Structure *14*, 1603–1609, November 2006 ©2006 Elsevier Ltd All rights reserved DOI 10.1016/j.str.2006.09.004

The Unmasking of Telomerase

Minireview

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Telomerase is a ribonucleoprotein complex that reverse transcribes a portion of its RNA subunit during the synthesis of G-rich DNA at the 3′ end of each chromosome in most eukaryotes. This activity compensates for the inability of the normal DNA replication machinery to fully replicate chromosome termini. The roles of telomerase in cellular immortality and tumor biology have catalyzed a significant interest in this unusual polymerase. Recently the first structures of two domains, the CR4/CR5 and pseudoknot, of human telomerase RNA (hTR) were reported, offering a structural basis for interpreting biochemical studies and possible roles of hTR mutations in human diseases. Structures of the stem II and stem IV domains of *Tetrahymena thermophila* TR as well as the N-terminal domain of the *T. thermophila* telomerase reverse transcriptase have also been determined. These studies complement previous biochemical studies, providing rich insight into the structural basis for telomerase activity.

The end-replication problem is the inherent inability of primer-based DNA replication to fully replicate linear DNA molecules (Olovnikov, 1973; Watson, 1972). Nature has evolved several solutions to the end-replication problem (Nosek et al., 2006), but the vast majority of all eukaryotes use the enzyme telomerase to ensure that the chromosome ends are completely replicated (Smogorzewska and de Lange, 2004). Telomerase has generated a significant level of attention because of its connection to cellular mortality. Telomere length, which is regulated by telomerase activity, regulates replicative aging, possibly as a mechanism to oppose the proliferation of cancer cells. In humans, telomerase activity is low or undetectable in somatic cells but is upregulated in the majority (~90%) of cancer cells (Shay and Bacchetti, 1997).

Telomerase activity was first identified in the ciliate *Tetrahymena thermophila* by Greider and Blackburn (1985). Based on the observed sensitivity to ribonuclease A, they determined that telomerase was an RNA-dependent enzyme. After the single RNA subunit was identified, mutagenesis studies confirmed that it harbored the telomerase template (Yu et al., 1990), establishing telomerase as a ribonucleoprotein complex that reverse transcribes a small portion of its integral RNA subunit. Since this seminal work, telomerase activity has been observed in a wide variety of eukaryotes, and its primary constituent parts have been identified (Autexier and Lue, 2006; Harrington, 2003).

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After first being discovered from *T. thermophila*, telomerase RNA (TR) was identified in several ciliates, yeast, and vertebrates (Theimer and Feigon, 2006). In each, the template, which is complementary to the telomeric DNA sequence, represented only a small portion of the total RNA sequence, foreshadowing a greater role for the RNA subunit in telomerase biochemistry. The telomerase protein subunits resisted identification until Lingner and Cech (1996) purified telomerase from the ciliate *Euplotes aediculatus*, and peptide sequences were obtained by the Mann laboratory (Lingner et al., 1997). Two proteins were identified: p43 and p123. Concomitantly, the Lundblad lab identified several genes from a yeast genetic screen for an *est* (ever shorter telomeres) phenotype (Lendvay et al., 1996). One of the gene products, Est2p, was homologous to p123, and both contained amino acid sequences that are hallmarks of reverse transcriptases (RT). Mutation of the conserved RT motifs in Est2p resulted in an *est* phenotype, confirming the identification of the telomerase catalytic subunit (Lingner et al., 1997), which is commonly referred to as telomerase reverse transcriptase (TERT) (Nakamura and Cech, 1998). This seminal work ushered in a new phase in telomerase biochemistry, as it allowed the identification of TERT homologs in a wide variety of organisms.

While TR and TERT are the minimal requirements for telomerase activity in vitro, additional components of the telomerase holoenzyme are required in vivo and have been identified by various approaches. When telomerase was purified from *E. aediculatus*, a homolog of the human La protein, p43, was identified (Lingner and Cech, 1996). Subsequently, the human La protein was found to coimmunoprecipitate with telomerase from human cells (Ford et al., 2001). In *E. aediculatus*, p43 is involved in nuclear retention of the telomerase complex (Mollenbeck et al., 2003) and its *T. thermophila* ortholog p65 is involved in telomerase assemblage (O'Connor and Collins, 2006). The yeast genetic screen for the *EST* mutants identified several other proteins besides Est2p, the yeast TERT (Lendvay et al., 1996). Two of these proteins, Est1p and Est3p, are demonstrably part of the yeast telomerase complex (Hughes et al., 2000; Lin and Zakian, 1995), and yeast Est1p regulates telomerase (Evans and Lundblad, 2002; Osterhage et al., 2006). Est1p appears to be a common telomerase subunit, as homologs have been identified in human (Reichenbach et al., 2003; Snow et al., 2003) and fission yeast (Beernink et al., 2003). Several other proteins have been identified as telomerase components, and the telomerase holoenzyme is the subject of other recent reviews (Autexier and Lue, 2006; Harrington, 2003).

This review discusses recent progress toward understanding the relation of the structure of telomerase to its function. The biochemical activities of telomerase have received substantial attention (Autexier and Lue, 2006; Harrington, 2003; Kelleher et al., 2002). Therefore, the focus here will be on the solution structures of catalytically and structurally essential regions of human (Leeper et al., 2003; Leeper and Varani, 2005; Theimer et al.,

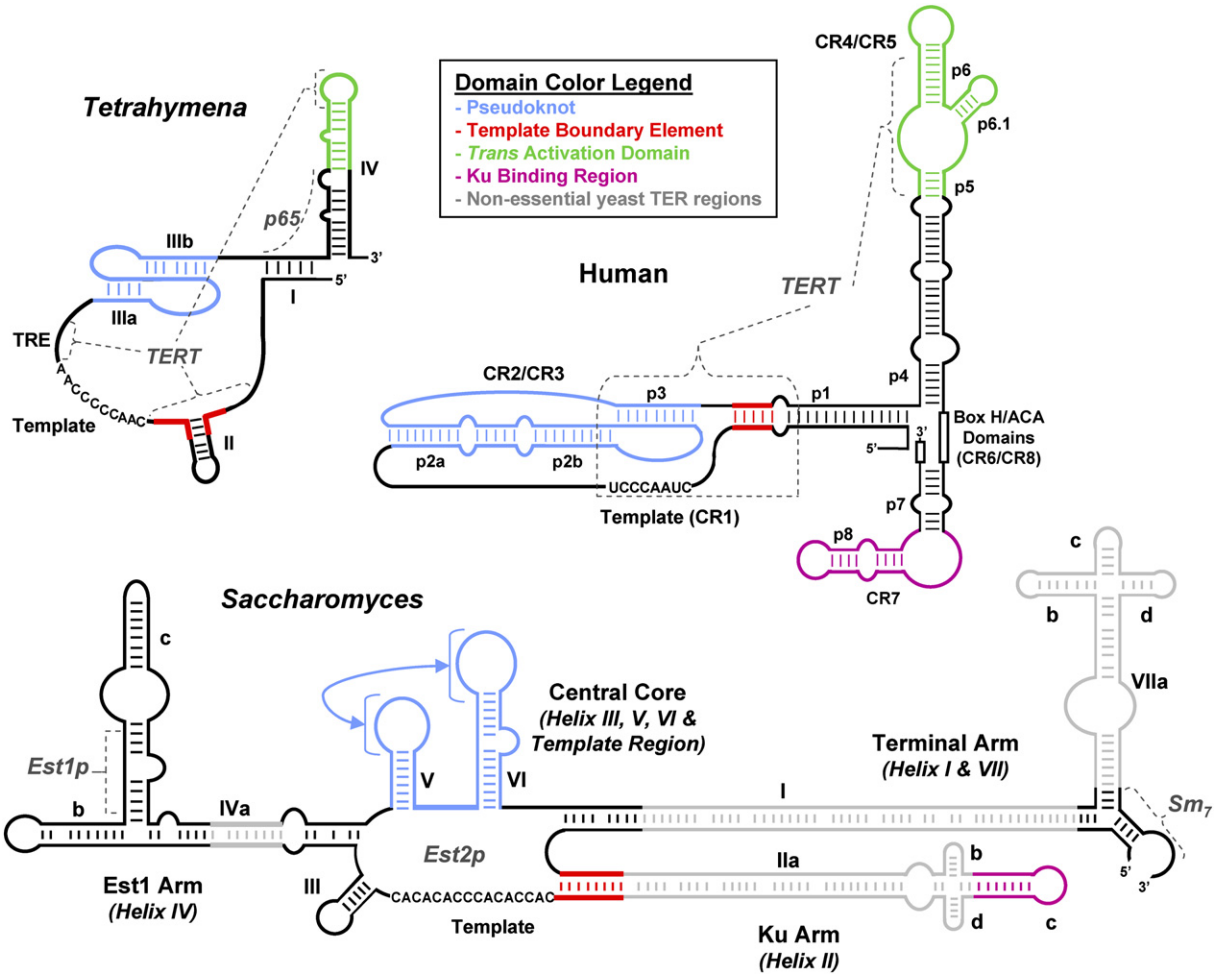

Figure 1. Secondary Structure of Telomerase RNAs

Elements common between at least two of the RNAs are highlighted by color as described in the legend. Dark gray brackets and text illustrate regions defined to interact with a specific protein. The putative *Saccharomyces* TR pseudoknot is depicted as two conserved stem-loops with a connector linking the possible pseudoknot-forming interaction. The light gray regions of yeast TR are dispensable for forming an active telomerase particle in vivo and in vitro as determined by Zappulla et al. (2005). The yeast TR nomenclature is as previously reported (Dandjinou et al., 2004; Lin et al., 2004; Zappulla and Cech, 2004).

2005) and *T. thermophila* telomerase RNA (Chen et al., 2006; Richards et al., 2006a) and the first crystal structure of the N-terminal domain of TERT (Jacobs et al., 2006). Given the current level of interest in telomerase, these studies represent significant advances for the field and portend future progress in telomerase biochemistry and rational drug design targeting the human telomerase complex.

Telomerase RNA

TRs from diverse species differ greatly in their size and share little sequence homology, but do appear to share common secondary structures (Figure 1). Important common features include a template, a 5′ template boundary element, a large loop including the template and putative pseudoknot, and a loop-closing helix. The ciliate (Romero and Blackburn, 1991), vertebrate (Chen et al., 2000, 2002), and yeast (Dandjinou et al., 2004; Lin et al., 2004; Zappulla and Cech, 2004) TR structures have been established by comparative phylogenetic and mutational analyses. Interestingly, the core structure of the large, 1157 nt, yeast TR can be reduced to a functional ~500 nt RNA (Zappulla et al., 2005). Recently, NMR spectroscopy has confirmed the proposed structures of several domains from ciliate and human TR (Chen et al., 2006; Leeper et al., 2003; Leeper and Varani, 2005; Richards et al., 2006a, 2006b; Theimer et al., 2005).

The Structure of Tetrahymena thermophila *Telomerase RNA*

The structures of two *T. thermophila* TR domains, stem-loop II of the template boundary element (Richards et al., 2006a) and the transactivating domain stem-loop IV (Chen et al., 2006; Richards et al., 2006b), were determined by NMR spectroscopy (Figure 2). The structure of stem-loop II is significant because its template proximal portion, specifically base pair G37·C19, is essential for proper template boundary definition. Stem-loop II forms a tight 7 nt long A-form helix containing six base pairs and one pair of opposing, un-base-paired adenines that stack into the middle of the helix. The pentaloop is well defined, with A29 in a rare *syn* conformation. Surprisingly, the distal portion of stem-loop II appears to be dispensable for activity and TERT binding (Lai et al.,

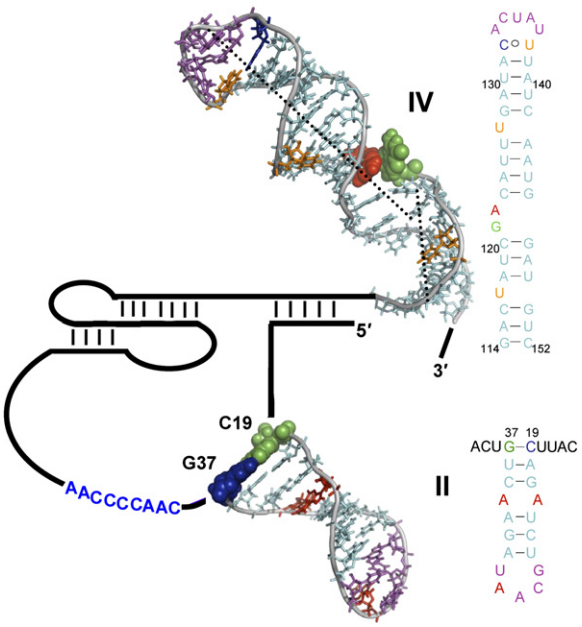

Figure 2. Solution Structures of *T. thermophila* Telomerase RNA Domains

The sequences for stem-loop II and IV are shown next to the atomic coordinates with the conserved template boundary element in stem II colored black. Nucleotides are colored as follows: canonical base pairs (cyan), loops (magenta), and nucleotides in unique configurations are colored as follows: A22, A29, A34, A122 (red), C19, C132 (blue), U117, U127, U138 (orange), and G37, G121 (green). The template is colored blue. The kink formed by the GA bulge in stem-loop IV is highlighted by dotted black lines. The GA121–122 bulge in stem-loop IV and the G37·C19 base pair in stem-loop II are draw as spheres to highlight their location. Coordinates for all structures were rendered using PyMOL (http://www.pymol.org).

2002; Licht and Collins, 1999). It is therefore unlikely that the entire stem-loop II is a necessary motif. Instead, it may function to facilitate high-affinity TERT binding by bringing together the two flanking template boundary elements present 3′ and 5′ of stem II, sequences 5′-UCA and 5′-CAUU, respectively (Lai et al., 2002; Licht and Collins, 1999). The absolute identity of these nucleotides as well as their location two residues 5′ of the template is required for tTERT binding (O'Connor and Collins, 2006) and for enzymatic activity (Autexier and Greider, 1995; Lai et al., 2002; Licht and Collins, 1999). This proposal is supported by the fact that *T. paravorax* contains these template boundary elements in close proximity, but lacks stem-loop II (McCormick-Graham and Romero, 1995).

More recently, the solution structure of the 43 nt stem-loop IV domain from *T. thermophila* TR was reported (Chen et al., 2006; Richards et al., 2006b). Stem-loop IV has been implicated in TERT binding (Lai et al., 2003), proper pseudoknot folding (Sperger and Cech, 2001), and processivity (Mason et al., 2003), and the proximal portion of stem-loop IV has recently been identified as a p65 binding site (Prathapam et al., 2005). Stem-loop IV forms a severely kinked structure capped by a structured 7 nt loop that is closed by a noncanonical C·U base pair (Figure 2). The eight base-pair helix preceding the apical loop is interrupted by a bulged U, which is identified as U127 in one population of structures and U126 in another population, suggesting that this region is in conformational flux. The unpaired GA bulge in the center of stem-loop IV severely kinks the structure by 40°–50°, confirming previous predictions (Bhattacharyya and Blackburn, 1994). Deleting the GA bulge, but not mutating its sequence, prevents reconstitution of telomerase activity, consistent with the proposal that stem-loop IV must be kinked for proper telomerase function (Sperger and Cech, 2001). Because stem IV can be added in *trans*, it seems that the GA-induced kink allows proper positioning of the distal stem-loop within the telomerase complex (Mason et al., 2003; Richards et al., 2006b).

Even though these NMR studies were conducted with isolated domains of tTR, they are pertinent because the features of the structures are consistent with chemical and enzymatic probing of full-length tTR in solution and in the telomerase complex (Bhattacharyya and Blackburn, 1994; Chen et al., 2006; Sperger and Cech, 2001; Zaug and Cech, 1995).

The Structure of Human Telomerase RNA

Human telomerase activity can be reconstituted by adding both the pseudoknot (nt 1–209) and the CR4/CR5 (nt 241–330) domains to hTERT in vitro and thus are the only TR domains required for catalytic activity (Tesmer et al., 1999). Furthermore, these domains bind independently to mammalian TERTs (Chen and Greider, 2003; Keppler and Jarstfer, 2004). Accordingly, they have been the focus of several structural studies, and the separate efforts of the Feigon and Varani groups have led to solution structures of portions of these domains (Leeper et al., 2003; Leeper and Varani, 2005; Theimer et al., 2005) (Figure 3).

The CR4/CR5 domain, including the p6a and p6b helices with an interconnecting loop (Leeper and Varani, 2005) and the essential p6.1 hairpin (Leeper et al., 2003), was reported by the Varani lab. The 32 nt structure of the p6a/J6/p6b hairpin contained 20 nt of the native sequence and evinces several important structural features of the human TR (Figure 3). Although phylogenetic analysis could not confirm base pairing in this region, the NMR-based model clearly shows a stable secondary structure with the two pairing regions, p6a and p6b, in standard A-form helices, consistent with footprinting data (Antal et al., 2002). The two helices are perturbed 20° from a coaxial alignment by loop J6. Though the role of J6 has not been extensively probed, it is a conserved element in all mammalian TRs (Chen et al., 2000) and the solution structure reveals a solvent-accessible tunnel. Interestingly, N3 of C290, which forms one wall of this tunnel, is protected from dimethyl sulfoxide in vivo (Antal et al., 2002) but not in vitro, suggesting that this tunnel is a receptor for hTERT or another component of the telomerase holoenzyme.

The p6.1 hairpin was first identified as an essential secondary structure of mammalian TRs by mutational analysis demonstrating the requirement of the stem region and two conserved residues, U307 and G309, (Chen et al., 2000, 2002). Subsequently, a role of the human p6.1 hairpin in binding hTERT and in enzymatic activity was established (Moriarty et al., 2004). The solution structure of the p6.1 hairpin contains four canonical base pairs in the helix capped by a wobble base pair U306·G310 (Figure 3). The p6.1 loop contains three

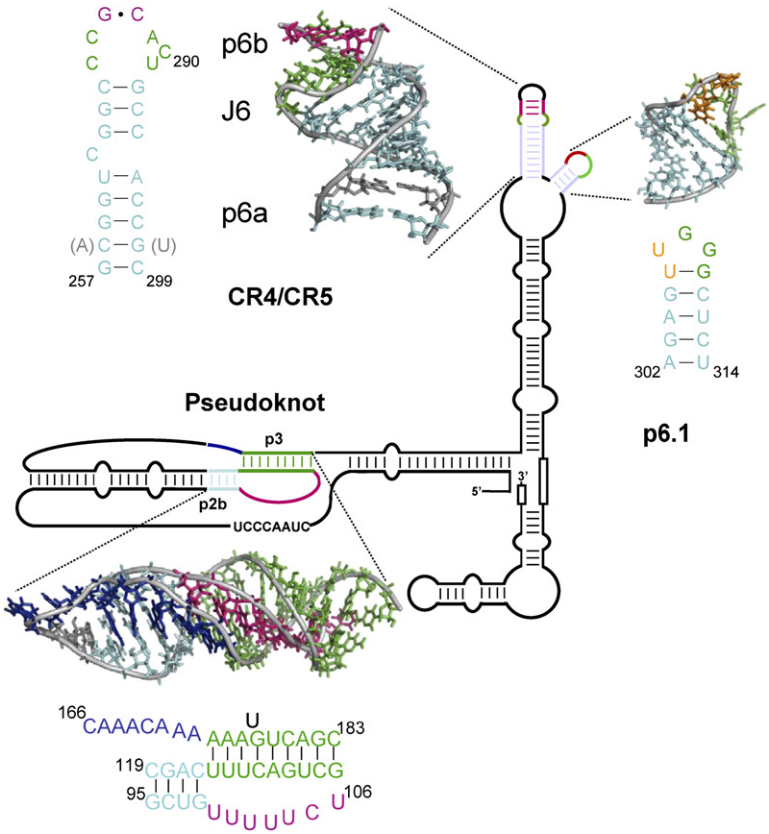

Figure 3. Solution Structures of Human Telomerase RNA Domains

Black dashed lines indicate the location of the individual structures in the full-length RNA. The full-length RNA and sequence of the individual structure elements are colored to match the ribbon diagrams. For the pseudoknot, the p2b helix is cyan, the p3 helix is green, the J2a/3 loop is blue, the J2b/3 loop is pink, and U177, which was deleted in the construct used for structure determination, is black. For the CR4/CR5 domain, the p6a helix is cyan, the J6 bulge is green, the p6b helix is pink, the p6.1 helix is cyan, uridines in the loop are orange, and guanosines in the loop are green. Residues in the NMR structures that are not native to human TR are colored gray.

nucleotides that present their Watson-Crick faces to the solvent. This solvent exposure is recapitulated by footprinting of full-length hTR, but these same residues are protected in vivo (Antal et al., 2002). Thus, these residues may be directly involved in protein interactions (Moriarty et al., 2004) or long-range interactions with the telomerase template (Ueda and Roberts, 2004).

The highly conserved pseudoknot domain of vertebrate TRs has been extensively investigated, owing to its predicted roles in telomerase functions and because mutations of this region of human TR are associated with several diseases (Chen and Greider, 2004; Theimer and Feigon, 2006). Recent solution structures of RNAs representing portions of the pseudoknot domain have greatly illuminated previous work and will continue to guide future efforts toward understanding the role of TR in telomerase function (Theimer et al., 2003a, 2003b, 2005). The structure of the human pseudoknot contains helices p2b and p3 and loops j2b/3 and j2a/3 including nt 93–121 and nt 166–174, with U177 deleted for stability reasons. These represent all of the residues required for formation of the conserved H-type pseudoknot (Theimer et al., 2005). The pseudoknot forms a well-ordered structure with the U-rich j2b/3 loop (U99–U106) residing in the major groove of helix p3 and the A-rich j2a/3 loop (C166–A173) located in the minor groove of helix p2b. Nucleotides U99–U101 of the j2b/3 loop form three U·A·U base triplets with the first three base pairs in helix p3, while A171 and A173 of the j2a/3 loop form two noncanonical base triplets. Each of these tertiary interactions was validated by mutational and thermodynamic studies on the stability of the pseudoknot. Importantly, telomerase activity correlated with the relative stability of these pseudoknot mutants (Theimer et al., 2005).

The formation of the pseudoknot in full-length human TR and within cells is not supported by chemical and enzymatic footprinting (Antal et al., 2002). This could be explained if the pseudoknot exists in an alternative, partially unfolded p2b hairpin that is in equilibrium with the pseudoknot structure, as previously proposed (Comolli et al., 2002; Theimer et al., 2003b). The structure of the p2b hairpin contains a unique series of polypyrimidine base pairs including three U·U base pairs and a water-mediated U·C base pair capped by a structured pentaloop (Theimer et al., 2003b). Interestingly, the dyskeratosis congenita-associated mutation GC(107-8)AG was found to stabilize the p2b hairpin and destabilize the pseudoknot conformation. Structurally, the basis for the increased stabilization is owed to a stabilizing YNMG-like tetraloop structure (Theimer et al., 2003a).

Telomerase Reverse Transcriptase

The catalytic subunit of telomerase, TERT, has been identified from several organisms and analysis of their sequences reveals a conserved primary structure that can be broken up into at least four domains (Autexier and Lue, 2006; Kelleher et al., 2002). A centrally located RT domain contains all seven of the universally conserved RT motifs, mutation of which disrupts telomerase activity. A high-affinity RNA-binding domain N-terminal of the RT motifs, the N-terminal proximal domain known as RID2, has been confirmed in both hTERT and tTERT (Bachand and Autexier, 2001; Bryan et al., 2000; O'Connor et al., 2005). A second N-terminal domain, distal to the RT motifs and referred to as RID1, TEN, or DAT,

has been implicated in primer binding (Lee et al., 2003; Moriarty et al., 2005), and mutations of hTERT within this domain separate in vivo function from catalytic activity (Armbruster et al., 2001). The fourth domain is the C-terminal domain, which is essential for hTERT and tTERT (Autexier and Lue, 2006) and has been implicated in promoting processive polymerization (Huard et al., 2003). This domain is absent in round worms, suggesting that its functions have been supplanted by other domains within TERT or other subunits of the telomerase complex in these organisms (Malik et al., 2000; Meier et al., 2006).

Recently, a high-throughput screen was conducted to identify domains of *T. thermophila* TERT that can be expressed in soluble form in *Escherichia coli* (Jacobs et al., 2005). The screen successfully identified a 23.5 kDa fragment representing residues 2–191, which encompasses the TERT essential N-terminal distal domain, TEN. Subsequently, Jacobs et al. (2006) crystallized this portion of tTERT to generate the first high-resolution structure of a domain from the catalytic subunit of telomerase (Figure 4). The structure of the TEN domain represents a unique protein fold and contains several residues that are conserved among all TERT sequences identified save TERT from round worms, which share only weak homology in the TEN domain (Meier et al., 2006). In TEN-containing TERTs, the invariant glycine residues G144 and G171 (*T. thermophila* numbering) seem to be involved in proper folding as they connect two different secondary structure elements via sharp turns. An important feature of the TEN structure constitutes a deep groove that runs from the C terminus to the center of the structure and is composed of several conserved residues that are important for catalytic activity, and the floor of this groove contains the invariant residue Q168.

The TEN domain has been implicated in DNA and RNA binding. However, the isolated domain only weakly binds single-stranded telomeric DNA. Photo-crosslinking was therefore used to demonstrate sequence-specific binding of TEN to telomeric DNA. Importantly, mutants Q168A, F178A, and W187A all abrogated crosslink formation to the TEN domain and greatly reduced telomerase activity in the context of full-length TERT. Because these residues reside near each other in the deep groove found in the TEN domain, it is tempting to speculate that this groove is involved in binding to and aligning the telomere into the telomerase active site and may be the anchor site that other studies have implicated (Hammond et al., 1997; Lue, 2005; Moriarty et al., 2005). The role of the TEN domain in affinity and specificity of telomere binding is further suggested by mutations within the N-terminal domain of hTERT that affect elongation of telomeric but not nontelomeric DNA primers (Armbruster et al., 2001; Lee et al., 2003), mutations in the TEN domain of hTERT that are rescued by increasing primer concentration (Moriarty et al., 2005), and deletion mutants of yeast TERT that affect elongation of primers forming only short duplexes with the telomerase template (Lue, 2005).

Telomerase Holoenzyme and Telomerase Subunits

The telomerase holoenzyme minimally contains TR and TERT. However, several other subunits are known to be a part of the telomerase holoenzyme and these have recently been reviewed (Autexier and Lue, 2006; Harrington, 2003). Most of these proteins were identified by

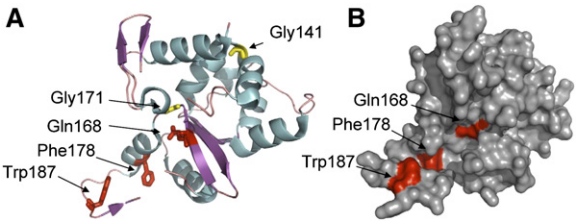

Figure 4. Crystal Structure of the Essential N-Terminal Domain of TERT from *T. thermophila*

(A) Ribbon diagram of the TEN domain with helices in cyan, sheets in purple, and loops in orange. Residues implicated in binding to the telomeric primer are highlighted in red. Conserved glycines are highlighted in yellow.

(B) Surface representation of the TEN domain is highlighted as in (A).

copurification, and many have been confirmed as telomerase components by exhibiting a telomere maintenance phenotype when mutated. Currently, the structural basis for holoenzyme assemblage is poorly understood, but we can expect further elucidation of the telomerase complex structure and confirmation of its subunits to follow. One approach that has been particularly productive in this regard is the use of an endogenously expressed, epitope-tagged TERT to facilitate tandem affinity purification of telomerase subunits in *T. thermophila*. In this way, the Collins laboratory identified four new subunits, two of which, p45 and p65, were required for telomere maintenance in vivo (Witkin and Collins, 2004).

One of the major structural questions regarding the telomerase holoenzyme is the oligomerization state of the complex. Biochemical data suggest that the human (Wenz et al., 2001) and yeast (Prescott and Blackburn, 1997) telomerase complexes exist in dimeric forms, whereas telomerase from *E. crassus* (Wang et al., 2002) can exist in both dimeric and higher order forms depending on the life-cycle stage of the organism. Interestingly, telomerase from *T. thermophila* exists as a monomer, suggesting that the inherent biochemical activity of telomerase does not require a dimeric complex (Bryan et al., 2003). Recently, direct evidence for the oligomerization state of *E. aediculatus* telomerase was provided by electron microscopy (Fouche et al., 2006). In this experiment, *E. aediculatus* telomerase was visualized at the ends of synthetic chromosomes as a dimer, with two types of structures primarily observed: single DNA molecules with a telomerase dimer at one end and two DNA molecules brought together by two telomerase dimers.

Conclusion

The importance of telomerase to stem-cell technology, anticancer therapies, and human disease ensures that the initial structures of this fascinating enzyme are only a beginning. As the field expands, we can anticipate the ability to address several important questions regarding the mechanism of telomerase. Foremost, what is the structural basis for template identification and accurate template utilization and related questions regarding orchestration of processive primer elongation and promotion of telomere association, given the limited base pairing between the template and telomere?

Increasing knowledge of the structure of human telomerase will facilitate rational drug design. Currently,

small-molecule telomerase inhibitors are being identified by random screening followed by optimization through chemical modification. While this has identified several potent molecules, the affinities of most are not high enough to warrant advancement into the clinic, even though an oligonucleotide-based template antagonist has entered clinical trials (Djojosubroto et al., 2005). Given the success of virtual screening to enhance the rational design of small-molecule ligands, it is clear that the field of telomerase-targeted therapeutics will rapidly expand as the structural biology of telomerase is advanced.

Acknowledgments

We would like to thank Yu Chen and Gabriele Varani for sharing coordinates prior to publication and Shawn Ahmed for helpful suggestions on the manuscript. M.B.J. is supported by grants from the National Science Foundation and the National Institutes of Health. A comprehensive list of references could not be included due to strict limitations on the length of this article.

Received: June 9, 2006
Revised: September 1, 2006
Accepted: September 8, 2006
Published: November 14, 2006

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