Figure 2.1) (Chen & Greider, 2003b). Extensive evidence has demonstrated the importance of the specific TR template sequence for telomerase enzymatic function, whereby alterations in the template

sequence alone changes the rate and processivity of telomeric DNA repeat synthesis (Drosopoulos et al, 2005; Förstemann et al, 2003; Gilley & Blackburn, 1996; Gilley et al, 1995; Qi et al, 2012). Additionally, telomerase has been shown to exhibit differential activity toward telomeric DNA primers with permuted sequences (Maine et al, 1999).

During telomere repeat synthesis, telomerase catalyzes nucleotide addition to the DNA primer which forms a duplex with the RNA template within the active site. Each nucleotide addition creates a discrete RNA/DNA duplex sequence inside the binding pocket of the catalytic TERT subunit. It has remained elusive how the telomerase active site handles this growing and dynamically changing RNA/DNA duplex during processive telomeric DNA repeat synthesis. In this study, we investigated how the human telomerase active site utilizes its specific RNA template during nucleotide polymerization. By employing a telomerase-free telomerase system and specific assay conditions, we discovered that hTR is embedded with a single-nucleotide signal to pause nucleotide addition at an exact position, defining a template boundary without the canonical P1 defined structural boundary.

2.2 Material and Methods

Oligonucleotides and RNA/DNA hybrid substrates

All RNA and DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT). The sequences of oligonucleotides used in each experiment are shown in the text or figures. The RNA/DNA duplexes were prepared by adding the RNA and complementary DNA oligos at a final concentration of 100 μM in 1x annealing buffer (100mM Tris–HCl pH 7.5, 500mM NaCl and 50mM EDTA). Duplex formation

was facilitated by incubation at 70°C for 3 min, followed by slowly cooling to room temperature.

Reconstitution of telomerase

Reconstitution of human telomerase *in vitro* was carried out in RRL as previously described (Xie et al, 2010). Briefly, hTERT was expressed from pNFLAG-hTERT using the TnT® T7 Quick coupled transcription/translation kit (Promega) according to the manufacturer's instruction. The hTR fragments, PK-TF (nt 64–184) and CR4/5 (nt 239–328), were transcribed *in vitro*, gel purified and added at a final concentration of 1 μM to assemble with hTERT in RRL.

Template-free telomerase activity assay

To analyze TF telomerases, 1 μL *in vitro* reconstituted TF telomerase was assayed in a 10 μL reaction containing 1X duplex reaction buffer (50 mM Tris- HCl, pH8.0, 50 mM KCl, 2 mM $MgCl_2$, 2 mM DTT and 1 mM spermidine), 50 μM pre-annealed RNA/DNA duplex, 2 μM dGTP, 0.165 μM α-^{32}P-dGTP (3000Ci/mmol, 10 mCi/ ml, Perkin-Elmer) and 0.5 mM dATP as indicated. The reaction was incubated at 20°C for 60 min and terminated by phenol/chloroform extraction, followed by ethanol precipitation. The DNA products were resolved on an 18% polyacrylamide/8 M urea denaturing gel, dried, exposed to a phosphorstorage screen and imaged on a Bio-Rad FX-Pro phosphorimager. The AMV RT duplex assay was identical to the TF telomerase duplex assays with the exception of substituting TF telomerase with 0.5 unit of AMV RT enzyme (Promega) and the products were analyzed directly by denaturing polyacrylamide gel electrophoresis. The size markers for template free duplex assay were prepared in a 10 μL reaction containing 1X reaction buffer (100 mM sodium cacodylate, pH 6.8, 1 mM $CoCl_2$ and 0.1

mM DTT), 10 μM oligonucleotide as indicated, 10 units of terminal deoxynucleotidyl transferase (TdT, Affymetrix) and 0.165 μM α-^{32}P-dGTP (3000Ci/mmol, 10 mCi/ml, Perkin-Elmer). The reaction was incubated at 30°C for 5 seconds and terminated by addition of 10 μL 2X formamide loading buffer (10 mM Tris-HCl, pH 8.0, 80% formamide, 2 mM EDTA, 0.08% bromophenol blue and 0.08% xylene cyanol).

Duplex competitive inhibition assay

One microliter of *in vitro* reconstituted template-free telomerase was assayed in a 10 μL reaction containing 1X duplex reaction buffer, 50 μM non-extendable duplex substrate (competitive inhibitor) as substrate, 2 μM dGTP, 0.165 μM α-^{32}P-dGTP (3000Ci/mmol, 10mCi/ ml, Perkin-Elmer). 50 μM of extendable P1 substrate was also added to reactions as indicated (+ on gel). The reaction was incubated at 20°C for 60min before termination with phenol/chloroform extraction followed by ethanol precipitation. The DNA products were resolved on an 18% polyacrylamide/8M urea denaturing gel, dried, exposed to a phosphorstorage screen and imaged on an FX-Pro phosphorimager (Bio-Rad).

2.3 Results

The duplex sequence defines nucleotide addition pausing.

To investigate how the telomerase active site interacts with different duplexes, we employed a human telomerase lacking the template region from hTR (Qi et al, 2012) and examined telomerase activity with pre-annealed RNA/DNA duplexes as substrates (Figure 2.1, right). This template-free (TF) telomerase was assayed with six permuted RNA/DNA duplexes, P1-P6, that represent the six distinct sequence registers formed

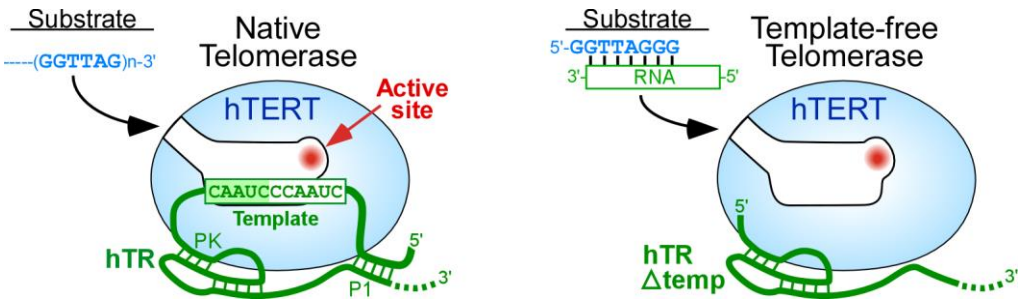

Figure 2.1: Schematic comparison of native (left) and template-free (right) human telomerases. In the native telomerase, the RNA template is tethered to the 5' P1 helix and the 3' pseudoknot structures. The TF telomerase was reconstituted *in vitro* with hTERT and a 5' truncated hTR (Δtemp) that lacks the template and the P1 helix. Substrates for the activity assay are single-stranded DNA for native telomerase or a pre-annealed RNA/DNA duplex for TF telomerase.

during nucleotide addition for the synthesis of a GGTTAG repeat (Figure 2.1B, left). Interestingly, TF telomerase exhibited distinct extension patterns and diverse activities with each permuted duplex. In the presence of ^{32}P-dGTP, the DNA primers from all duplex substrates were extended by only one nucleotide (Figure 2.2, lanes 1, 3, 5, 7 and 11), aside from P5 which was nearly inactive (Figure 2.2, lane 9). With the addition of dATP to the reaction, P1, P2, and P6 were extended by three nucleotides, reaching the end of the 3-nt RNA template as expected of a conventional RT (Figure 2.2, lanes 2, 4 and 12). Unexpectedly, TF telomerase was only able to extend P3 by two nucleotides and P4 by a single nucleotide (Figure 2.2, lanes 6 and 8). The same premature pausing was also seen with longer templates only when the telomeric sequence was used (Figure S2.1). The differences in the extension pattern between these permuted duplexes suggest that the duplex sequence alone determines the pausing site during nucleotide addition.

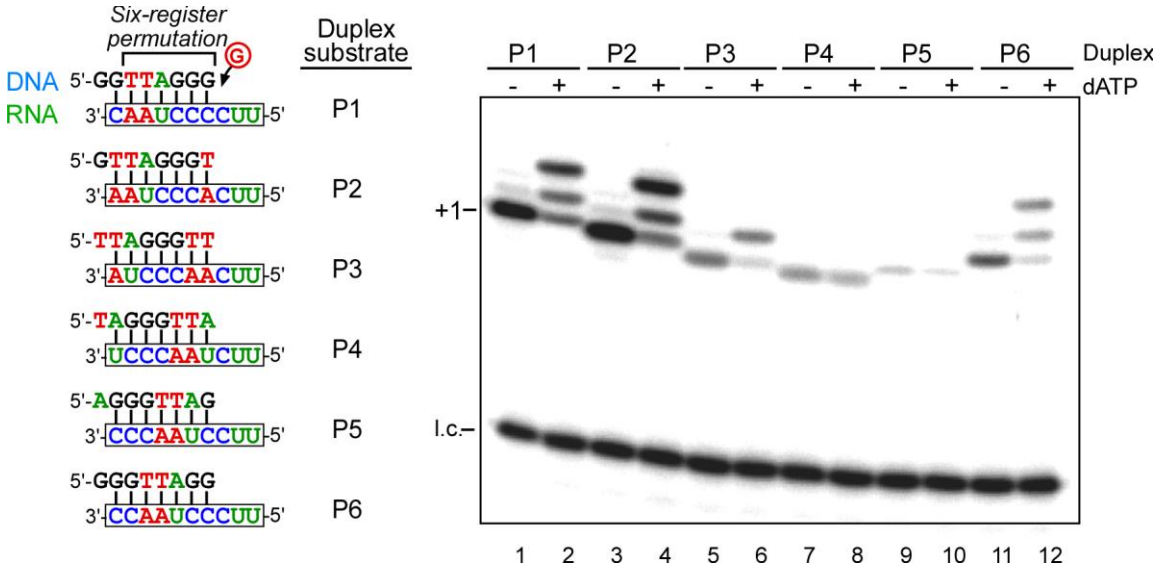

Figure 2.2: A single nucleotide in the RNA/DNA duplex signals a pause in nucleotide addition with template-free telomerase. (*left*) Sequences of permuted telomeric RNA/DNA duplexes P1-P6 or P4 substitution variants. (*right*) Activity assay of *in vitro* reconstituted TF telomerase with various duplex substrates. Substrates were extended by the enzyme with α-^{32}P-dGTP in the presence (+) or absence (-) of 0.5 mM dATP as denoted above the gel. An α-^{32}P end-labeled 18-mer oligonucleotide was used as a loading control (l.c.).

A single base pair in the duplex defines the nucleotide addition pause site.

We further examined which base pair(s) in the duplex signals for this unanticipated nucleotide addition pausing. Transversion substitutions were introduced for each base pair in the P4 duplex and each variant was assayed with TF-telomerase (Figure 2.3A, left). All P4 substitutions retained the pause signal with the exception of P4e, which had the first rA:dT base pair changed to rU:dA. P4e permitted the addition of a second nucleotide, shifting the pause site from +1 to +2 (Figure 2.3A, lane 12). However, P4e did not allow the addition of the third nucleotide, pausing nucleotide addition prior to reaching the end of the RNA template. We suspected that the neighboring rA:dT base pair in the duplex potentially became a new pause signal. Indeed, transversion

substitutions at both rA:dT base pairs completely abolished the sequence-defined pausing regardless of sequence permutation tested (Figure 2.3B). Thus, the first rA:dT base pair formed in the duplex induced a pause in DNA synthesis after incorporating three additional base pairs.

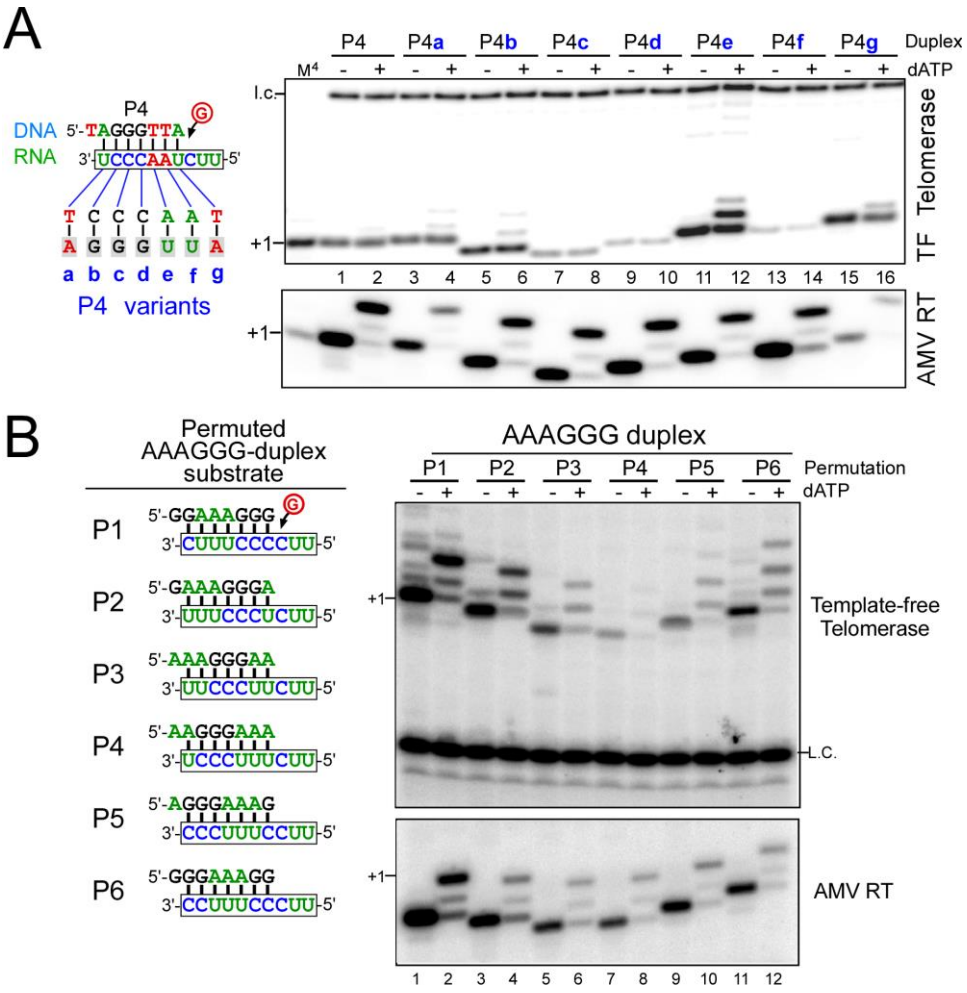

Figure 2.3: A single rA:dT base pair in the RNA/DNA duplex signals a pause in nucleotide addition with template-free telomerase. (A) Gel image courtesy of Xiaodong Qi. (A-B, *left*) Sequences of RNA/DNA duplexes used. (A-B, *right*) Activity assay of *in vitro* reconstituted TF telomerase (upper panel) and AMV RT (lower panel) with various duplex substrates. Substrates were extended by the enzyme with α-^{32}P-dGTP in the presence (+) or absence (-) of 0.5 mM dATP as denoted above the gel. An α-^{32}P end-labeled 18-mer (A) or 5-mer (B) oligonucleotide was used as a loading control (l.c.). The DNA primer TAGGGTTA (M_4) extended by one ^{32}P-dGTP with terminal deoxynucleotidyl transferase (TdT) were included as size markers.

To discern the functional groups in the rA:dT base pair necessary to induce the pause in nucleotide addition, we designed variants of duplex P4 with specific functional groups modified in the first rA:dT base pair (Figure 2.4, top). The TF telomerase assays revealed that transition substitutions, rA:dU, rG:dC, or rP:dT (rP = 2-6-diamino-purine-ribose), which altered individual functional groups in the major and minor grooves of the duplex, had only slight effects on the position of pause site (Figure 2.4, lanes 5-10). However, the transversion substitution of the first rA:dT base pair in P4 to rU:dA (P4-UA) showed a noticeable shift in the pause site (Figure 2.4, lane 3-4). This result suggests that any ribonucleotide purine:deoxyribonucleotide pyrimidine base pair, rR:dY, is sufficient to signal pausing. Thus, it appears that the telomerase active site does not recognize specific functional groups in the signaling rA:dT base pair. Moreover, extensive mutagenesis of surface residues in the duplex binding pocket of TERT, predicted by homology modeling with the *Tribolium castaneum* TERT crystal structure, failed to identify specific residues responsible for sensing the rA:dT pause signal in template free (Figure S2.2). To see if RNA in the nearby pseudoknot could play a role, we also tested various pseudoknot truncations for an effect on pause signaling, but no such change was observed (Figure S2.3). The apparent lack of specificity for nucleic acid functional groups, together with our inability to discern specific sensing residues in TERT, suggests an indirect readout of the rR:dY pause signal by the TERT protein.

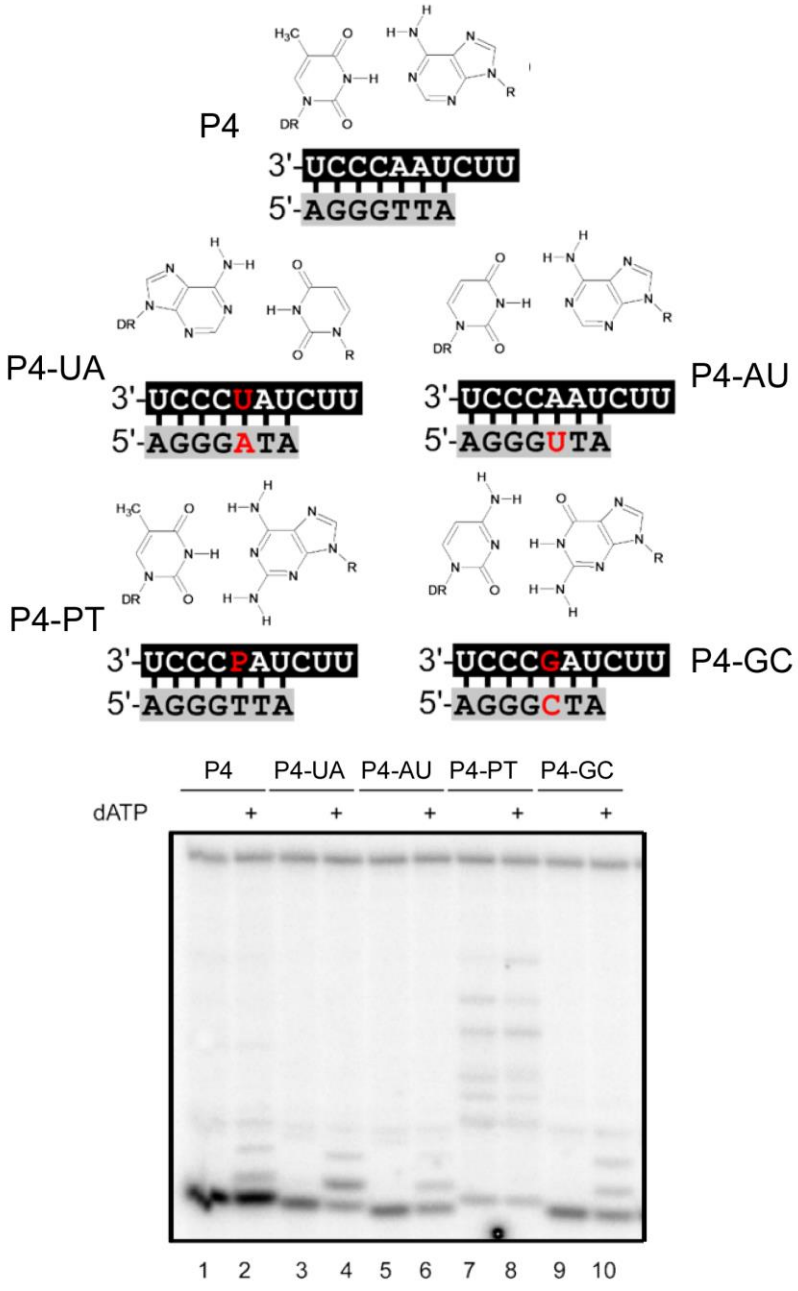

Figure 2.4: A ribonucleotide purine:deoxyribonucleotide pyrimidine base pair makes up the sequence defined boundary. (*above*) Sequences of permuted telomeric RNA/DNA duplexes P4 variants with substitutions of the first rA:dT base pair. P = 2-6-diamino-purine. (*below*) Activity assay of *in vitro* reconstituted TF telomerase with variant duplex substrates. Substrates were extended by the enzyme with α-^{32}P-dGTP in the presence (+) or absence (-) of 0.5 mM dATP as denoted above the gel. An α-^{32}P end-labeled 18-mer oligonucleotide was used as a loading control (l.c.).

P5 inactivity results from catalysis deficiency

In addition to the distinct extension patterns, the six permuted RNA/DNA duplexes also exhibited markedly different activities with TF-telomerase (Figure 2.2). Among the six duplexes, P5 consistently displayed little to no activity with TF-telomerase. This is consistent with a previous report that Chinese hamster telomerase failed react with a DNA primer ending in the exact register as P5, GGTTAG (Maine et al, 1999). The inactivity of the P5 duplex with TF telomerase presumably results from the inability of telomerase to either bind the P5 duplex or catalyze nucleotide addition onto this substrate. To discern the relative binding affinity of P5 to the telomerase active site, we performed a competitive inhibition assay with the six permuted telomeric duplexes as inhibitors competing against a single duplex substrate for binding to the telomerase active site (Figure 2.5). The RNA template for these telomeric duplex inhibitors was blocked with an rG residue, preventing incorporation of dGTP and therefore could not be extended when bound to the telomerase active site. Thus, the inhibition of telomerase activity with the non-telomeric substrate correlates with telomerase binding affinity for the duplex inhibitors. Interestingly, all duplex permutations showed fairly similar levels of inhibition and did not correlate with observed activities of the P1-P6 duplexes (Figure 2.5). While a direct measurement of relative K_d values of each duplex is needed to confirm these results, they suggest that P5 inactivity may result from inefficient catalysis, such as improper positioning of DNA 3'-OH within the catalytic site rather than decreased affinity to the active site.

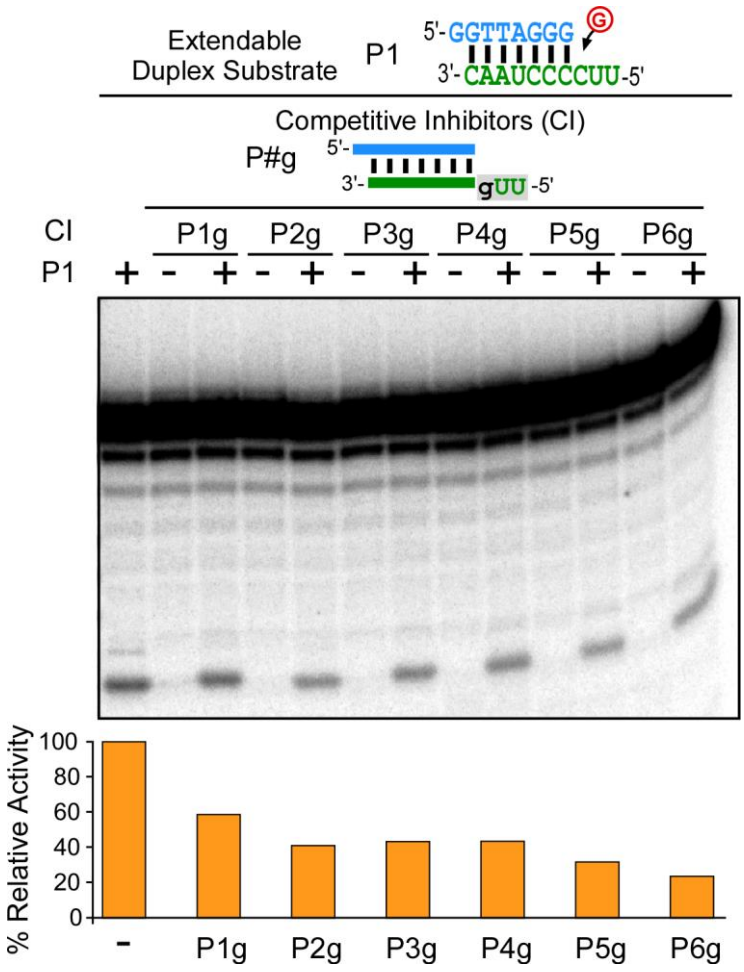

Figure 2.5: Competitive inhibition assay of P1-P6 duplex substrates. *In vitro* reconstituted TF telomerase with non-extendable duplex competitor variants (P1g-P6g) with or with P1 substrate. Substrates were extended by the enzyme with α-^{32}P-dGTP. A ^{32}P end-labeled 15-mer oligonucleotide was used as a loading control (l.c.). Quantitation of the relative activity in the presence of competitor is displayed below the gel.

Duplex length and DNA overhang effect nucleotide-addition processivity

In addition to the sequence of the duplex effecting nucleotide addition processivity, previous work has shown that the length of a duplex can have drastic effects on activity of duplex substrates through enzyme turnover as well as altering nucleotide addition processivity (Qi et al, 2012). When examine the nucleotide addition processivity

of short RNA/DNA duplexes, a 5 bp duplex substrate could only be extended by a single nucleotide regardless of the template length provided (Figure 2.6A, lanes 1-3). In contrast, a 6bp hybrid substrate can be extended 2 nt (Figure 2.6A, lanes 4-6). We suspect the longer duplex might transiently generate a single-stranded DNA or RNA overhang at the duplex end distal the active site. The potential effects of overhangs in the RNA/DNA hybrid substrates on nucleotide-addition processivity were assayed with substrates containing a 5bp hybrid and either a 3′ RNA or 5′ DNA overhang (Figure 2.6B). The duplex substrates with 5′ DNA overhangs were extended by the addition of two nucleotides in the presence of dTTP, while the substrate with the 3′ RNA overhangs failed to extend beyond a single nucleotide (Figure 2.6B, lanes 4, 6, 8 and 10). This suggests that a putative TERT active site proximal anchor site binds the 5′ DNA overhang on the 5-bp RNA/DNA hybrid to facilitate nucleotide-addition processivity. In addition, the K_m^{app} of the 5-bp substrates with the 2-nt 5′ DNA overhang is ∼5 μM, substantially lower than the 15 μM for the substrate with a single nucleotide DNA overhang (Figure 2.6C). Thus, the presence of a DNA residue at the −7 position is important for binding affinity of the 5-bp RNA/DNA hybrid to the active site (Figure 2.6C).

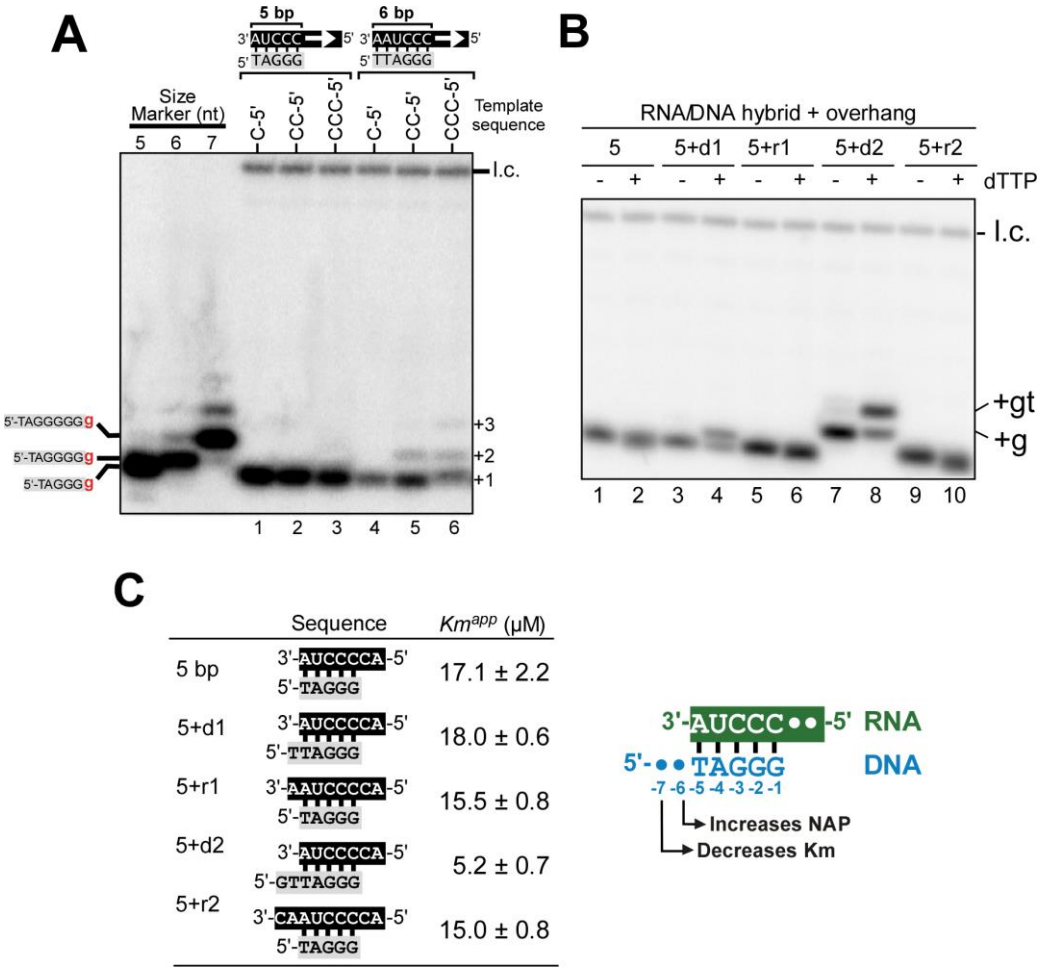

Figure 2.6: Effect of the RNA/DNA hybrid length and overhangs on Km rate and nucleotide-addition processivity. (A) I*n vitro* reconstituted template-free telomerase was assayed on ice for 30 min using telomeric DNA/RNA hybrids of 5 bp (lanes 1-3) or 6 bp (lanes 4-6). The RNA template sequence contains 1, 2, or 3 cytosines as indicated above the gel. DNA size markers (5, 6 and 7 nt) were generated by 3'-end labeling of DNA oligonucleotides (5'-TAGGG, 5'-TAGGGG, and 5'-TAGGGGG) with terminal deoxynucleotidyl transferase and α-^{32}P-dGTP. A ^{32}P end-labeled 18-nt DNA oligonucleotide was used as the loading control (l.c.). (B) Effects of overhangs of RNA/DNA hybrid substrates on nucleotide-addition processivity. The RNA/DNA hybrid substrates contain a 5 bp duplex and a 1–2 nt overhang at either the 5' DNA (5+d1 and 5+d2) or the 3' RNA (5+r1 and 5+r2) end. A ^{32}P end-labelled 15 nt DNA oligonucleotide was used as a loading control (l.c.). (C) K_m of RNA/DNA hybrid substrates with and without overhangs. Sequences and structures of the RNA/DNA hybrid substrates are shown. Diagram of RNA/DNA hybrid substrate indicating nucleotides important for nucleotide-addition processivity (NAP) and K_m.

Processivity factors POT1 and TPP1 don't effect RNA/DNA duplex usage

It has been well established that the telomerase accessory proteins POT1 and TPP1 have a drastic stimulatory effect on telomerase processivity (Latrick & Cech, 2010; Wang et al, 2007). The primary mechanism for this effect has been suggested to be due to delaying the dissociation of single stranded DNA products during processive repeat addition (Latrick & Cech, 2010). However, since the affinity of telomerase to its RNA/DNA duplex has been shown to be correlated with telomerase processivity, it is possible that these accessory proteins might affect how telomerase handles RNA/DNA substrates. To test this, we purified recombinant hPOT1 and hTPP1 and tested them with both the native and template free telomerase assay for any stimulatory effect on activity and processivity. As expected, when added to the native telomerase reaction, TPP1 showed a slight increase in processivity, but when both POT1 and TPP1 were added the processivity increased dramatically (Figure 2.7, lanes 1-3). However, no discernable effects were observed when repeated with template free telomerase using a pre-annealed RNA/DNA duplex substrate (Figure 2.7, lanes 4-6). This suggests that the processivity effects of POT1 and TPP1 are independent of interactions with the RNA/DNA duplex.

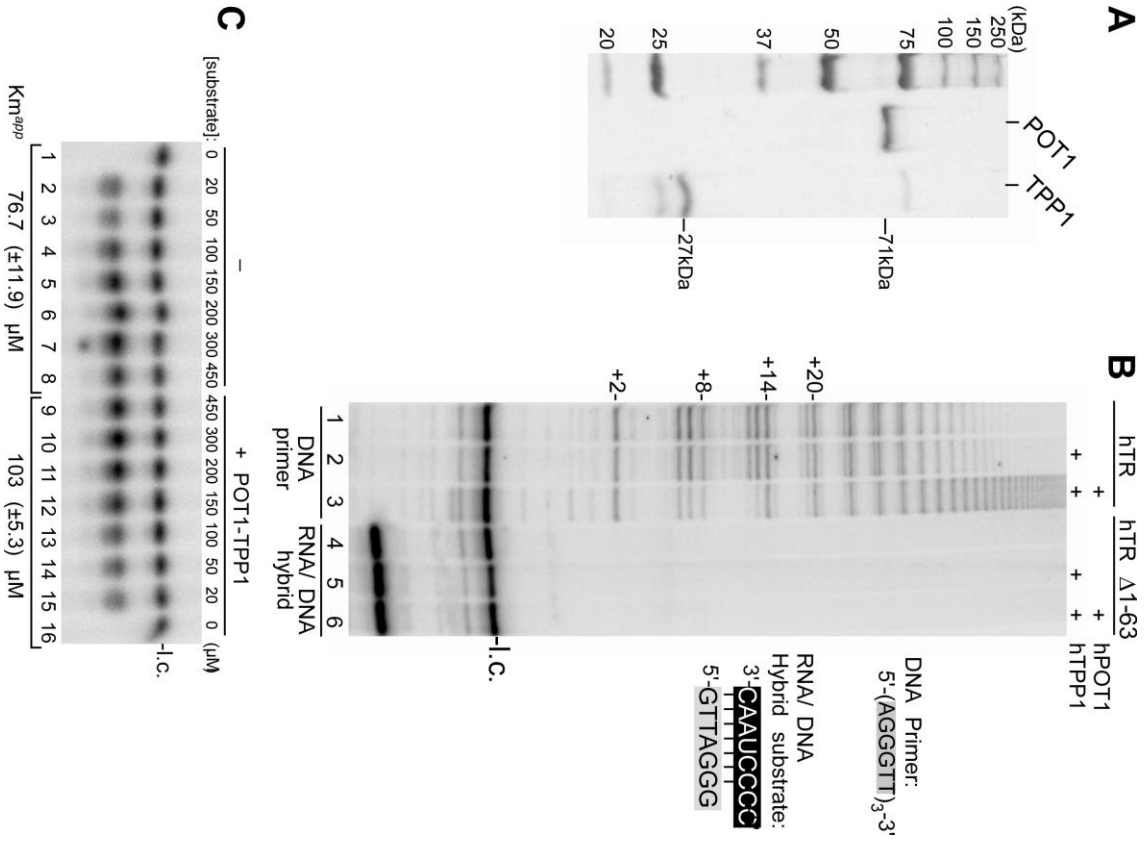

Figure 2.7: Effect of POT1-TPP1 on duplex binding to template-free telomerase. (A) SDS-PAGE analysis of purified POT1 and TPP1 proteins. The hPOT1 protein was purified from baculovirus infected sf9 cells as described previously (Lei et al., 2004). The hTPP1 protein fragment (a.a. 89-334) was expressed in E. coli cells and purified as described previously (Wang et al., 2007). Molecular weight (in kilodaltons, kDa) of protein marker (lane 1) is indicated to the left side of the gel and the size of POT1 (71kDa) and hTPP1N (27kDa) are indicated to the right side. (B) Effects of POT1-TPP1 on the activity of template-containing and template-free telomerases. Direct telomerase assay was performed in the presence of 1 μM TPP1 (lanes 2 and 5) or 1μM POT1-TPP1 (lanes 3 and 6) with telomerase in vivo reconstituted from either template containing hTR (lanes 1 to 3) or template-free hTRΔ1-63 (lanes 4 to 6). The template-containing telomerase was assayed with single-stranded DNA primer, while the template-free telomerase was assayed with a 7 bp RNA/DNA hybrid substrate. A 32P end-labeled 15-nt DNA oligonucleotide was used as the loading control (l.c.). (C) The K_m of template-free telomerase to the 7 bp RNA/DNA hybrid substrate in the absence or presence of POT1-TPP1. The template-free telomerase activity assay was performed with a substrate concentration of 0 to 450 μM in the absence (lanes 1 to 8) or presence (lanes 9 to 16) of 1μM of POT1-TPP1. The activity was normalized and plotted against the substrate concentration and the K_m^{app} was determined by the best fit to the Michaelis–Menten equation. Average K_m^{app} and standard deviations were calculated from two independent experiments.

2.4 Discussion

Our results shed new insights into the telomerase catalytic site, specifically how telomerase handles the RNA/DNA duplex during nucleotide addition. While the process of template translocation to regenerate the template has been a hot area of study, relatively little attention has been given to the process of nucleotide addition. Taken together, the data produced here can be used to create a more detailed model of the initiation, continuation, and termination of nucleotide addition in telomerase. In this model, a previously annealed RNA/DNA duplex composed of the TR template and the 3' end of the telomeric DNA will bind to the active site, facilitated though interactions between a template proximal DNA anchor site and the unpaired single stranded DNA 5' of the duplex. After each nucleotide addition occurs, these single stranded DNA interactions help translocate the RNA/DNA duplex through the active site, repositioning the newly formed 3' end into the active site so that subsequent nucleotide addition can occur. Nucleotide addition will then continue until telomerase detects an rR:dY base pair in the correct position relative to the active site (4 nucleotides away). Once this is detected, nucleotide addition terminates by a pausing in polymerization due a defect in the catalysis of the P5-like duplex.

While this data can only support this model in the context of the template free telomerase system, the fact that the sequence dependent pause corresponds to the exact sequence register of the native telomerase is strong evidence for the relevance of this mechanism in the template containing enzyme. A functional sequence dependent boundary mechanism is one explanation for why the template sequence register 5'-GGTTAG-3' is so well conserved (Chen & Greider, 2003b; Podlevsky et al, 2008). It

also would explain why previous experiments which disrupt the P1 defined structural template boundary only showed an increase in template boundary read through instead of a complete elimination of template definition (Chen & Greider, 2003b). This data introduces a completely novel mechanism for regulating nucleotide addition and defining a template boundary not seen before in telomerase or other polymerases.

2.5 References

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CHAPTER 3

NATIVE TELOMERASE USES A TEMPLATE DEPENDENT PAUSING SIGNAL TO PRODUCE HOMOGENEOUS TELOMERIC ENDS

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3.1 Introduction

Unique from traditional reverse transcriptases, the catalytic protein component of telomerase (TERT) has coevolved with an integral telomerase RNA component (TR), which contains, in part, the RNA template used to extend telomeres. This coevolution of protein and RNA has produced a reverse transcriptase which has become specialized in adding short DNA repeats onto single stranded telomere ends. However, we have recently shown that the telomerase active site has retained the ability to bind double stranded RNA/DNA duplex like a conventional reverse transcriptase and that this ability is essential to repeat addition processivity in human telomerase (Qi et al, 2012). Our investigation revealed that a telomerase enzyme without the templating region of the TR could bind and extend an external RNA/DNA duplex with both a telomeric and non-telomeric sequence. However, while telomerase RNA from diverse species have evolved to be drastically different in size and primary sequence, most RNA's have conserved a similar template sequence, encoding for a canonical GGTTAG or similar repeat in most studied species (Podlevsky et al, 2008). One theory for this conservation of template sequence is the coevolution of telomerase with important telomere binding proteins which are specialized to bind T/G rich DNA and help cap the chromosome ends (Shakirov et al, 2009). Apart from the function of the telomere, there has been evidence that specific templating residues play a greater role in the biochemistry of telomerase then just acting as a template for nucleotide addition. Mutation of the RNA template in *Tetrahymena thermophila* revealed that specific templating residues effected enzyme fidelity and product dissociation not explainable by its ability to base pair with the DNA primer (Gilley & Blackburn, 1996; Gilley et al, 1995). In human telomerase, mutation of

the RNA template found that changes in the template sequence could lead to significant changes in nucleotide addition rate which, again, could not be attributed solely to the base pairing of the RNA template with the DNA primer (Drosopoulos et al, 2005).

The importance of a well-defined telomere sequence is highlighted by numerous studies that show even single-nucleotide variations in the repeat sequence is enough to compromise the protective function of the telomeres, culminating in deleterious genome instability and cell death (Guiducci et al, 2001; Kim et al, 2001; Li et al, 2004; Marusic et al, 1997; Stohr & Blackburn, 2008; Stohr et al, 2010; Vulliamy et al, 2006). Since telomerase only uses a short region of its TR as a template, proper definition of the template ends is crucial for proper telomere synthesis. TRs from different phylogenetic groups have evolved slightly different methods to define their template ends, but is usually a RNA structural elements, or template boundary elements (TBE), which prevents polymerization past the template boundary (Podlevsky & Chen, 2012). In humans, the TBE is composed of a helix formed upstream of the template, called helix P1. The length of the single stranded RNA between the 5' end of the template and the P1 helix has been shown to define the template end and prevent read through (Chen & Greider, 2003b).

In previous work (chapter 2), we showed that when separated from other protein-RNA or protein-DNA interactions, template-free telomerase will pause during nucleotide addition at the native telomeric end sequence of GGTTAG. These observations suggest that telomerase can read the sequence of the telomeric repeat during synthesis to regulate its biochemical function. In this study, we seek to investigate how this unique sequence dependent pause in nucleotide addition functions in the context of the native, template

containing telomerase. We show that the observed sequence dependent pause site works cooperatively with the structurally defined template boundary to preclude incorporation of non-telomeric nucleotides from residues outside the template region. Additionally, this sequence-defined pausing mechanism prevents premature arrest of nucleotide synthesis and is the predominate mechanism for generating the characteristic 6-nt ladder banding-pattern of telomeric DNA products *in vitro*. In the absence of the pausing signal, telomerase stalls nucleotide addition at multiple sites along the template, generating DNA products with diverse repeat registers at the termini. Our findings demonstrate a unique self-regulating mechanism of the human TR template for high fidelity synthesis of DNA repeats.

3.2 Material and Methods

In vitro reconstitution of human telomerase.

The hTERT protein was expressed in rabbit reticulocyte lysate (RRL) from pNFLAG-hTERT plasmid DNA using the TnT T7 Quick coupled kit (Promega) following the manufacturer's instruction. The hTR pseudoknot (32-195 nt) and CR4/5 (239-328 nt) fragments were *in vitro* transcribed, gel purified and assembled with hTERT in RRL at a final concentration 1.0 μM.

In vivo reconstitution of human telomerase.

293FT cells were grown in DMEM medium (Corning) supplemented with 10% FBS, 1X Penicillin-Streptomycin-Amphotericin B mix (Lonza) and 5% CO2 at 37°C to 80–90% confluency. Cells in a 6-well plate were transfected with 0.4 μg of pcDNA-NFLAG-hTERT, 1.6 μg of pBS-U1-hTR (Cristofari et al, 2007) and 6 μL of Fugene HD

transfection reagent (Promega) following manufacturer's instruction. Cells were harvested 48 hours post transfection and homogenized in lysis buffer (10 mM Tris–HCl pH 7.5, 200 mM NaCl, 1 mM EGTA, 0.5% CHAPS, 1 mM $MgCl_2$, 10% glycerol, 5 mM β-mercaptoethanol and 1x complete protease inhibitor cocktail (Roche)). Fifty microliters of cell lysate was combined with 5 μL Anti- FLAG® M2 Magnetic Beads (Sigma) pre-washed with 1X TBS buffer (50 mM Tris-HCl pH 7.4 and 150 mM NaCl), and incubated at 4°C with gentle rotation for 1 hour. The beads were washed three times with 500 μL of 1X TBS buffer and once with 100 μL 1X telomerase reaction buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 3 mM $MgCl_2$, 2 mM DTT and 1 mM spermidine).

Telomerase activity assay

For analyzing native telomerase, 5 μL immuno-purified *in vivo* reconstituted telomerase on beads was assayed in a 10 μL reaction containing 1X telomerase reaction buffer, 1 μM DNA primer, 1 mM dTTP, 1 mM dATP, 2 μM dGTP, and 0.165 μM α-^{32}P-dGTP (3000Ci/mmol, 10 mCi/ml, Perkin-Elmer). The reaction was incubated at 30°C for 60 min and terminated by phenol/chloroform extraction followed by ethanol precipitation. The telomerase assay was also performed with 2 pmol of 5' ^{32}P-end labeled primer $(TTAGGG)_3$ without the addition of α-^{32}PdGTP. The DNA products were resolved on a 10% polyacrylamide/8 M urea denaturing gel, dried, exposed to a phosphorstorage screen and imaged on a Bio-Rad FX-Pro phosphorimager. The size markers for telomerase assays were prepared in a 10 μL reaction containing 1X reaction buffer (100 mM sodium cacodylate, pH 6.8, 1 mM $CoCl_2$ and 0.1 mM DTT), 10 μM oligonucleotide as indicated, 10 units of terminal deoxynucleotidyl transferase (TdT, Affymetrix) and 0.165 μM α-^{32}P-dGTP (3000Ci/mmol, 10 mCi/ml, Perkin-Elmer). The

reaction was incubated at 30°C for 5 seconds and terminated by addition of 10 μL 2X formamide loading buffer (10 mM Tris-HCl, pH 8.0, 80% formamide, 2 mM EDTA, 0.08% bromophenol blue and 0.08% xylene cyanol).

Northern blotting

RNA was extracted from 5 μL immuno-purified *in vivo* reconstituted telomerase on beads with 50 μL 1X SDS elution buffer (25 mM Tris-HCl, pH8.0, 200 mM NaCl, 5 mM EDTA and 1% SDS) followed by phenol/chloroform extraction and ethanol precipitation. Extracted RNA was resolved on a 4% polyacrylamide/8 M urea denaturing gel, electrotransferred and UV crosslinked to a Hybond-XL membrane (GE Healthcare). The hTR riboprobe and hybridization were performed as previously described (Xie et al, 2010).

POT1 exonuclease protection assay

The hPOT1 protein was purified from baculovirus infected sf9 cells as described previously (Lei et al, 2004; Qi et al, 2012). Prior to exonuclease addition, ^{32}P-end-labeled DNA oligonucleotides were incubated at a concentration of ~10 nM in 1x PE reaction buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM DTT, 3 mM $MgCl_2$, and 1 mM spermidine) with purified hPOT1 protein at a concentration of 4μM at room temperature for 15 minutes. After incubation, 5 U of Exonuclease T (5U/μL, NEB) in 1 x PE reaction buffer was mixed with the prebound oligonucleotides and POT1 and incubated at room temperature for another 10 minutes. Reaction was terminated by ethanol precipitation. Resuspended products were resolved on a 10% polyacrylamide/8 M urea denaturing gel, dried, exposed to a phosphorstorage screen and imaged on a Bio-Rad FX-Pro phosphorimager.

3.3 Results

Native telomerase exhibits sequence-defined pausing

Within the full-length hTR, the sequence-defined pause site would coincide with the physical template boundary. To uncouple and separate the potential sequence-defined pause from physical template boundary in native telomerase, we reconstituted telomerase in 293FT cells with full-length hTR that contains permuted template sequences which would separate the sequence-defined pause site from the physical boundary. Telomerases containing permuted template sequences, hTR T1-T6, were immuno-purified from 293FT cells and assayed for activity with permuted 7-mer DNA primers which anneal to the same position on the template relative to the physical boundary (Figure 3.1, left). The T1-T6 templates were each flanked at 5' and 3' by guanosine residues to further define each template region and prevent incorporation of non-telomeric nucleotides from outside the template with the omission of dCTP from the reaction. In accordance with the TF-telomerase results, all template-permuted telomerases demonstrated major sequence-defined pausing at a position three nucleotides following the first rA:dT base pair (Figure 3.1, white and red arrowheads). In T2-T6, minor bands corresponding to pausing at the physical boundary were visible (Figure 3.1 and Figure 3.2, blue arrowheads). In the presence of dCTP, minor bands corresponding to products derived from incorporation of non-telomeric DNA from outside the template were visible, indicating that the structure-defined template boundary, when uncoupled from the sequence-defined pause, is insufficient to prevent template boundary bypass (Figure 3.1, compare even and odd lanes). The inadequacy of the structure-defined template boundary and the predominate pause at the sequence-defined pause site were consistently observed. Since different

permutations of primers were used with each of the permuted template telomerases, we assayed the same mutants with a single permutation of the telomeric primer (5'-GGTTAGGG-3') to ensure that the primers were not the cause of the altered banding patterns (Figure 3.2). As expected, regardless of the primers initial alignment position on the template, all mutants showed a major band at the +4 position, corresponding to the native template boundary end sequence GGTTAG (Figure 3.2, short primer). Only minor bands corresponding to the P1 structurally defined boundary were seen. To be thorough, we tested all six template permutations with all six primer permutations and observed the same template dependent stops in all combinations (Figure S3.1). The same experiment was repeated using a long 18-mer primer to ensure single stranded DNA binding would not affect the results, but the same banding patterns were observed (Figure 3.2, long primer). As was done with the template free system, extensive mutagenesis of the TERT interior surrounding the duplex binding pocket in search of a TERT interaction responsible for duplex sensing were tested for disruption of the sequence defined stop in the T4 mutant (Figure S3.2). Unfortunately, none of the mutants tested showed a significant difference in the pausing site.

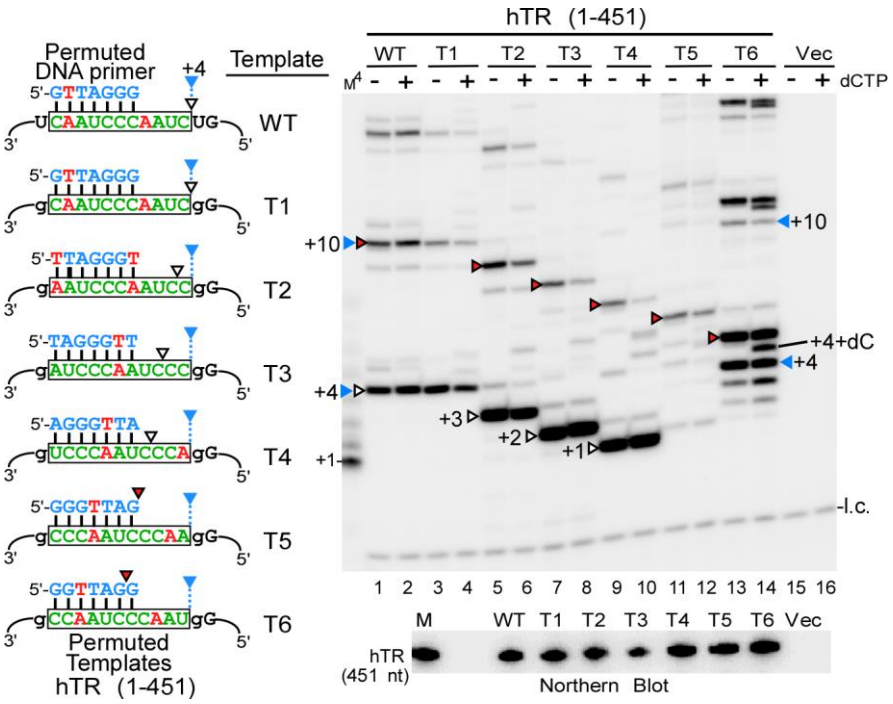

Figure 3.1: *In vivo* reconstituted telomerases with permutated templates exhibit sequence-defined nucleotide addition pausing. (*left*) Sequences of the DNA primer and the hTR template variants used. The sequence-defined pausing sites before (white arrowhead) or after (red arrowhead) template translocation are denoted. The pausing sites at structure-defined template boundary are also denoted by blue arrowhead. (*right*) Activity assay for telomerase with template permutations. Telomerases reconstituted in 293FT cells with full-length hTR containing permuted template sequences were assayed with similarly permuted DNA primers in the presence (+) or absence (-) of 0.5 mM dCTP. The DNA primer AGGGTTA extended by one α-^{32}P-dGTP with TdT was included as a size marker (M_4). The vector-only transfected cells (Vec, lanes 15-16) were included as a negative control. Northern blot (courtesy of Josh Podlevsky) for hTR co-immuno-purified with FLAG-tagged TERT is shown under the activity gel. T7-transcribed hTR (451 nt) was included as a size marker (M).

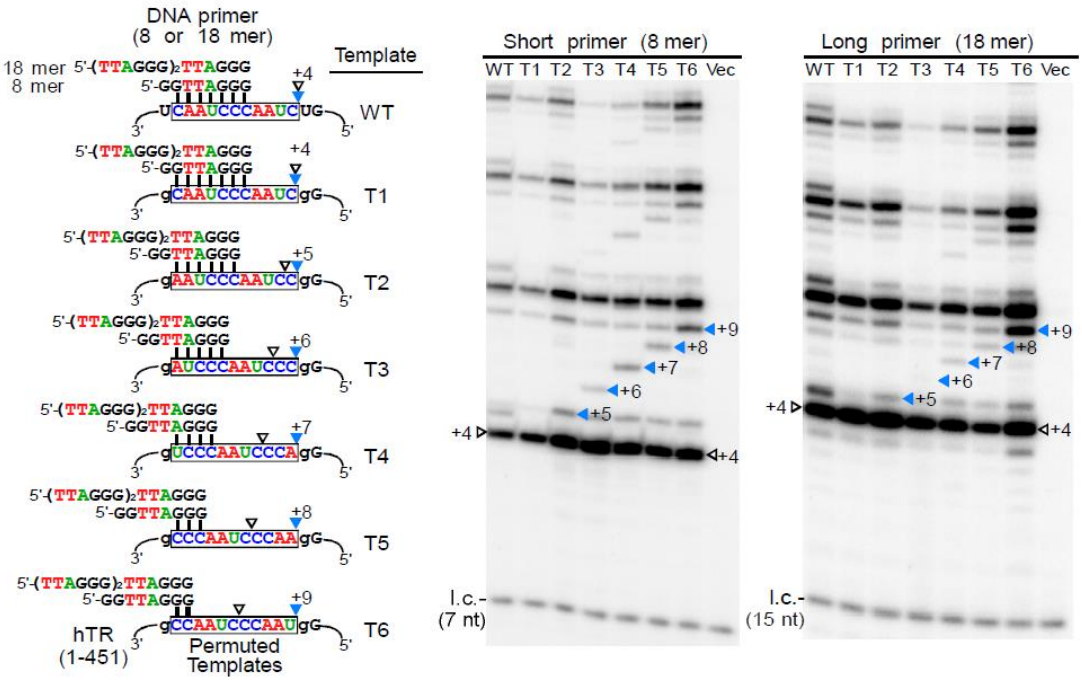

Figure 3.2: Native telomerases with permutated templates exhibit sequence-defined nucleotide addition pausing. (*left*) Sequences of the DNA primers and the TR template variants assayed. Permuted templates (T1-T6) are flanked by guanosine (g) substitutions and the remaining TR (black line) sequence is shown. The sequence-defined pausing site (white arrowhead) and the physical template boundary (blue arrowhead) are denoted. (*right*) Activity assay for telomerase template permutation mutants. *In vivo* reconstituted telomerases with full-length hTR containing permuted templates were assayed with single-stranded DNA primers. A ^{32}P end-labeled 7-mer or 15-mer oligonucleotide was used as a loading control (l.c.).

Telomerase generates the characteristic 6 nt ladder banding-pattern of DNA products presumably by releasing products at the template boundary during processive DNA repeat synthesis. However, in light of the coincidence in position of the sequence-defined pause site and physical template boundary, we investigated which mechanism is principally responsible for DNA product release. We employed our uncoupled sequence-defined pause site and physical template boundary template permutated hTR variants and performed a unique product release assay. Telomerases reconstituted *in vivo* with hTR

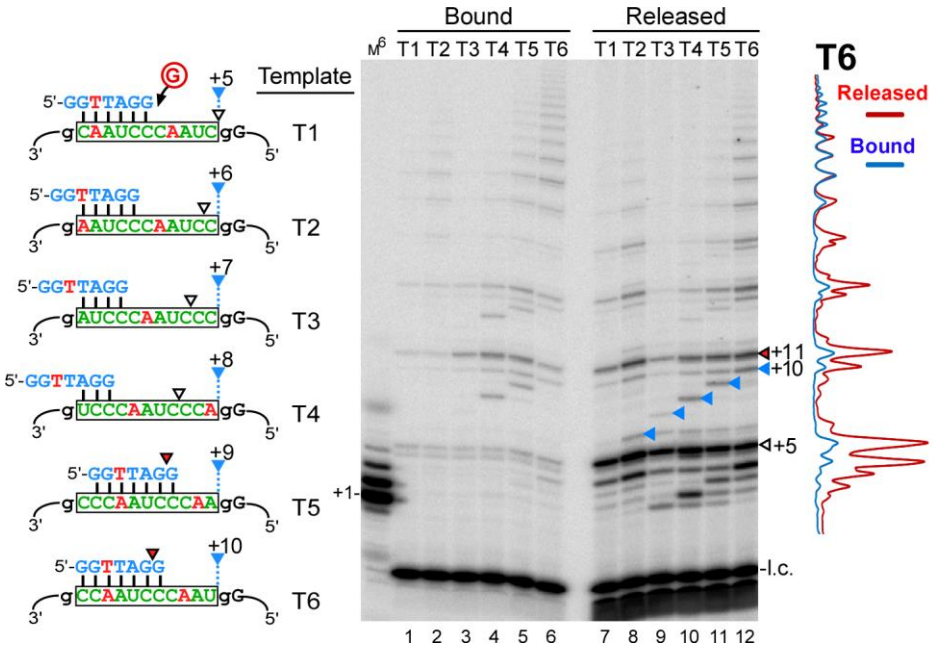

Figure 3.3: Products from sequence defined boundary are fully released from enzyme during repeat synthesis. (*left*) Sequences of the DNA primer and the hTR template variants used. The sequence-defined pausing sites before (white arrowhead) or after (red arrowhead) template translocation are denoted. The pausing sites at structure-defined template boundary are also denoted by blue arrowhead. (*right*) Analysis of bound and released DNA products. *In vivo* reconstituted telomerases (T1-T6) were immobilized on anti-FLAG beads and assayed with a 7-mer DNA primer. DNA products released were separated from those bound to the immobilized enzyme and analyzed by gel electrophoresis. A 6-mer ^{32}P end-labeled oligonucleotide was added to the reaction mix prior to separation from the beads and a secondary 7-mer ^{32}P end labeled oligonucleotide was added during phenol/chloroform extraction. Intensity traces of released (red) and bound (blue) products from the T6 template mutant are shown to the right of the gel. The DNA primer GGTTAGG extended by one α-^{32}P-dGTP with TdT was included as a size marker (M_6).

variants, T1-T6, and FLAG-tagged hTERT were immobilized on beads and assayed with a 7-mer DNA primer, 5'-GGTTAGG-3' (Figure 3.3, left). Following the telomerase extension reaction, DNA products remained bound to the immobilized telomerases were separated from the released products and analyzed. The results showed that telomerase

released DNA products predominately at the sequenced-defined pause site, and also evidently at the structure-defined boundary (Figure 3.3, lanes 7-12). Therefore, nucleotide addition arrest induced by either mechanism is sufficient for product release. Notably, lower molecular weight products had a higher release rate (Figure 3.3, right, T6 trace lines), likely due to less sufficient interactions between the short DNA primer and TERT DNA anchor sites (Finger & Bryan, 2008; Wyatt et al, 2007).

Processive repeat synthesis does not require sequence-defined pausing

Precise product release is an important component of processive telomeric DNA repeat addition. Since sequence-defined pausing leads to product release, we explored whether it also contribute to repeat addition processivity of telomerase. A telomerase template mutant 48u49u, which had the pausing signal completely eliminated, was analyzed for repeat addition processivity and DNA extension patterns (Figure 3.4). When boundary bypass is prevented by the absence of dCTP, template variants, T2 and T6, arrested nucleotide addition at the sequence-defined pause site (Figure 3.4, lanes 5 and 7). In contrast, the 48u49u mutant paused at multiple sites along the template, producing an evenly distributed banding pattern (Figure 3.4, lanes 11 and 12) (Drosopoulos et al, 2005). This suggests that the sequence-defined pausing mechanism prevents premature stalling during nucleotide addition and promotes pausing specifically at the sequence-defined site. Thus, the sequence-defined pausing is the predominate mechanism for generating the characteristic 6 nt ladder banding-pattern of telomerase products. Surprisingly, the 48u/49u mutant had a higher level of repeat addition processivity than the wild-type enzyme, suggesting that sequence-defined pausing is not essential for

telomerase repeat addition processivity (Figure 3.4, lane 11). Similar results were also observed when the P1 linker was extended instead of deleted (Figure S3.3).

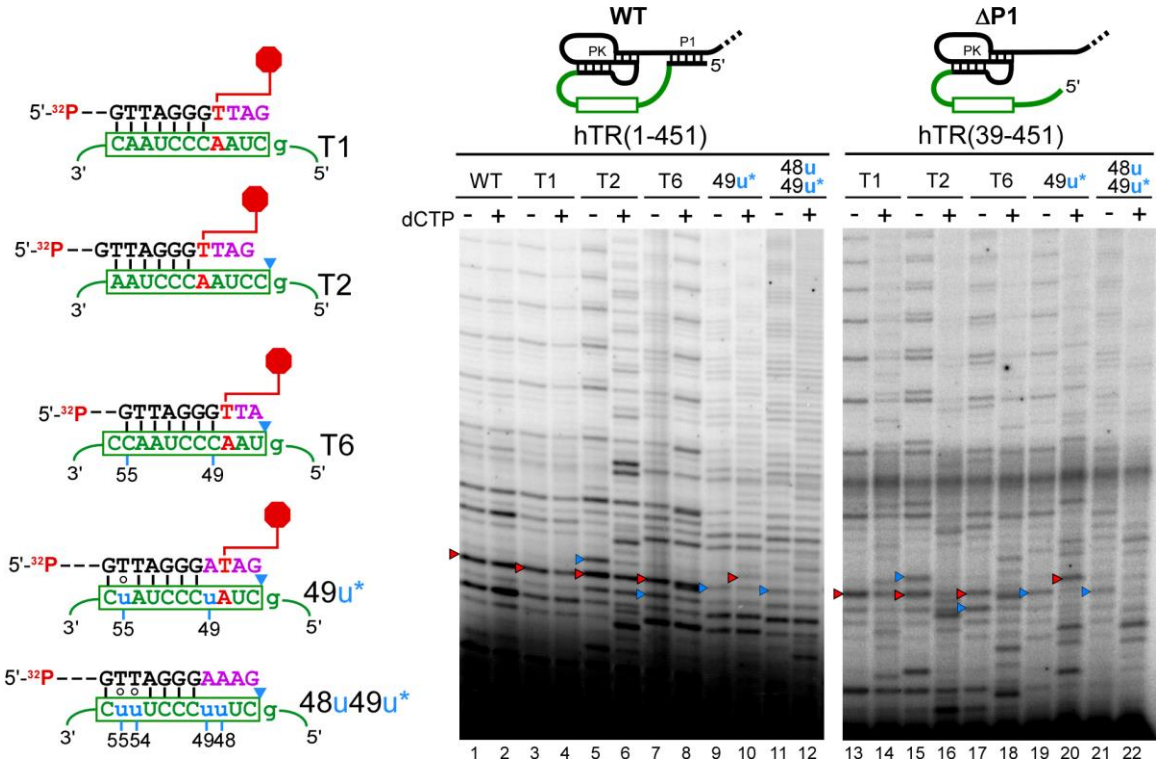

Figure 3.4: Activity assay of *in vivo* reconstituted telomerases that lack the sequence-defined pausing signal or the P1-defined physical boundary. (*left*) Sequences of the DNA primer and the hTR template variants T1, T2, T6, 49u and 48u49u. The sequence-defined pausing site is denoted (red hexagon). The P1-defined physical boundary is denoted with blue arrowhead. The asterisk (*) denotes that the 49u and 48u49u mutants contain additional mutations (55u and 54u55u, respectively) in the primer alignment region to permit processive repeat addition. (*right*) Telomerase reconstituted *in vivo* with hTR variants containing either permuted (T1, T2 and T6) or mutated (49u* and 48u49u*) templates were assayed with the 18-mer ^{32}P end-labeled DNA primer $(TTAGGG)_3$ in the presence (+) or absence (-) of 0.5 mM dCTP. The sequence- and structure-defined pause sites are denoted by red and blue arrowheads, respectively. The secondary structure of full-length hTR (1-451) with P1 and truncated hTR (39-451) without P1 are shown above the gel.

In vertebrate TR, a long-range base paired region, termed helix P1, defines the physical template boundary by limiting access to the template flanking sequence (Chen &

Greider, 2003b). The complete removal of the P1 helix did not impair telomerase processivity, when boundary bypass was prevented by the omission of dCTP from the reaction, and the template variants, T2 and T6, paused nucleotide addition at the expected positions (Figure 3.4, lanes 13, 15, 17, 19 and 21). However, in the presence of dCTP, the addition of non-telomeric nucleotides from outside the template boundary drastically impaired telomerase processivity (Figure 3.4, lanes 14, 16, 18, 20 and 22). Therefore, while important for defining the template boundary, tethering the template to the P1 helix is however not necessary for telomerase repeat addition processivity so long as the non-telomeric nucleotide incorporation is prevented.

POT1 requires exact telomeric repeats at the 3' end of DNA to protect from exonucleases

With such a unique sequence dependent mechanism to produce uniform telomeric ends, it is likely that there is an important biological function for these uniform ends. As expected from our findings, telomerase-positive cells have been shown to have a higher frequency of the GGTTAG register at chromosome termini compared to other permutation registers (Sfeir et al, 2005). Since a primary biological function of the short telomeric repeats at the ends of chromosomes is to bind shelterin proteins to protect the ends, we looked at the POT1 shelterin protein for its interactions with telomeric ends of different sequence registers. It has already been shown that POT1 binds with high specificity to the 10 nucleotide permutation of telomeric sequence 5′-TTAGGGTTAG (Lei et al, 2004). If the telomere end is even a single nucleotide short of this register, POT1 will not bind well to the extreme termini of the chromosome leaving a 10 nt overhang at the 3' end that could be recognized by DNA damage response proteins. However, if the extreme chromosome termini is a few nucleotides longer than the POT1

binding sequence, it is unknown if POT1 can protect a short overhang beyond its binding site. To test this, we assayed POT1 bound to telomeric oligonucleotides of various lengths for its ability to protect the oligonucleotides from exonuclease digestion (Figure 3.5). As expected from previous data, the 11-mer oligonucleotide which was one nucleotide short of the POT1 binding sequence was not protected from the exonuclease due to no POT1 binding (Figure 3.5, lanes 13-15). With all other oligonucleotides tested, POT1 was able to protect only a single nucleotide past its canonical binding sequence and any overhang longer was chewed back by the exonuclease (Figure 3.5, lanes 1-12). These results show that POT1 has very limited ability to protect telomere ends behind its canonical binding site and even a couple nucleotides beyond can lead to exposed DNA ends.

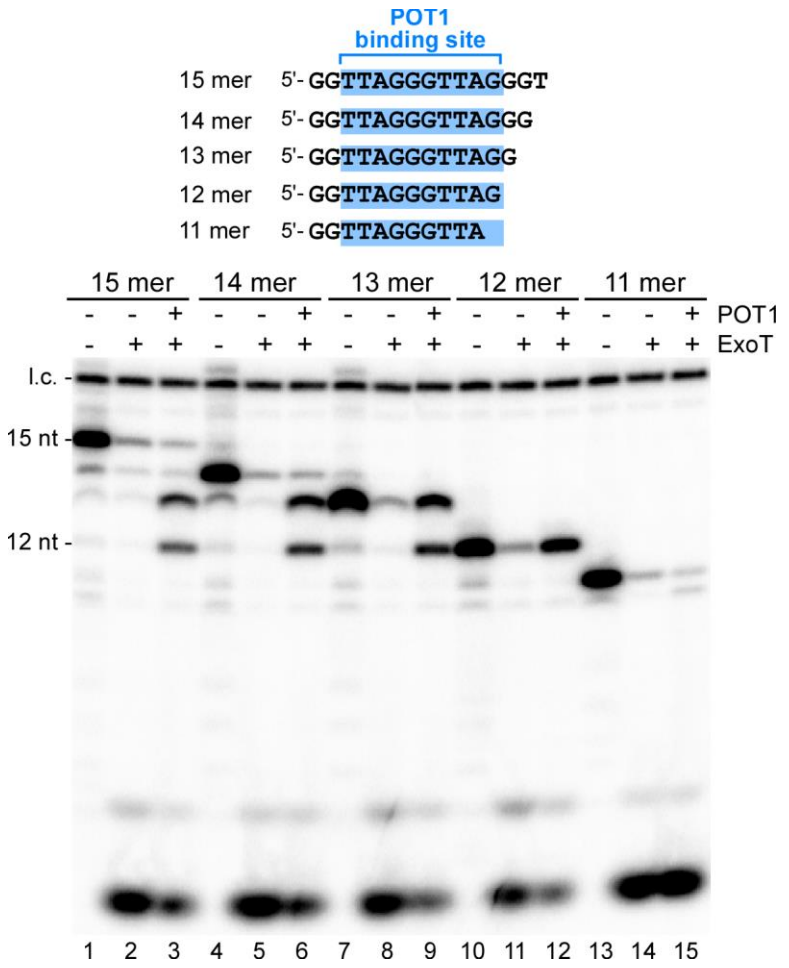

Figure 3.5: Protection of telomeric DNA ends through POT1 protection. ^{32}P-end-labeled oligonucleotides (*above*) were incubated were incubated either with or without hPOT1 (+ or -) and further incubated either with or without Exonuclease T (+ or -). Blue background of oligonucleotides show expected POT1 binding site. An 18-mer ^{32}P-end-labeled oligonucleotide was added after the reaction was completed to act as a loading control (l.c.).

3.4 Discussion

Telomerase synthesizes telomeric DNA repeats by iteratively copying the intended template sequence from an integral RNA component. The template region from the vastly larger TR must be precisely defined to avoid incorporation of non-telomeric nucleotides from the template flanking region. The sequence-defined pausing mechanism we have

discovered in human telomerase provides new insights into the template boundary definition mechanism for vertebrate telomerase. In our current working model of the telomerase catalytic cycle, the single-residue sequence-defined pause signal functions synergistically with the structure defined template boundary. Following the processive addition of six nucleotides to the DNA primer, the first rA:dT base pair in the duplex signals a pause in DNA synthesis at the end of the template region which is redundantly safeguarded by the P1 helix (Figure 3.6, step a). This pausing permits RNA/DNA duplex dissociation from the active site (Figure 3.6, steps b-d). Our model explains the high fidelity by which telomerase synthesizes exact 6-nt DNA repeats, allowing complete product release and template translocation selectively at the end of template.

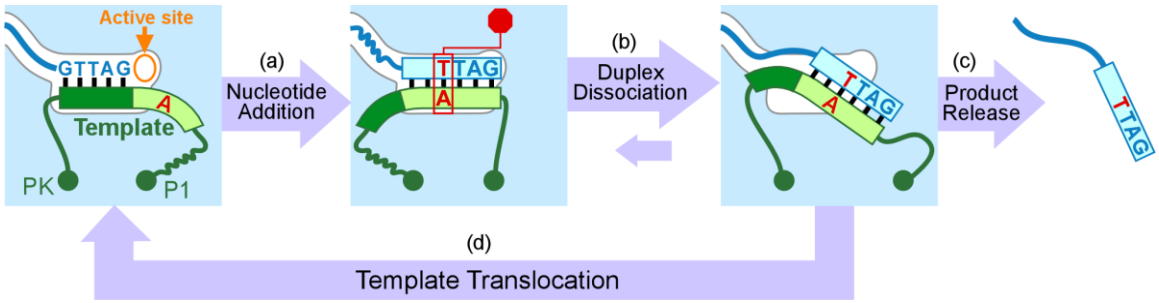

Figure 3.6: A model of the telomerase sequence-defined catalytic cycle. A duplex of the RNA template and DNA primer is bound to telomerase. Nucleotide addition (step a) proceeds, specified by the template sequence. After addition of three nucleotides past the pause signal (red), nucleotide addition is arrested. Duplex disassociation (step b) leads to an unbound duplex with 5' DNA overhang bound to the TERT anchor site. The strand-separation of RNA/DNA duplex results in either complete DNA product release (step c) or template translocation (step d) that aligns and regenerates the template/primer duplex bound by TERT, ready for further nucleotide addition (return to step a).

In a processive telomerase reaction, RNA/DNA duplexes formed immediately prior to and following template translocation have the sequence register GGTTAG, which is identical to the P5 duplex (chapter 2). In the context of TF telomerase, we previously

showed that the P5 duplex is inactive, despite the presence of a DNA 5' overhang. However, the processive addition of DNA repeats by native telomerase indicates that the P5 duplex formed after template translocation is extended. Moreover, telomerase reconstituted with full-length template-permuted hTR T5 is active with the DNA primer GGGTTAG which forms a P5-like duplex with the RNA template (Figure 3.2). Therefore, in the context of native telomerase, additional mechanisms are required for telomerase to extend the P5 duplex following template translocation. When comparing TF and template-containing telomerases, the principle difference is the tethering of the RNA template to the core enzyme. It has been previously proposed for Tetrahymena telomerase that changes in the tension of the 5' and 3' template-flanking RNA regions during nucleotide addition facilitates proper template translocation (Berman et al, 2011). However, human telomerase variants lacking the P1 helix retained processive repeat addition when template boundary bypass was prevented (Figure 3.4, lane 13). This is consistent with a previous report that mouse telomerase with a longer template is processive despite the lack of a P1 helix (Chen & Greider, 2003b). These observations suggest that the 3' template-flanking RNA linker is responsible for telomerase activity with the P5 duplex. Potentially, this 3' template-flanking linker is in a stretched state following template/primer realignment, which then promotes correct positioning or stable binding of the P5 duplex to the telomerase active site, reinitiating nucleotide addition. It is also conceivable that differences in the template-flanking regions prior to and following template translocation induce conformational changes in telomerase for extending the P5 duplex. Future studies are necessary to explore each of these putative mechanisms for P5 activity in native telomerase.

Comparative studies of the sequence-defined pausing in telomerases from various species also provides insights into the fundamental differences in TR template boundary elements between vertebrates/invertebrates and other species. Sequence-defined pausing is conserved in vertebrate and invertebrate telomerases, while not found in ciliate or fungal telomerases (Figure S3.4). The lack of this sequence-defined pausing mechanism in ciliate and fungal telomerases is presumably offset by their distinct and more robust structure-defined template boundaries. Ciliate and fungal TR templates have boundaries rigidly defined by protein binding and immediately adjacent helical structures, respectively (Lai et al, 2002; Miller et al, 2000; Qi et al, 2013; Tzfati et al, 2000). Vertebrate and invertebrate TR templates are tethered by a flexible linker to the distal P1 helix (Chen & Greider, 2003b; Li et al, 2013). The vertebrate/invertebrate P1-tethering mechanism appears less stringent in defining the template boundary which necessitates an ancillary mechanism, such as sequence-defined pausing, to facilitate precise synthesis of telomeric DNA repeats.

The sequence-defined pausing mechanism potentially explains the higher degree of TR template sequence conservation in vertebrate and invertebrate species (Chen et al, 2000; Podlevsky et al, 2008; Qi et al, 2013). The sequence-defined pause generates the exact GGTTAG register at the chromosome termini, evident by the characteristic 6-nt ladder banding pattern of telomerase-generated DNA products *in vitro*. Consistent with this finding, a previous study reported that telomerase-positive cells exhibit a markedly higher frequency of the GGTTAG register at chromosome termini (Sfeir et al, 2005), implying there is a biological significance for the terminal GGTTAG sequence *in vivo*. Moreover, the telomeric DNA motif bound and protected by the single-stranded

telomeric DNA binding protein, POT1, also ends with GGTTAG (Lei et al, 2004). Leaving even a couple nucleotide overhang past the POT1 binding site can expose the single stranded DNA to harmful proteins such as exonucleases (Figure 3.5) The conservation of terminal register GGTTAG as the product of telomerase DNA synthesis and as the moiety bound by POT1 implies a functional connection and potential co-evolution between telomerase and POT1, whereby telomerase synthesizes DNA with the specific terminal repeat for precise binding by POT1.

Our findings shed light on the mechanism behind the conservation of the vertebrate TR template for synthesizing the specific GGTTAG sequence register (Chen et al, 2000) and the acutely deleterious effects from template mutations that impair the sequence-defined pausing mechanism (Drosopoulos et al, 2005; Stohr et al, 2010). The fidelity of telomeric repeat synthesis by telomerase is crucial for telomere function and genome stability in germ line and stem cells as well as in cancer. This sequence-defined pausing mechanism is a novel attribute of human telomerase to self-define the template region from adjacent sequences, ensuring the synthesis of exact GGTTAG repeats with exquisitely high fidelity, thereby contributing to genome stability.

3.5 References

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CHAPTER 4

NOVEL PROTEIN AND RNA RESIDUES AND INTERACTIONS IMPORTANT FOR TELOMERASE ACTIVITY AND PROCESSIVITY

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Huang J., Brown A. F., Wu J., Xue J., Bley C. J., Rand D. P. , Wu L., Zhang R., Chen J. L., & Lei M. (2014) Structural Basis for Protein-RNA Recognition in Telomerase. *Nature structural & molecular biology*. (In Press)

4.1 Introduction

While the telomerase enzyme functions to catalyze the polymerization of DNA using an RNA template like a traditional reverse transcriptases, its very specific biological role has caused telomerase to evolve several unique differences that set it apart from any other known reverse transcriptase. One of the most significant of these differences is that telomerase has evolved into a ribonucleoprotein which minimally requires both the catalytic protein component (TERT) and the template containing RNA subunit (TR) to function (Greider & Blackburn, 1987). In addition to providing the template for DNA synthesis, the TR subunit of telomerase contains multiple structural domains important for catalytic activity, localization in the cell, and assembly of important assembly proteins (Podlevsky & Chen, 2012). Unlike the relatively well conserved TERT protein, the TR is wildly divergent sequence, size, and structure between different phylogenetic groups (Podlevsky et al, 2008). Despite this divergence, several conserved structures have been identified in a variety of distantly related species that are important for telomerase function. These include a pseudoknot-template domain, composed of a pseudoknot RNA structure with a unique triple helix in close spatial proximity to the templating region, and a distal stem-loop moiety, usually consisting of a three-way junction structure (Chen & Greider, 2004; Qi et al, 2013). To accommodate this new RNA subunit, the TERT protein had to co-evolve with the RNA and develop new domains to recognize and bind specific conserved structural regions of the divergent RNA.

Another significant difference between telomerase and conventional reverse transcriptases is the novel property of telomerase to reiteratively copy a short region of its

RNA template in a processive manner. This distinctive property of telomerase is known as its repeat addition processivity. While this property has been fairly well studied *in vitro*, it was previously unclear how significant it was *in vivo*. While telomerase from nearly all phylogenetic groups currently known have been shown to be processive, there are several key examples of telomerase which have little to no processivity *in vitro* such as yeasts *K. lactis* and *S. cerevisiae* and vertebrates such as mouse (Cohn & Blackburn, 1995; Fulton & Blackburn, 1998; Prowse et al, 1993). Recently, several novel human TERT mutations have been linked to human diseases and the primary defect caused by these mutations were found to be in its repeat addition processivity (Alder et al, 2011; Gramatges et al, 2013). These findings indicate that unlike yeasts or rodents, human telomerase required high repeat addition processivity to properly maintain telomere length *in vivo*.

In this study, we set out to use mutagenesis of the TERT and TR subunits to characterize various novel protein and RNA residues for their role in telomerase activity. In particular, we identify and characterize a universally conserved adenosine residue in the junction of the TWJ in vertebrate and fungal TRs. We also employ mutagenesis of the human motif T in hTERT to elucidate the role of motif T in repeat addition processivity observed with the disease mutants T567M and K570N.

4.2 Material and Methods

Native Telomerase activity assay

To test the activity and processivity of the telomerase mutants, we expressed each of them *in vitro* and quantified function using a direct telomerase activity assay as

previously described (Xie et al, 2010). All telomerase variants were reconstituted using the TnT (transcription and translation) Quick Coupled rabbit reticulocyte lysate system (Promega) following manufacturer's instructions. Briefly, recombinant N-FLAG tagged hTERT was expressed in 10 µL of TNT lysate at 30°C for 60 minutes. To obtain active telomerase, *in vitro* transcribed hTR pseudoknot (nt 32–195) and CR4-CR5 (nt 239–328) fragments were each added to a concentration of 1 µM and incubated at 30°C for 30 minutes. To assay the activity and processivity of each telomerase variant, a 10 µl reaction was carried out using 3 µl of *in vitro* reconstituted telomerase in the presence of 1x PE buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM DTT, 3 mM $MgCl_2$, and 1 mM spermidine), 1 µM DNA primer, 0.5 mM dTTP, 0.5 mM dATP, 5 µM dGTP, and 0.165 µM α-^{32}P-dGTP (3000Ci/mmol, 10 mCi/ml, Perkin-Elmer) at 30°C for 1 h. Reactions were terminated by phenol-chloroform extraction followed by ethanol precipitation before being resolved on a 10% denaturing polyacrylamide gel.

Template free telomerase activity assay

The hTERT protein was expressed in rabbit reticulocyte lysate (RRL) from pNFLAG-hTERT plasmid DNA using the TnT T7 Quick coupled kit (Promega) following the manufacturer's instruction. The template free hTR pseudoknot (64- 184 nt) and CR4/5 (239-328 nt) fragments were *in vitro* transcribed, gel purified and assembled with hTERT in RRL at a final concentration 1.0 µM. For the direct assay, 1 µL *in vitro* reconstituted TF telomerase was assayed in a 10 µL reaction containing 1X duplex reaction buffer (50 mM Tris- HCl, pH8.0, 50 mM KCl, 2 mM $MgCl_2$, 2 mM DTT and 1 mM spermidine), 50 µM pre-annealed RNA/DNA duplex, 2 µM dGTP, 0.165 µM α-^{32}P-dGTP (3000Ci/mmol, 10 mCi/ ml, Perkin-Elmer) and 0.5 mM dATP as indicated. The reaction was incubated at

20°C for 60 min and terminated by phenol/chloroform extraction, followed by ethanol precipitation. The DNA products were resolved on an 18% polyacrylamide/8 M urea denaturing gel, dried, exposed to a phosphorstorage screen and imaged on a Bio-Rad FX-Pro phosphorimager.

Medaka, S. pombe, and N. crassa telomerase in vitro reconstitution and telomerase activity assay

The medaka, *S. pombe* and *N. crassa* telomerases were *in vitro* reconstituted as previously described (Bley et al, 2011; Qi et al, 2013). The TERT protein was synthesized in rabbit reticulocyte lysate (RRL) using the TnT Quick Coupled Transcription/Translation System (Promega) and then assembled with 1 μM of *in vitro* transcribed RNA fragments in the RRL. Medaka TERT was assembled with medaka TR fragments PK (1-150) and CR4/5 (170-220), *S. pombe* TERT was assembled with *S. pombe* TR fragments T/PK (nt 83–957) and TWJ (nt 1037–1082), while *N. crassa* TERT was assembled with *N. crassa* TR RNA fragments T/PK (nt 225- 1515, Δ463-1288/GGAC, Δ256-433/GAAA) and TWJ (nt 1813-1877). The telomerase assay was carried out with 2 μL of *in vitro* reconstituted telomerase in a 10 μL of reaction. For *S. pombe* telomerase assays, the reaction contained 1X reaction buffer [50 mM Tris- HCl (pH 8.0), 0.5 mM $MgCl_2$, 1 mM spermidine, 2 mM DTT], 100 μM dATP, 100 μM dCTP, 100 μM dTTP, 5 μM dGTP, 0.165 μM α-^{32}P-dGTP (3000 Ci/mmol, 10 mCi/ml, PerkinElmer) and 1 μM DNA primer 5'-GTTACGGTTACAGGTTACG-3'. For *N. crassa* and Medaka telomerase assays, the reaction contained 1X reaction buffer, 1 mM dATP, 1 mM dTTP, 5 μM dGTP, 0.165 μM α-^{32}P-dGTP (3000 Ci/mmol, 10 mCi/ml, PerkinElmer) and 1 μM DNA primer 5'-$(TTAGGG)_3$-3'. The reactions were incubated at

30°C for 60 min and terminated by phenol/chloroform extraction, followed by ethanol precipitation. Telomerase extended products were resolved on a denaturing 8M urea/10% polyacrylamide gel. The dried gel was exposed to a phosphor storage screen and analyzed with a Bio-Rad FX Pro Molecular Imager.

4.3 Results

The TRBD-CR4/5 recognition mechanism is common to species from yeast to human

One major obstacle to our understanding of the telomerase enzyme and how the protein and RNA work together to synthesis new telomere ends is the low abundance of high quality structural information. While a full length TERT-TR co-crystal remains elusive in the telomerase field, recent structural studies have been able to isolate specific regions of the TERT and TR to produce high resolution structures of these fragments. Most recently, a collaborator has been able to obtain a co-crystal of the TERT TRBD motif of the Medaka fish telomerase bound to the three-way junction CR4/5 domain of its TR using x-ray crystallography (Figure 4.1). Using this high resolution crystal structure, many different protein and RNA interactions were identified between the two fragments. While some of these interactions had been previously identified as important for RNA-Protein binding, many were new with no biochemical data as to their role or importance to the telomerase enzyme (Bley et al, 2011).

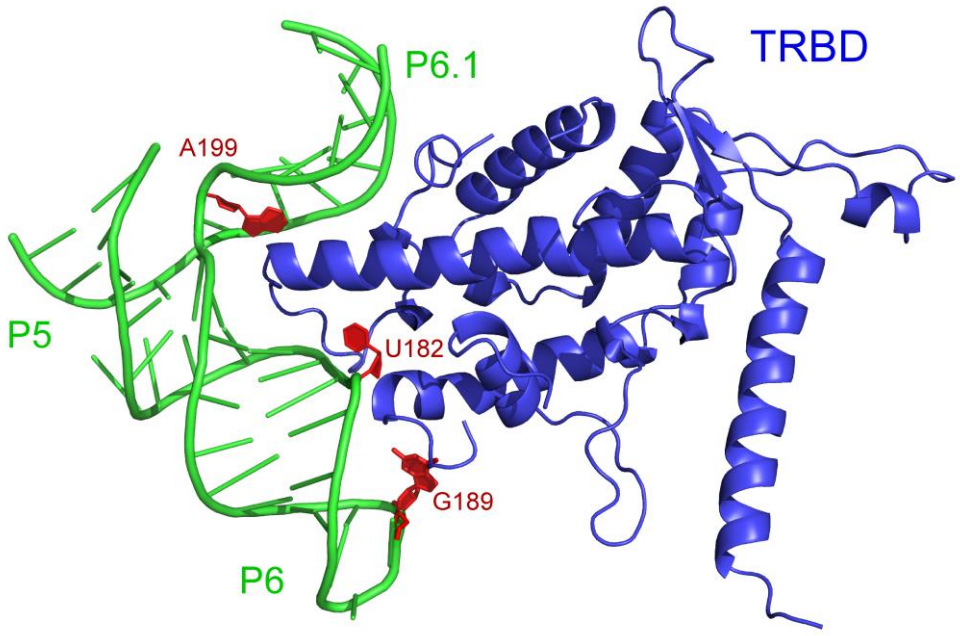

Figure 4.1: Overview of the CR4/5-TRBD complex structure. (A) Stereo view of the overall structure of the CR4/5-TRBD complex. TRBD and CR4/5 are colored in blue and green, respectively. TRBD interacting residues U182, G189, and A199 in the CR4/5 are denoted in red.

Among the novel protein-RNA interactions found between the TRBD and CR4/5 domain, the significance of the interaction with the RNA residue A199 was of particular interest. This is because structure-based sequence analysis of all vertebrate CR4/5 and yeast TWJ domains clearly shows that junction J6/6.1 invariably contains only a single nucleotide, adenine (A199 in medaka CR4/5) (Figure S4.1). Furthermore, the guanine residue at the 5' terminus of junction J5/6.1 (G213 in medaka CR4/5) is also highly conserved across species from yeast to human (Figure S4.1). This observation strongly suggests that formation of an unusual A•G pair at the junction between stems P6 and P6.1 is a conserved feature of all CR4/5 and TWJ of TRs, and that the TRBD-CR4/5 recognition mechanism revealed in the medaka crystal structure is very likely common to all species whose TR contains a CR4/5 or TWJ structure. To test this hypothesis, we

examined the interaction between TRBD and TWJ from the telomerase of the medaka fish and the fission yeast *Schizosaccharomyces pombe*. Wild-type *S. pombe* TRBD binds to *S. pombe* TWJ with an affinity comparable to that of the medaka TRBDCR4/ 5 interaction (Huang et al, 2014). Substitution of the junction nucleotide A199 in medaka or A1060 of *S. pombe* TWJ with any other nucleotide either severely impaired its binding to the TRBD or disrupted the folding of TWJ (Huang et al, 2014). To demonstrate the effects of these substitutions in the activity of the enzymes, we reconstituted medaka and *S. pombe* telomerase from *in vitro* generated TERT protein and TR fragments (T/PK and TWJ) and examined telomerase activities using a direct primer extension assay. In Medaka telomerase, substitutions in the previously identified U182 and G189 produced moderate reductions in telomerase activity *in vitro* while the A199 substitutions produced a much more drastic decrease (Figure 4.2A). Titrations of the different CR4/5 RNA fragments shows that the decreases in activity is related to defects in the binding of the fragments (Figure S4.2). Substitutions in the analogous A1060 residue in *S. pombe* also caused greatly impaired *in vitro* telomerase activities, suggesting that the junction adenine is important for the proper assembly of an active yeast telomerase RNP complex (Figure 4.2B). To expand these results, we also examined the TWJ of the filamentous ascomycete *Neurospora crassa* using *in vitro* reconstituted TERT and TR in our direct telomerase assay. Like with Medaka and *S. pombe*, substitution of the junction nucleotide A1848 with any other nucleotide also led to decreased telomerase activities *in vitro*, although to a lesser extent compared to that of the previous two (Figure 4.2C). Notably, an A to G mutation of the junction nucleotide A1130 in TR from the budding yeast *Kluyveromyces lactis* resulted in growth defect and shortened telomere length,

indicating that this linker nucleotide is also crucial for telomere function in cells (Brown et al, 2007).

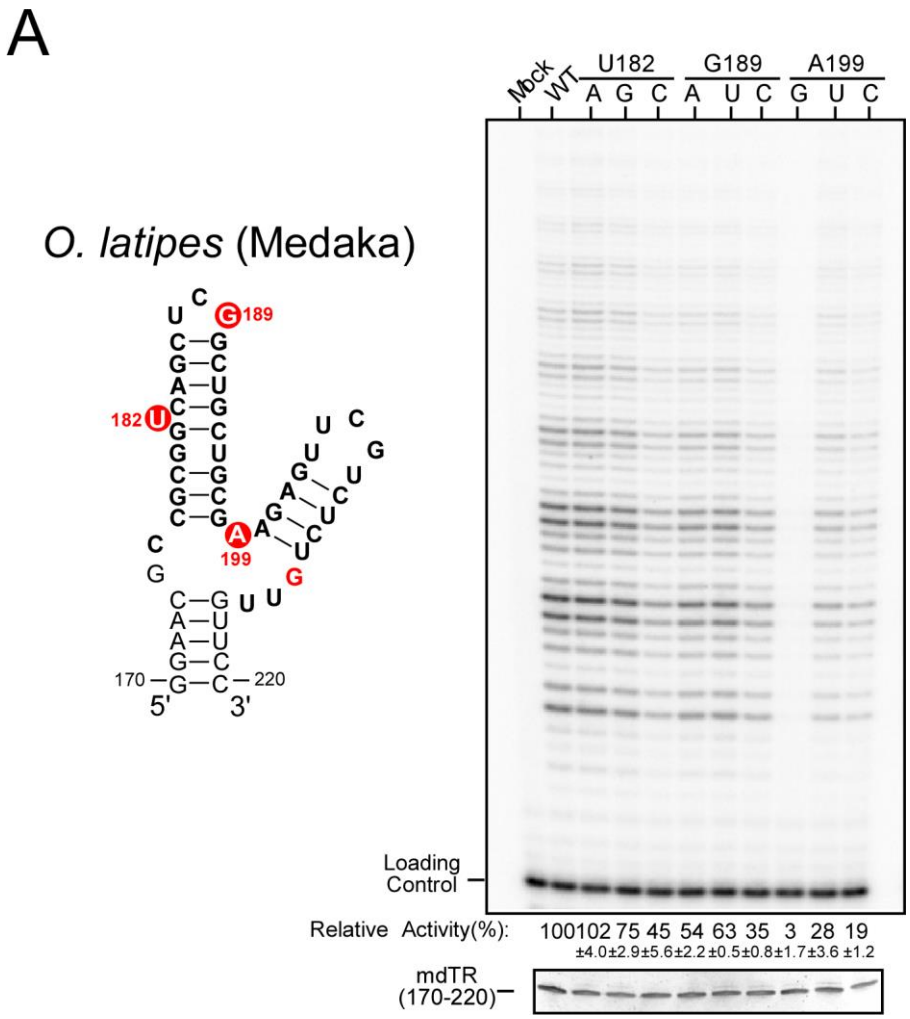

Figure 4.2A: Telomerase primer-extension assay of wild type and mutant Medaka (*O. latipes*) telomerases. Activities of mutants are normalized to wild type and show below the gel as relative activities. 1 µg of *in vitro* transcribed RNA of each TWJ fragment as run on a 4% PAGE gel and visualized below the gel.

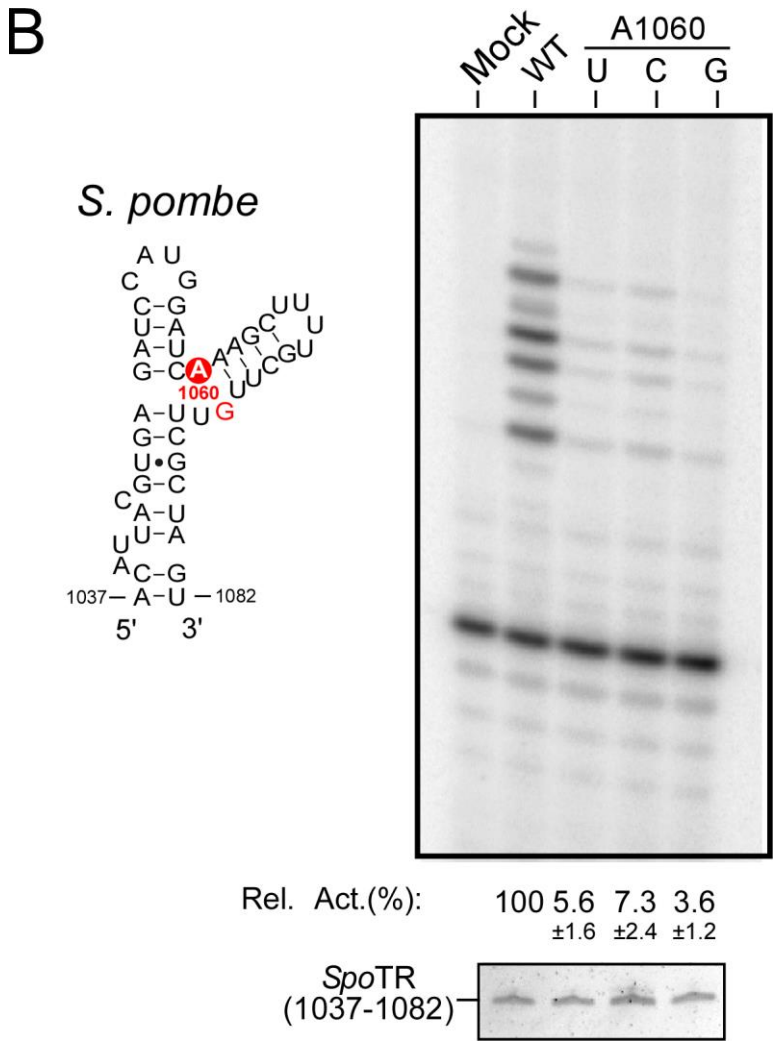

Figure 4.2B: Telomerase primer-extension assay of wild type and mutant *S. pombe* telomerases. Activities of mutants are normalized to wild type and show below the gel as relative activities. 1 µg of *in vitro* transcribed RNA of each TWJ fragment as run on a 4% PAGE gel and visualized below the gel.

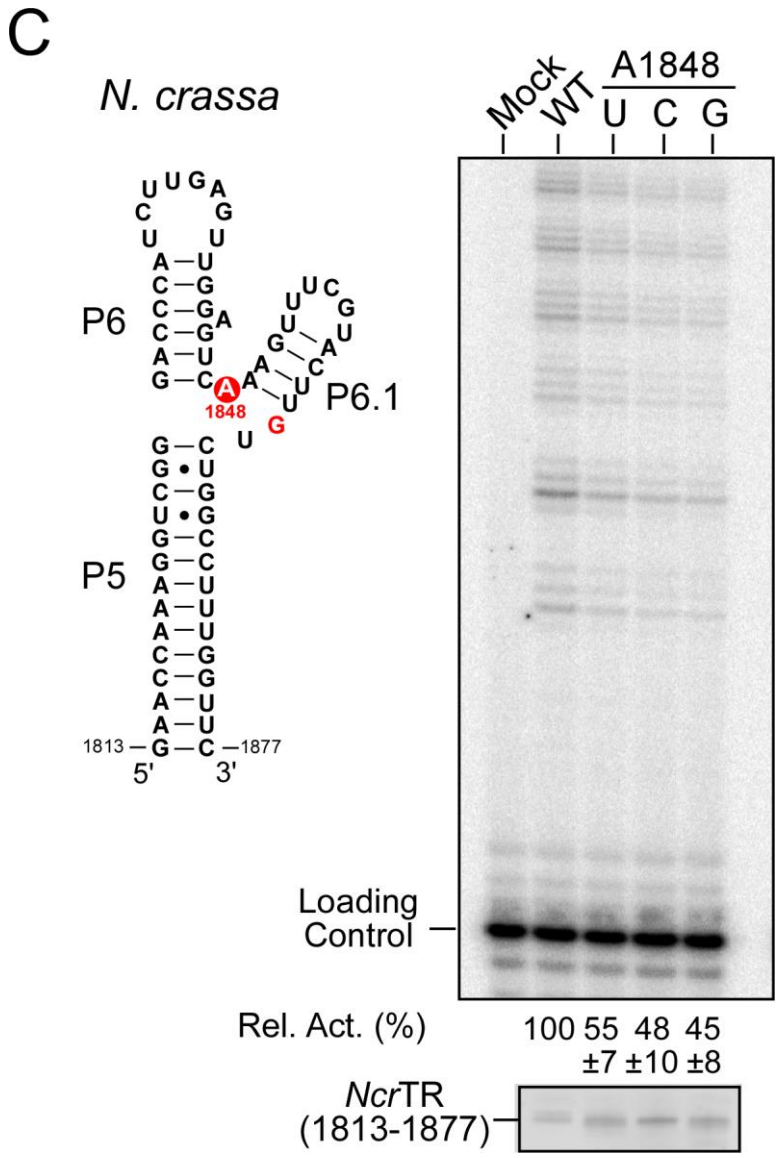

Figure 4.2C: Telomerase primer-extension assay of wild type and mutant *N. crassa* telomerases. Activities of mutants are normalized to wild type and show below the gel as relative activities. 1 µg of *in vitro* transcribed RNA of each TWJ fragment as run on a 4% PAGE gel and visualized below the gel.

Mutation of Motif T residue K570 displays defects in duplex translocation

Recently, a collaborator identified two disease mutations were in the motif T region of the human TRBD: T567M and K570N (Gramatges et al, 2013; Xin et al, 2007). Analysis of these mutations reviled that the primary defect caused by these mutations is in the repeat addition processivity of telomerase. Unlike previous processivity linked disease mutations which were in the catalytic RT domain, these mutations were in a region primarily attributed to binding the TR (Alder et al, 2011). However, these results were consistent with previous finds that found that a conserved region inside motif T also effects rate of repeat addition (Drosopoulos & Prasad, 2010). While these suggest that motif T has an involvement in repeat addition processivity, the mechanism by which this involvement occurs is still unknown.

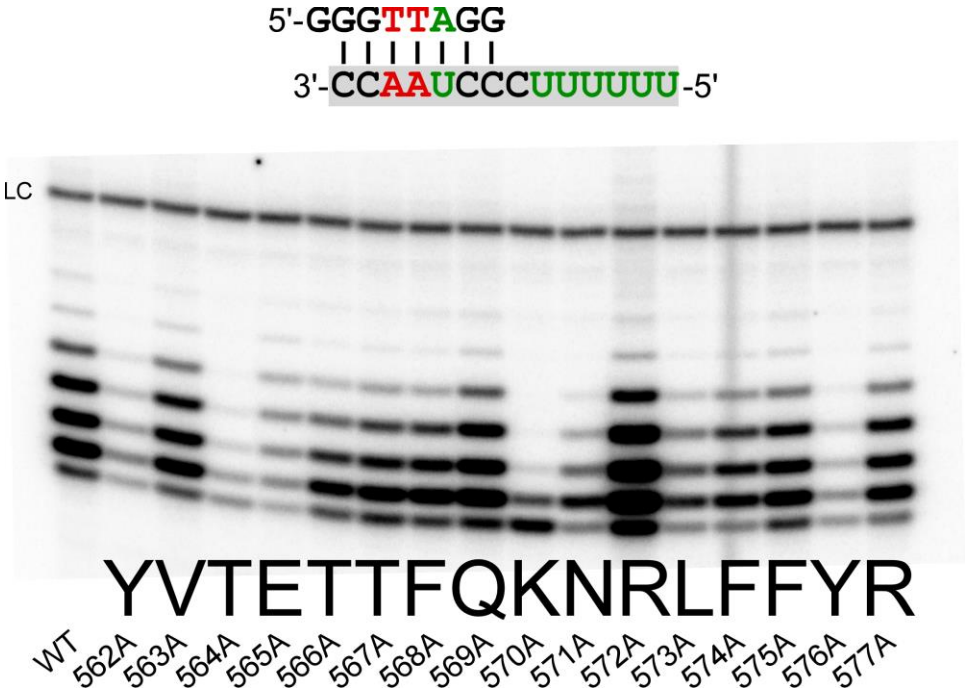

Figure 4.3: Alanine screening of human motif T with template free telomerase. Alanine substitution TERT mutants were reconstituted *in vitro* with template free hTR fragments. RNA/DNA duplex used in the assay is shown above the gel. L.C. was a ^{32}P-end-labeled 18-mer DNA primer.

To investigate possible roles of this motif in the telomerase reaction, we examined its location in the *T. castaneum* TERT crystal structure in relation to the bound RNA/DNA duplex in the active site (Mitchell et al, 2010). The position of motif T in this structure makes it unlikely that it can directly interact with the single stranded DNA to facilitate template translocation efficiency without significant conformational change. However, it is easy to imagine that the small motif could interact with the 5' single stranded RNA template. In an attempt to elucidate any potential interactions between this motif and the single stranded RNA template, we created alanine substitution mutants of the motif T residues and tested them for activity in our template-free telomerase system with an RNA/DNA duplex with a long 5' RNA overhang (Figure 4.3). While various mutations showed different levels of telomerase activity, interestingly the K570A mutant, similar to the K570N disease mutant previous described, had dramatically decreased nucleotide addition processivity. Like the K570N disease mutant, the K570A substation also displayed dramatically decreased repeat addition processivity when tested with native template containing telomerase, regardless of the primer used in the assay (Figure 4.4). Although the K570 mutant does not appear to be significantly worse than the wild type enzyme at reaching the end of the first repeat, which requires nucleotide addition processivity, the connection between decreased repeat addition processivity in the template free system and repeat addition processivity in the native system is interesting.

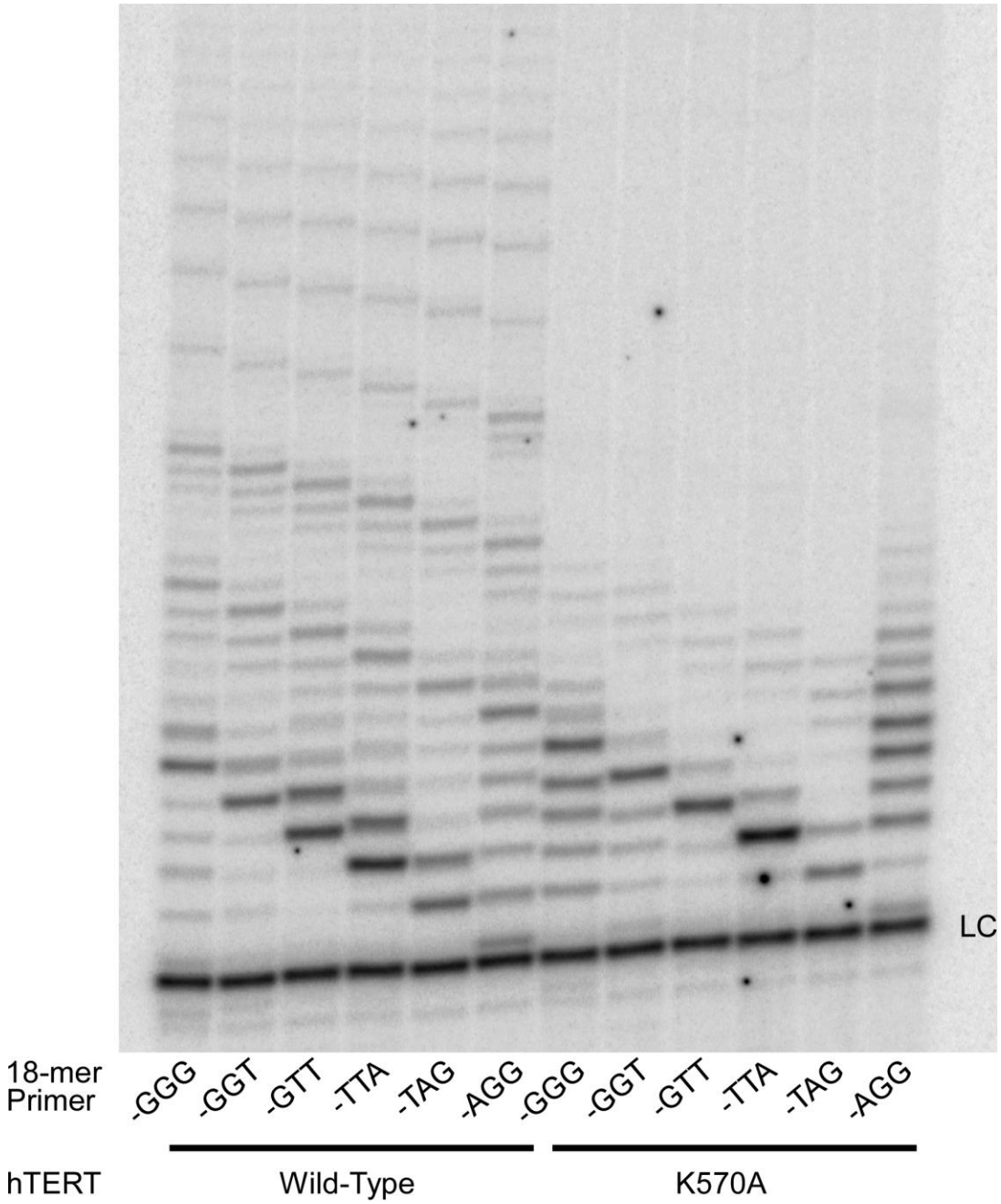

Figure 4.4: Activity assay of K570A telomerase mutant with all 6 permutations of telomeric DNA primer. Primers used were all three full repeats of the telomeric sequence with the permutation as indicated below the gel. A 15-mer ^{32}P end-labeled oligonucleotide was included as a loading control (LC).

To see if any potential single stranded RNA interactions were sequence specific or required a certain length of RNA overhang, we tested the K570A mutant with identical

RNA/DNA duplexes but with varying template length or sequence (Figure 4.5). While the longer template did allow slightly higher nucleotide addition processivity compared to the short template, all K570A mutant reactions had drastically impaired processivity compared to the wild type enzyme with the same substrate. The consistent impairment in nucleotide addition processivity suggests that the K570 residue could be important for duplex translocation through the active site and/or proper positioning of the duplex in the active site. Our previous work has shown that the sequence of the duplex can also produce a drastic difference in nucleotide addition processivity (see chapter 3). To test how the sequence of the duplex affects the activity and processivity of the K570A mutant, we tested it with all six circular permutation duplexes in the template free system (Figure 4.6). In contrast to wild type TERT, the K570A mutant did not show any differences in activity or processivity with any of the duplex sequences. This suggests that the mechanism of motif T facilitating duplex translocation is unique from the sequence dependent pause seen with the wild type protein.

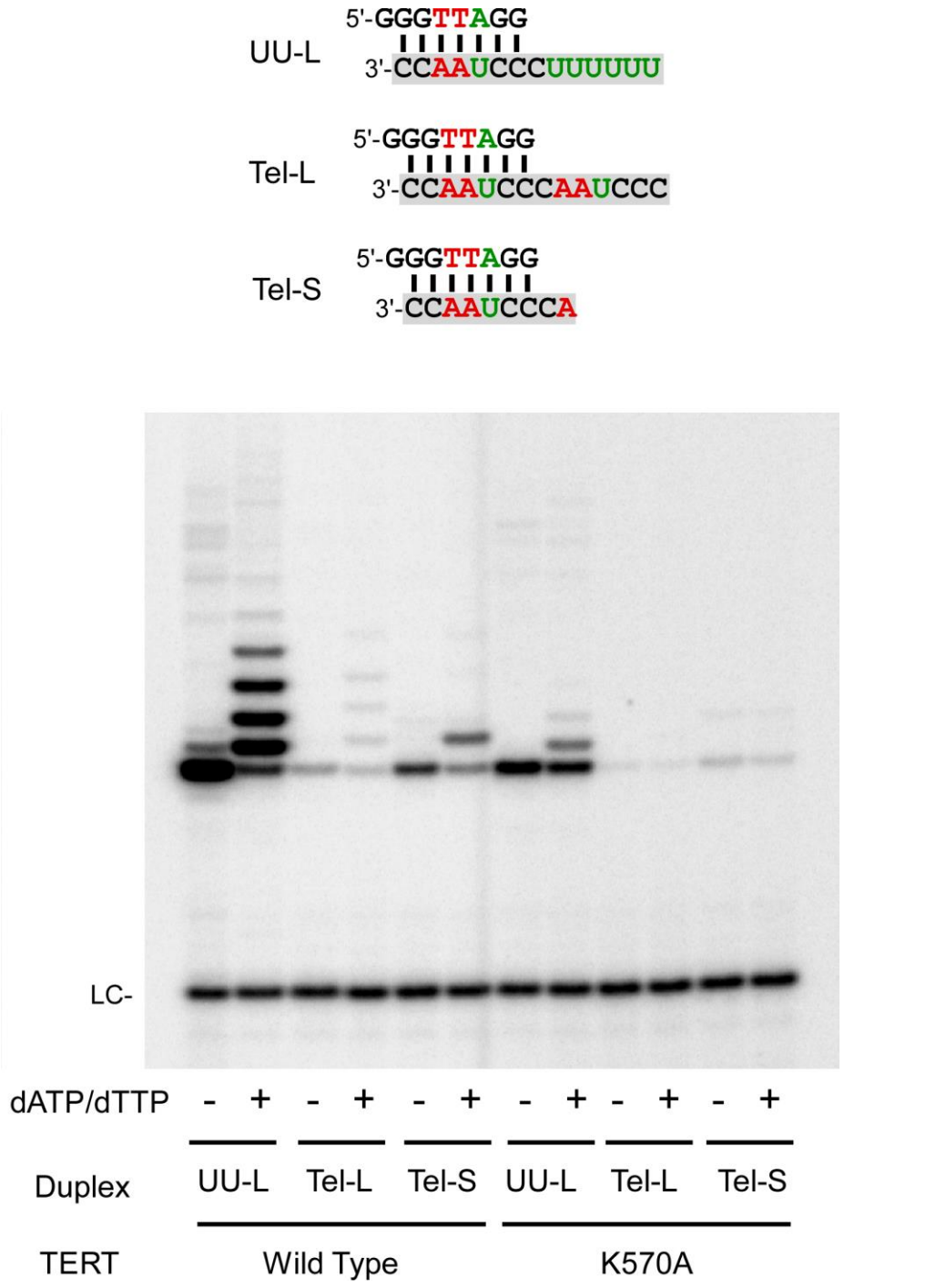

Figure 4.5: Effects of template length and sequence on K570A mutant. Template free telomerase composed of either wild type or K570A mutant TERT was assayed with three 7 bp RNA/DNA duplexes with varying template length and sequence (shown above gel). Addition of 0.5 mM dATP and dTTP is denoted below the gel (+). A ^{32}P-end-labeled 5-mer was used as a loading control (LC).

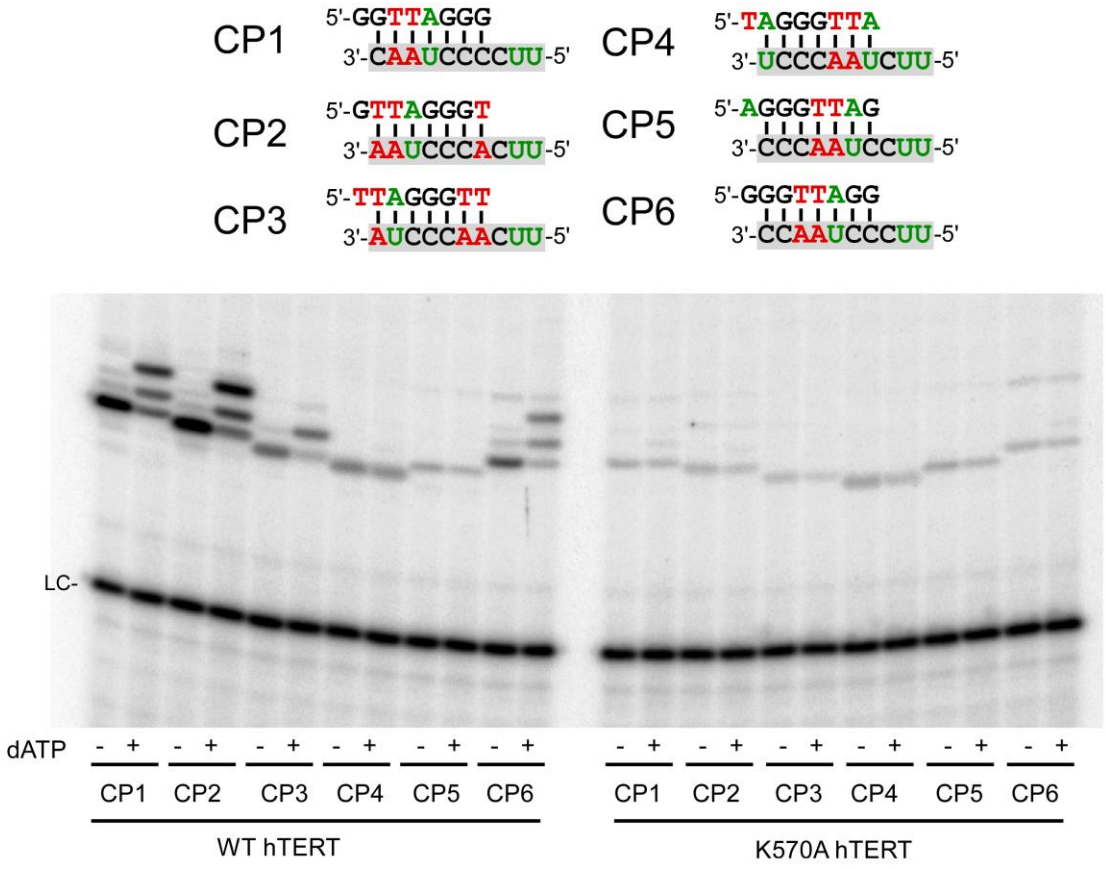

Figure 4.6: K570A TERT mutant shows defects in nucleotide addition processivity using circularly permuted duplex substrates. Template free telomerase composed of either wild type or K570A mutant TERT was assayed with all 6 circular permutations of the telomeric 7 bp RNA/DNA duplex sequence (shown above the gel). Addition of 0.5 mM dATP is denoted below the gel (+). A ^{32}P-end-labeled 5-mer was used as a loading control (LC).

4.4 Discussion

While our knowledge of the telomerase enzyme and its unique mechanism has grown drastically over the last few decades, there are still a larger number of things we still do not understand. This project aims to fill in these gaps though characterizing the functional role of several different TERT and TR residues through mutational analysis.

Using the medaka TRBD and CR4/5 co-crystal to predict important interactions, we confirmed several residues in the medaka CR4/5 are vital for proper recognition and binding by the TERT protein. While the junction residue, A199, is absolutely conserved in TWJ structures from other TRs, the bulge residues, U182 and G189 , which mediate the specific interaction between medaka CR4/5 and TRBD are only conserved in closely related species, such as the fugu fish *Takifugu rubripes* (Podlevsky et al, 2008; Xie et al, 2008). This is consistent with previous observations that fugu T/PK and CR4/5 domains can reconstitute telomerase activity with medaka TERT (Xie et al, 2008). In contrast, *S. pombe* TWJ contains a much shorter stem P6 with no bulged nucleotide, suggesting that *S. pombe* TRBD must recognize TWJ through some features that are different from U182 and G189 of medaka CR4/5. Collectively, the structural and biochemical analyses suggest that CR4/5 (TWJ) is bound by TRBD through two types of interactions. The helical conformation of stems P6 and P6.1 and the conserved adenine at the junction J6/6.1 define the overall shape of CR4/5 (TWJ) essential for the TRBD-CR4/5 binding in all vertebrates and yeasts. The species-incompatibility of the TRBD-CR4/5 (TWJ) interaction presumably results from the co-evolution of the protein and RNA components of telomerase along different eukaryotic lineages.

When structural information is not available, which is usually the case with human telomerase, mutagenesis can still be used to elucidate valuable data on the function of different regions of telomerase. By characterizing disease mutants, several novel residues have been discovered to be important for telomerase repeat addition processivity and show that this unique property of telomerase is important for *in vivo* function (Alder et al, 2011; Gramatges et al, 2013). In particular, several disease

mutations in the motif T domain of TERT have been shown to effect telomerase processivity (Gramatges et al, 2013). Through biochemical analysis of these mutants in our template free system, we found that the defects of the K570A mutant in repeat addition processivity is linked to an inability to possessively add nucleotides to an RNA/DNA duplex or to distinguish sequence of the bound duplex substrates. This interaction seems to be duplex specific and not dependent on sequence or length of the RNA overhang. Previously, correlation between duplex binding and enzyme processivity in telomerase has been shown with motif 3 mutants, but this is the first instance of a mutation where duplex translocation has been tied to repeat addition processivity. Taken together, these results have shed new light on how telomerase uses numerous unique protein and RNA interactions to regulate its complex catalytic cycle.

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APPENDIX A

SUPPLEMENTARY DATE FOR CHAPTER 2

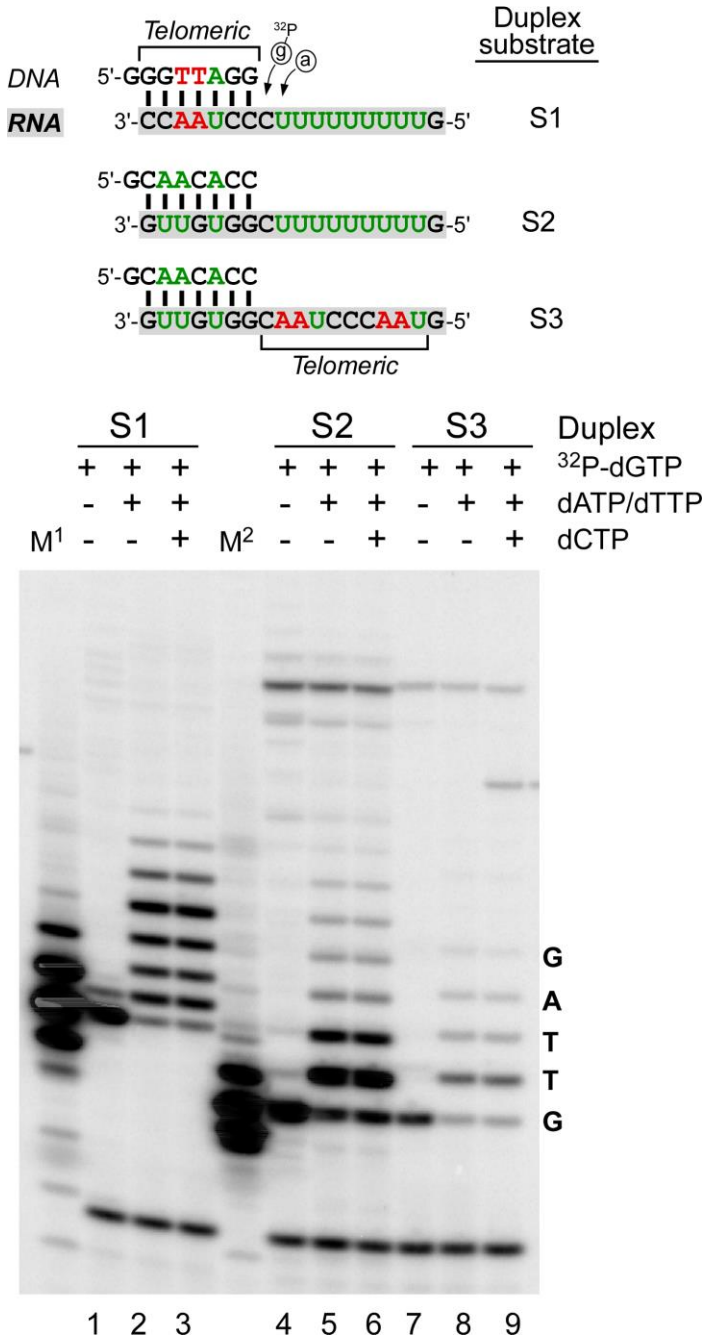


Figure S2.1: A telomeric template is sufficient for sequence defined pausing. (*top*) Sequences of RNA/DNA duplexes used. (*bottom)* Activity assay of *in vitro* reconstituted TF telomerase (upper panel) with various duplex substrates. Substrates were extended by the enzyme with α-^{32}P-dGTP in the presence (+) or absence (-) of 1 mM dATP, dTTP, or dCTP as denoted above the gel. An α-^{32}P end-labeled 5-mer oligonucleotide was used as a loading control (l.c.). The DNA primers GGGTTAGG (M_1) or GCAACACC (M_2) extended by one ^{32}P-dGTP with terminal deoxynucleotidyl transferase (TdT) were included as size markers.

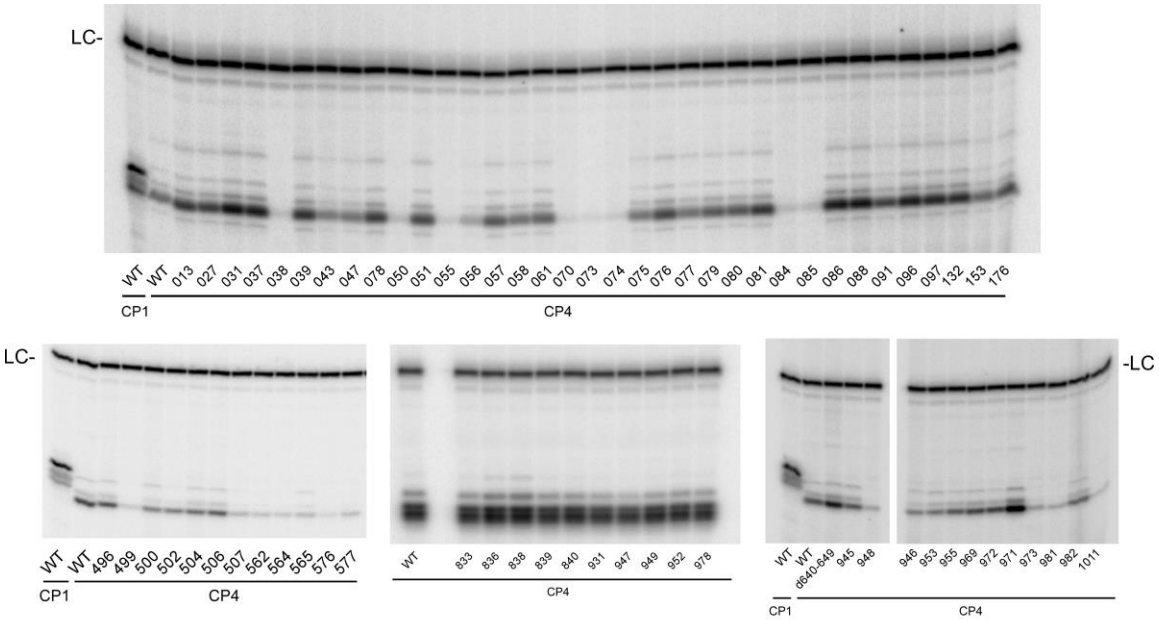
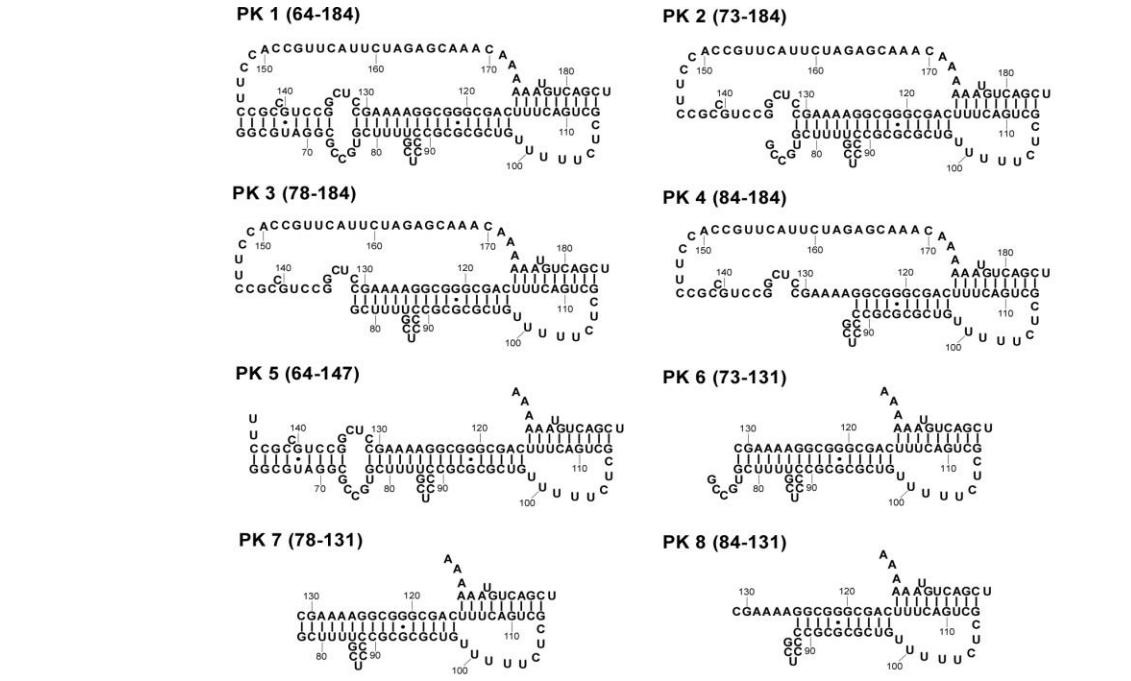


Figure S2.2: Alanine screening of hTERT residues with potential duplex interactions. Activity assays of various hTERT mutants reconstituted *in vitro* with template free telomerase RNA fragments for direct assay analysis. All reactions were performed with 5 μM dGTP and 0.5 mM dATP. A 15-mer ^{32}P-end-labeled oligonucleotide was included as a loading control (LC).



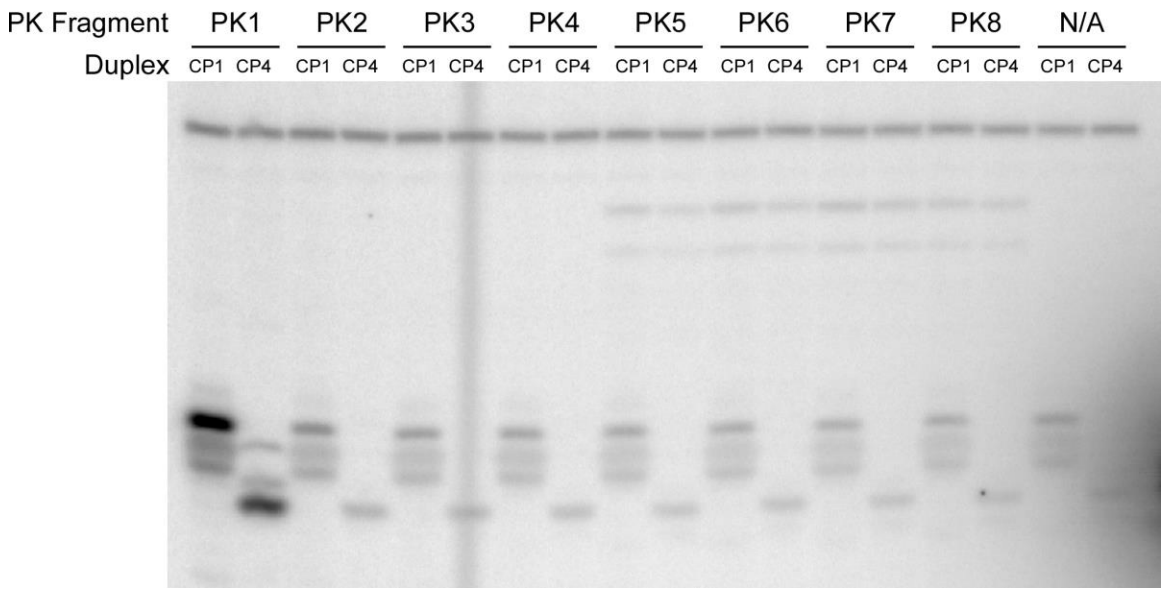


Figure S2.3: Effect of pseudoknot truncations on sequence dependent pausing. hTERT was *in vitro* reconstituted with various pseudoknot fragments (shown above gel). Resulting telomerase mutants were assayed with duplex substrates P1 or P4 in the presence of both α-^{32}P-dGTP and 1 mM dATP. A 15-mer ^{32}P-end-labeled oligonucleotide was included as a loading control (LC).

APPENDIX B

SUPPLEMENTARY DATA FOR CHAPTER 3

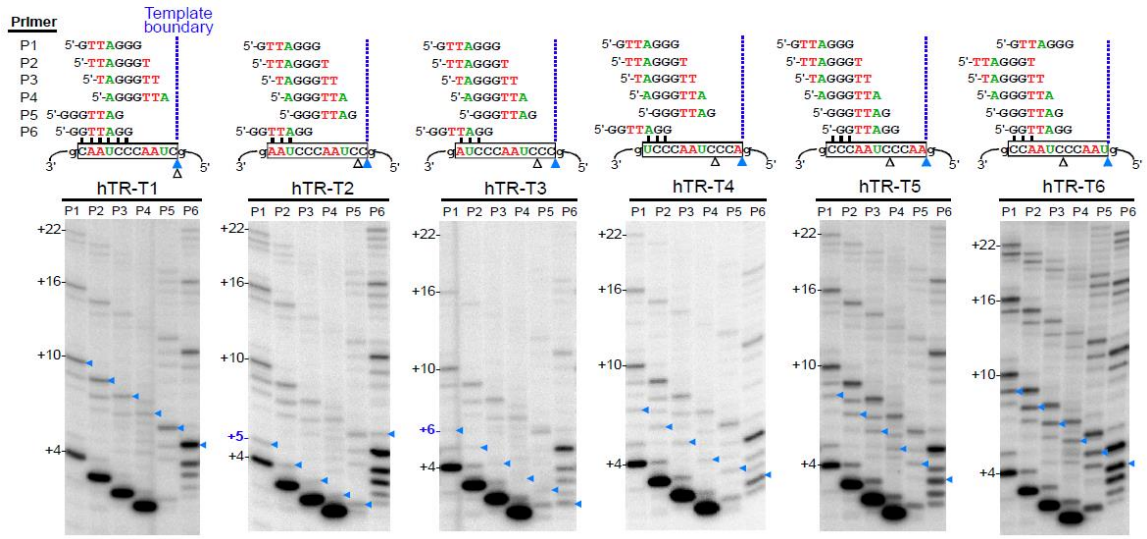


Figure S3.1: Native telomerases with permutated templates exhibit sequence-defined nucleotide addition pausing. Figure courtesy of Mingyi Xie. (*top*) Sequences of the DNA primers and the TR template variants assayed. Permuted templates (T1-T6) are flanked by guanosine (g) substitutions and the remaining TR (black line) sequence is shown. The sequence-defined pausing site (white arrowhead) and the physical template boundary (blue arrowhead) are denoted. (*bottom*) Activity assays for telomerase template permutation mutants. *In vivo* reconstituted telomerases with full-length hTR containing permuted templates were assayed with single-stranded DNA primers. A ^{32}P end-labeled 7-mer or 15-mer oligonucleotide was used as a loading control (l.c.).

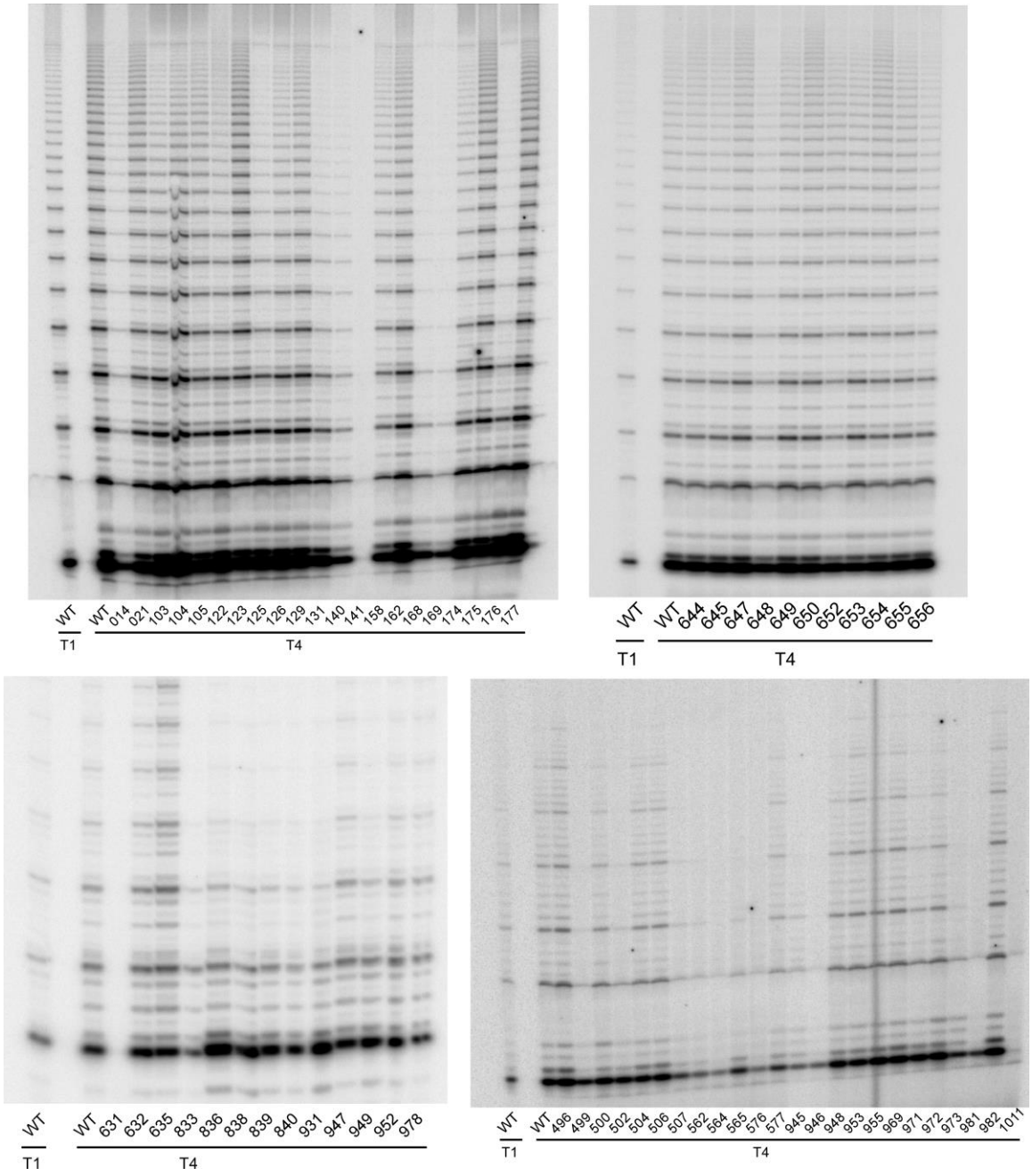


Figure S3.2: Alanine screening of hTERT residues with potential duplex interactions. Activity assays of various hTERT mutants reconstituted *in vitro* with telomerase RNA fragments for direct assay analysis with telomeric primer 5'- $(TTAGGG)_3$ -3'. A 15-mer ^{32}P-end-labeled oligonucleotide was included as a loading control (LC).

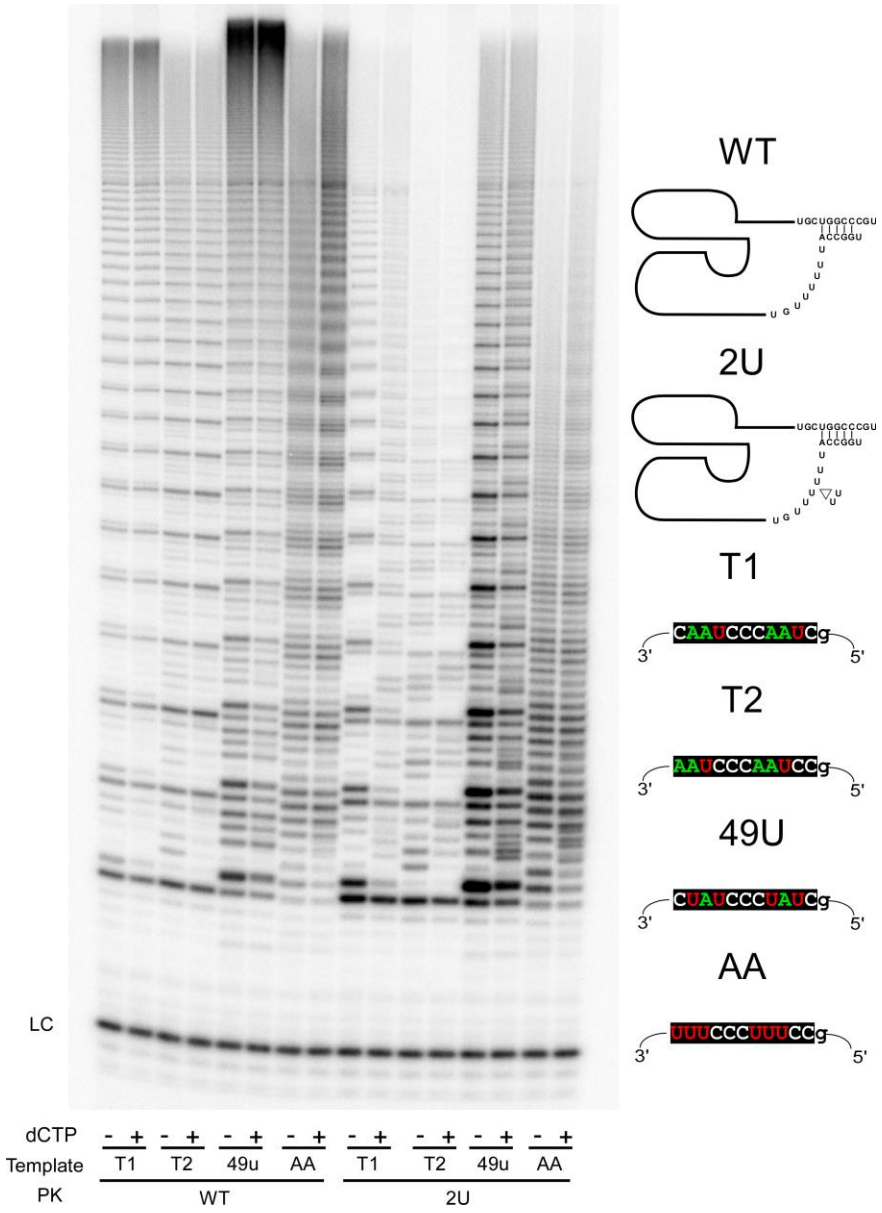


Figure S3.3: Activity assay of *in vitro* reconstituted telomerases that lack the sequence-defined pausing signal or the P1-defined physical boundary. (*right*) Structure of pseudoknot structures used and sequences of the hTR template variants T1, T2, 49u and 48u49u (AA). (*left*) Telomerase reconstituted *in vitro* with hTR with variants templates and/or P1 linker were assayed with the 18-mer DNA primer $(TTAGGG)_3$ in the presence (+) or absence (-) of 0.5 mM dCTP. The secondary structure of full-length hTR (1-451) with P1 and truncated hTR (39-451) without P1 are shown above the gel. A ^{32}P end-labeled 15-mer oligonucleotide was used as a loading control (l.c.).

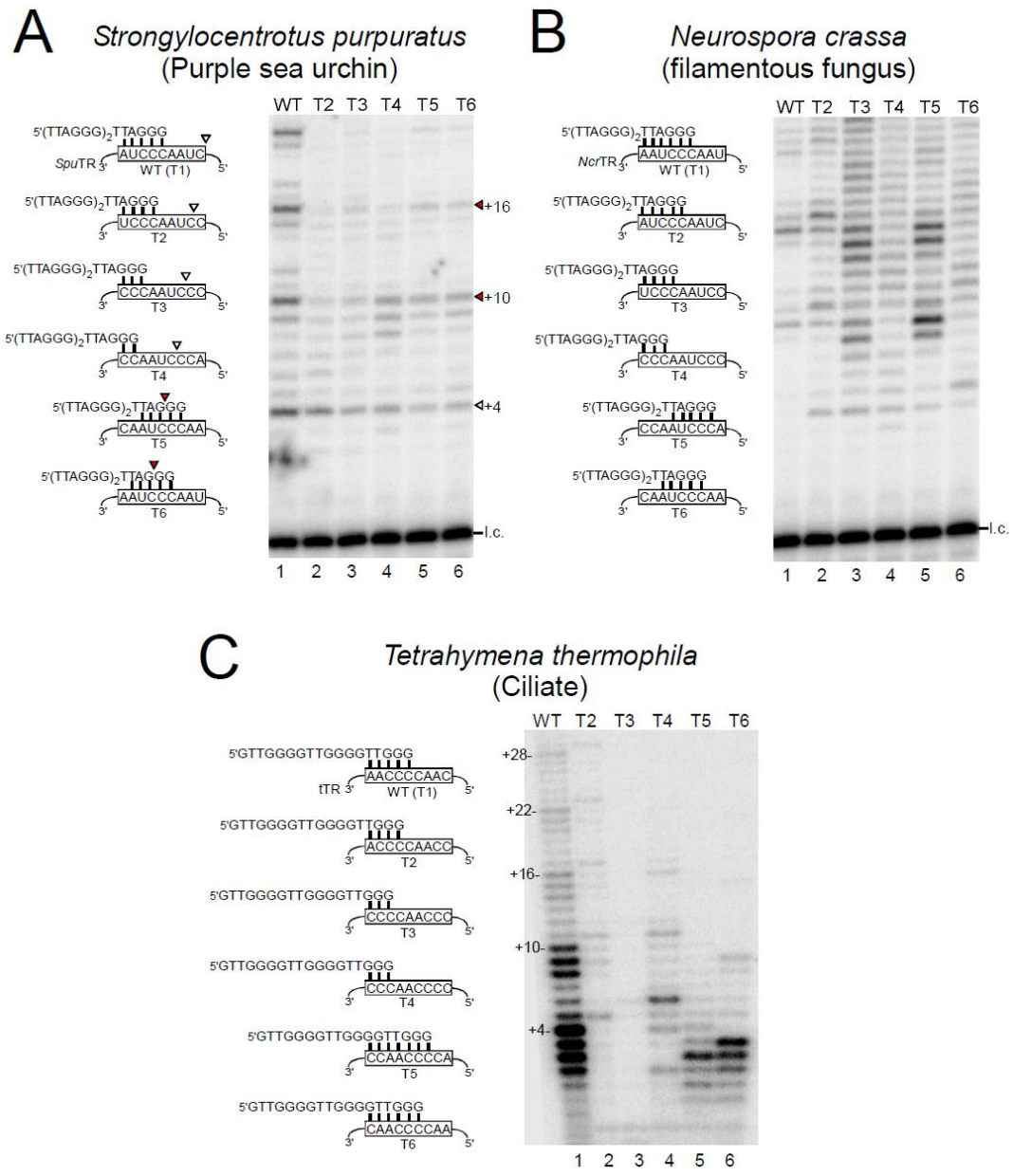


Figure S3.4: Evolutionary conservation of telomerase sequence-defined pausing. (A-C, left) Sequences of the DNA primers and the TR template variants assayed for *S. purpuratus*, *N. crassa* and *T. Thermophilia*, respectively. Sequence of permuted templates (T1-T6) are shown. (A-C, right) Sequence-defined pausing sites before (white arrowhead) and after (red arrowhead) template translocation are denoted. Activity assay for telomerase template permutation mutants. Substrates were labeled by the enzyme with α-^{32}P-dGTP. A ^{32}P end-labeled 18-mer oligonucleotide was used as a loading control (l.c.).

APPENDIX C

SUPPLEMENTARY DATA FOR CHAPTER 4

Figure S4.1: Sequence alignments of the CR4/5 (TWJ) domain of TR. Sequence alignment of the vertebrate CR4/5 domains and the fungi TWJ domains, based on predicted secondary structures. Conserved RNA secondary structures are denoted. The conserved nucleotide A at the junction J6/J6.1 as well as its interacting nucleotide G are highlighted in orange.

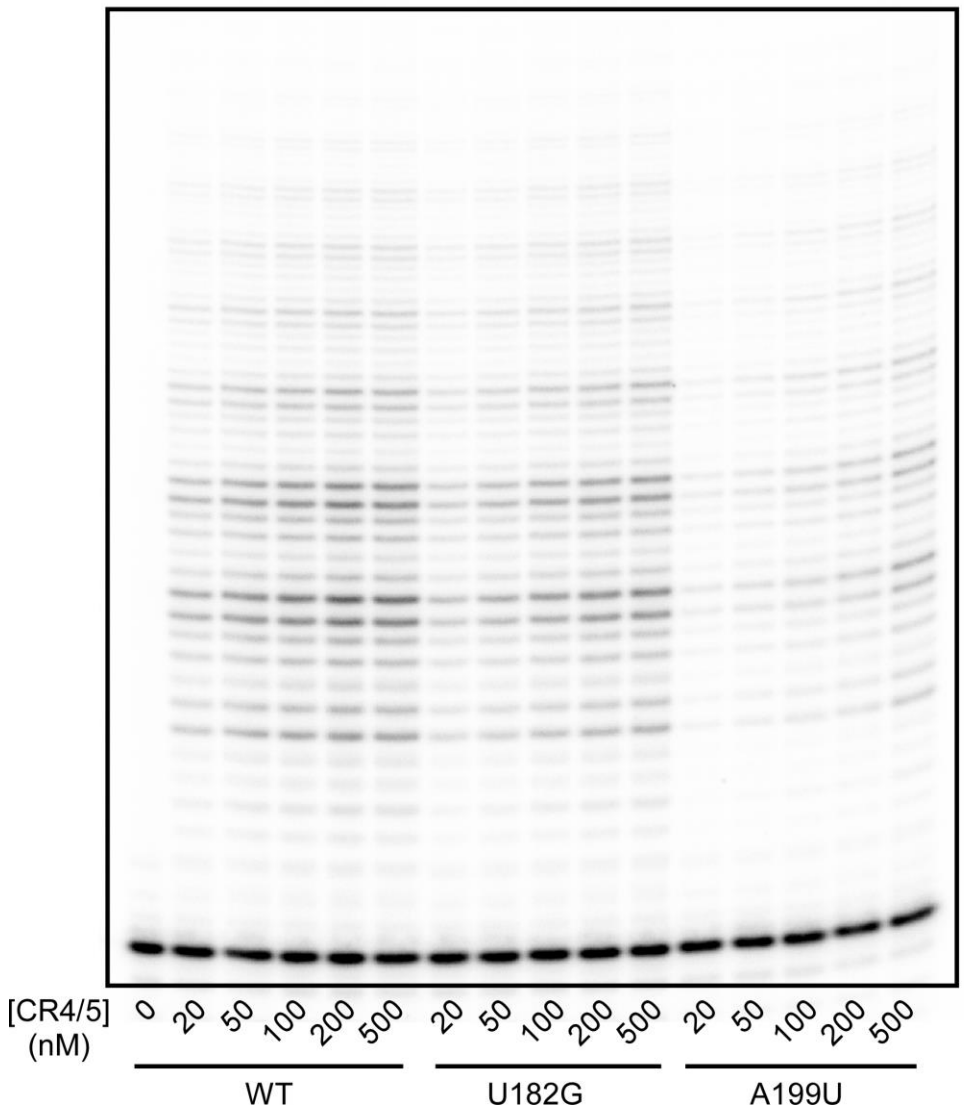


Figure S4.2: Titration of medaka CR4/5 RNA fragments. Medaka TERT was reconstituted with various concentrations of WT or mutant medaka CR4/5 fragments ranging from 0 to 500 nM. Concentrations of the pseudoknot fragment and all other reaction conditions were kept constant between trials. A ^{32}P-end-labeled 15-mer oligonucleotide was used as a loading control (LC).

APPENDIX D

LIST OF ABBREVIATIONS

Abbreviation	Meaning
bp	base pair
CR4/5	Conserved Region 4 and 5
CTE	C-terminal extension
DKC	Dyskeratosis Congenita
IFD	insertion in fingers domain
l.c.	loading control
NAP	nucleotide addition processivity
nt	nucleotide
POT1	protection of telomeres 1
RAP	repeat addition processivity
RID	RNA interacting domain
RT	Reverse transcriptase
sca	small cajal body-specific
sno	small nucleolar
TBE	template boundary element
TEN	telomerase N-terminal
TERT	Telomerase Reverse Transcriptase
TF	template-free
TR	Telomerase RNA
TRBD	telomerase RNA binding domain
TWJ	Three way junction
VSR	vertebrate specific region

APPENDIX E

CO-AUTHOR APPROVAL AND CONTRIBUTIONS

I verify that the following co-authors have approved of my use of our collaborative work in my dissertation.

Xiaodong Qi: Provided gel image of Figure 2.3A.

Mingyi Xie: Provided gel image for Figure S3.1 and preliminary data for experiments in chapters 2 and 3.

Josh Podlevsky: Performed northern blot of hTR mutants in Figure 3.1. Also provided Figure 1.1 and Figure 3.6 as well as helped with research and organization of introduction.

Chris Bley: Purified POT1 protein used in Figure 2.7 and Figure 3.5.

Jing Huang: Provided .pdb file of medaka crystal structure used in Figure 4.1 prior to publication.

APPENDIX F

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