

SURVEY AND SUMMARY

InTERTpreting telomerase structure and function

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ABSTRACT

The Nobel Prize in Physiology or Medicine was recently awarded to Elizabeth Blackburn, Carol Greider and Jack Szostak for their pioneering studies on chromosome termini (telomeres) and their discovery of telomerase, the enzyme that synthesizes telomeres. Telomerase is a unique cellular reverse transcriptase that contains an integral RNA subunit, the telomerase RNA and a catalytic protein subunit, the telomerase reverse transcriptase (TERT), as well as several species-specific accessory proteins. Telomerase is essential for genome stability and is associated with a broad spectrum of human diseases including various forms of cancer, bone marrow failure and pulmonary fibrosis. A better understanding of telomerase structure and function will shed important insights into how this enzyme contributes to human disease. To this end, a series of high-resolution structural studies have provided critical information on TERT architecture and may ultimately elucidate novel targets for therapeutic intervention. In this review, we discuss the current knowledge of TERT structure and function, revealed through the detailed analysis of TERT from model organisms. To emphasize the physiological importance of telomeres and telomerase, we also present a general discussion of the human diseases associated with telomerase dysfunction.

INTRODUCTION

Telomeres are essential nucleoprotein structures that define the terminal segments of linear chromosomes. In eukaryotes, telomeres are essential for genome stability, functioning to prevent chromosome ends from being recognized and processed as *bona fide* DNA double-strand (ds) breaks. Importantly, telomeres also provide a solution

to the ‘end-replication problem’, which was first proposed by Olovnikov and Watson in the early 1970s (1–3). This model predicts that during the process of DNA replication, a small amount of DNA from the 3′-ends of linear chromosomes is left unreplicated. As a result, chromosome 3′-ends progressively shorten during consecutive cell divisions, which limits cellular lifespan (1–3). Chromosome ends that lack sufficient telomeric repeats are prone to recombination and fusion with other pieces of genomic DNA, events that can interfere with normal cell cycle progression and promote genetic instability. Thus, telomeres provide a protective cap for the ends of linear chromosomes.

One universal feature of telomeric DNA is the organization into a C/A-rich strand and a complementary G/T-rich strand. Telomeric DNA almost always contains tandem repeats of simple, species-specific sequences that are 6–8 nucleotides (nt) long [e.g. (TTAGGG)_n in mammals]. Another conserved feature of telomeric DNA is the organization into a ds segment with a single-stranded (ss) 3′-overhang. Electron microscopic analysis of psoralen cross-linked human and mouse telomeric DNA revealed large lariat-like structures containing thousands of TTAGGG repeats (4). These structures are known as telomere-loops (t-loops) and are postulated to be formed and stabilized by invasion of the telomeric 3′-overhang into the duplex repeat array (4). Telomere loops have also been detected in trypanosomes (5), ciliates (6), plants (7), nematodes (8) and some strains of yeast (9,10). The existence of t-loops provides an attractive model that could explain how ss chromosome ends are protected from degradation, recombination and fusion (11). However, the molecular mechanism(s) that regulate t-loop formation *in vivo* remain to be elucidated. If living cells do contain t-loops, one interesting possibility is that telomeres may adopt alternative conformations at specific stages of the cell cycle (12). The identification of novel, cell-cycle-specific telomere-associated proteins will provide important insights into telomere dynamics and length regulation *in vivo*.

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Various proteins have been shown to associate with telomeric DNA and are implicated in the formation and maintenance of telomere architecture. These proteins are the focus of excellent reviews by Linger and Price (13) and Palm and de Lange (11), and will not be discussed in detail in this article. One general concept, however, relates to the evolution of telomere-associated proteins. Although telomere function is conserved in diverse organisms, the architecture and composition of telomere-associated proteins is remarkably varied and seems to have changed rapidly during evolution (13). For example, human telomeres are bound by a six-protein complex called shelterin, comprised of TRF1, TRF2, POT1, RAP1, TIN2 and TPP1, which interacts with ss and ds telomeric DNA (11). In contrast, budding yeast telomeres are bound by two separate protein complexes (13). One contains the Cdc13, Stn1 and Ten1 proteins and binds the G-rich 3'-overhang (14). The other, which binds duplex telomeric DNA, is minimally composed of Rap1, Rif1 and Rif2 (15). Despite these differences in composition, however, the telomere-associated proteins have a conserved function, which is to assemble a protective cap that ensures telomeres are maintained at an appropriate length and are protected from being recognized and processed as broken DNA. The ability to perform this task is facilitated by additional species-specific protein-protein interactions (11,13).

De novo synthesis of telomeric DNA in most eukaryotes is performed by the cellular ribonucleoprotein reverse transcriptase (RT) telomerase. Originally discovered by Carol Greider and Elizabeth Blackburn in the ciliate *Tetrahymena thermophila* (16–18), telomerase is a unique RT that contains a catalytic protein subunit, the telomerase RT (TERT), the telomerase RNA (TR) and species-specific accessory proteins. Telomere synthesis involves TERT-catalyzed reverse transcription of a small template region within TR and telomerase activity can be reconstituted in rabbit reticulocyte lysates by co-expressing the TERT and TR subunits (19–21). Importantly, species-specific accessory proteins regulate telomerase biogenesis, subcellular localization and function *in vivo*. For example, mass spectrometric analysis of affinity-purified telomerase from HeLa cells has identified integral protein components of human telomerase: dyskerin, NHP2, NOP10, pontin/reptin and TCAB1 (22–25). Dyskerin, NHP2 and NOP10 are required for the stability and accumulation of human telomerase RNA (hTR) *in vivo* (22). Similarly, pontin and reptin are two closely related ATPases necessary for the stability of dyskerin and hTR *in vivo* (24). The current model is that dyskerin, pontin and reptin form a scaffold that recruits and stabilizes hTR, and assembles the telomerase ribonucleoprotein particle. Once this complex is formed, pontin and reptin are thought to dissociate from the complex and yield the catalytically active enzyme (24). The subcellular localization of telomerase appears to be regulated by the recently identified protein TCAB1 (25). Further studies are needed to elucidate the biochemical and molecular significance of the intricate network of protein-protein and protein-nucleic acid interactions within the telomerase holoenzyme. Moreover, it will be

important to investigate whether the composition of the holoenzyme changes in specific stages of the cell cycle. This information will provide important insight into how human telomerase functions *in vivo*. Notably, the composition of the telomerase holoenzyme varies significantly between species and the reader is referred to Collins (26) and Gallardo and Chartrand (27) for excellent reviews of this topic.

THE TELOMERASE RT SUBUNIT

The catalytic core of telomerase is composed of the RNA subunit (TR) and the catalytic protein subunit (TERT), whereas the holoenzyme contains additional species-specific accessory proteins. The first TERT subunits were identified through genetic screens in yeast (28) and the biochemical purification of *Euplotes aediculatus* telomerase (29,30). The *E. aediculatus* protein was identified as a homolog of the yeast protein and sequence comparison with prototypical RTs revealed an evolutionarily conserved RT domain in both proteins (30). The first direct evidence that TERT was the catalytic subunit of telomerase came from *in vivo* studies showing that substitution of evolutionarily conserved residues within the RT catalytic triad of the yeast protein caused telomere shortening and cellular senescence *in vivo* and eliminated enzymatic activity *in vitro* (30). TERT homologs were subsequently identified in humans, rodents and ciliates (21,31–35). Further evidence that TERT was the catalytic subunit of human and *T. thermophila* telomerase came from studies showing that telomerase activity could be reconstituted *in vitro* by co-expressing wild-type TERT and TR in rabbit reticulocyte lysates (19–21). These studies were also the first to demonstrate that TERT and TR were sufficient to reconstitute telomerase activity *in vitro* (19–21), although it is important to note that rabbit reticulocyte lysates contain endogenous proteins that interact with telomerase to facilitate its assembly and activity [e.g. heat shock (36) and chaperone (37) proteins].

Bioinformatics and mutational studies have collectively established that TERT contains three main structural elements: (i) a long N-terminal extension that contains conserved DNA- and RNA-binding domains; (ii) a central catalytic RT domain; and (iii) a short C-terminal extension (38). To exemplify this domain organization, the predicted linear architecture of human TERT (hTERT) is illustrated in Figure 1A. Although this organization defines almost all TERT proteins, there are a few notable exceptions. First, certain insect and nematode TERTs harbor a truncated N-terminus that does not contain the telomerase essential N-terminal (TEN) domain (39,40). Second, the C-terminal region is absent from *Giardia lamblia* and nematode TERTs (40). Third, *Plasmodium falciparum* TERT contains an abundance of hypervariable insertions between the conserved domains and is at least three times larger than all other TERT proteins (41). The biological significance of this divergent architecture is not clear and it will be interesting to

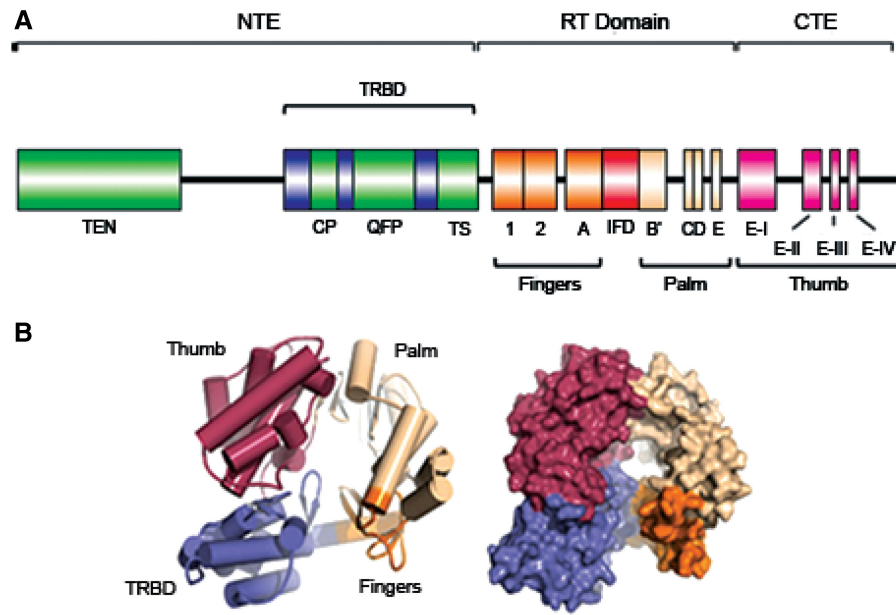


Figure 1. Structural organization of the TERT. (A) Predicted linear architecture of hTERT. In most organisms, TERT contains a long N-terminal extension (NTE), a central catalytic RT domain and a short C-terminal extension (CTE). Green boxes indicate the predicted locations of the telomerase essential N-terminal (TEN) domain and the telomerase-specific motifs CP, QFP and TS. Blue boxes indicate the TRBD, containing the CP motif, QFP motif and part of the TS motif. An unstructured linker region connects the TEN domain and TRBD. Orange and tan boxes represent the seven evolutionarily conserved motifs in the RT domain (1, 2, A, B', C, D, E) and a red box illustrates the telomerase-specific IFD. The CTE contains four blocks of conserved amino acids, which are shown as pink boxes (E-I, E-II, E-III, E-IV). (B) Domain organization of the protein predicted to be *T. castaneum* TERT (cartoon and surface representation), reprinted with permission from ref. (42). The protein is organized into a ring-shaped structure, containing hallmark 'thumb' (red), 'palm' (tan) and 'fingers' (orange) motifs. The telomerase-specific TRBD is shown in violet. The color scheme used in (A) corresponds to that shown in (B).

determine if other TERT domains and/or cellular proteins compensate for the missing domains *in vivo*.

A potential advance in our understanding of telomerase architecture came from the recent atomic-resolution structure of a protein thought to represent full-length *Tribolium castaneum* (flour beetle) TERT, in complex with an 18-nt telomeric oligonucleotide (42). However, whether this protein truly represents a telomerase is currently controversial. Formal proof that this *T. castaneum* protein represents the catalytic TERT subunit remains to be demonstrated, which is an important question to address given the complex evolution of telomerase and telomere-maintenance in insects (39,43). For example, Dipteran insects (e.g. mosquitoes) lack telomerase activity and maintain their telomeres *via* recombination-based mechanisms, whereas Arthropods (e.g. silkworm) have pentanucleotide (TTAGG)_n telomeric repeats and contain detectable levels of telomerase activity (39,43). Interestingly, the predicted *T. castaneum* TERT lacks a significant portion of the N-terminus that is critical for telomerase activity in ciliates, yeast and humans (42). One possibility is that *T. castaneum* TERT may be inactive, which would be supported by the absence of canonical telomeric repeats in this insect. Another prediction, which cannot be excluded in the absence of biochemical support for telomerase activity, is that the putative TERT represents a non-telomerase RT. Despite these potential caveats, the crystal structure reported by Skordalakes and colleagues (42) represents an important

snapshot of a TERT-like protein and provides the basis for structural comparisons with other TERTs.

The *T. castaneum* TERT structure suggests a ring-shaped protein held together by extensive hydrophobic interactions between the N- and C-terminal regions (Figure 1B) (42). This architecture is congruent with previous biochemical studies of human and *Euplotes crassus* TERT, which revealed physical interactions between N- and C-terminal domains (44–46). The ring-like structure of TERT is akin to that of other nucleic acid polymerases, including that of human immunodeficiency virus (HIV), and contains the hallmark 'thumb', 'palm' and 'fingers' motifs (Figure 1A and B) (42). Notably, the interior dimensions of the TERT ring structure predict that it can accommodate seven or eight base pairs of ds nucleic acid (42). This is consistent with previous biochemical studies demonstrating that the RNA–DNA duplex in the telomerase active site is maintained at a length of seven to eight base pairs (47,48). The ring's interior is lined with amino acids that mediate protein–nucleic acid interactions, nucleotide binding and DNA synthesis in yeast and hTERT (49–54). An important future challenge will be to determine the high-resolution structure of TERT in complex with its integral RNA subunit and/or additional protein components. Towards this goal, recent studies allowed the determination of the crystal structure of the *T. castaneum* TERT in complex with a short RNA–DNA hybrid designed to resemble the putative RNA-templating

region and complementary DNA sequence (55). One potential caveat to this study, however, is that the RNA component of this telomerase is not known and therefore, the physical contacts observed in this structure may be significantly different than those that occur in the presence of the biological RNA subunit and telomeric chromatin. An equally important challenge will be to solve the crystal structure of full length TERT from an organism with well-characterized telomerase activity.

The TERT N-terminal extension

The N-terminal extension of most TERTs contains two conserved domains, the TEN domain and telomerase RNA-binding domain (TRBD) (Figure 1A). High-resolution structures of the *T. thermophila* TEN domain and TRBD indicate that both of these regions represent novel protein folds involved in binding ss nucleic acids (56,57). In between these domains is a relatively long and unstructured linker region that may be important for conformational flexibility within the holoenzyme (Figure 1A). The N-terminal extension also contains several conserved telomerase-specific motifs, including the CP, QFP and TS motifs (Figure 1A) (58,59). These regions are important for TERT-TR binding interactions and the rate of template copying during telomere synthesis (19,58,60–69), and may thus be amenable to therapeutic interventions that inhibit or augment telomerase activity.

The crystal structure of the *T. thermophila* TRBD reveals that it is organized into two asymmetric lobes that contain residues from the CP and TS motifs (Figure 2A) (56). The two halves are connected by extended loops that impart the domain with structural flexibility (56). Hydrophilic and hydrophobic CP and TS residues form an extended RNA-binding groove on the surface of the domain. The dimensions of this cavity reveal a relatively wide hydrophilic pocket that could accommodate dsRNA and a narrow hydrophobic pocket that could accommodate ssRNA (56). In the context of full-length *T. castaneum* TERT, the RNA-binding groove is located on the side of the ring and faces the interior of the ring in close proximity to the active site (Figure 2B) (42). This is believed to permit the RNA 5'-end to enter the ring's interior where the active site is located. The structural data is supported by biochemical studies with ciliate, yeast and human telomerase that have identified the CP and TS motifs as being critical for TR-binding and telomerase activity *in vitro* and *in vivo* (19,58,60–68).

The most N-terminal domain in TERT (the TEN domain) exhibits binding affinity for ss telomeric DNA and contains residues that are essential for telomerase activity (38,57,70–75). Furthermore, a specific leucine in the TEN domain of ciliate TERT (L14) has been identified as a critical determinant of the enzyme's ability to add multiple telomeric repeats to a single telomeric primer (74). These observations indicate that the TEN domain has an important role in DNA substrate recognition and elongation. Interestingly, the TEN domain also displays a weak non-specific RNA-binding activity (57). Although the significance of this interaction is presently unclear,

it is possible that in the context of the full-length protein, the TEN domain and TRBD co-operate to ensure sequence-specific TERT-TR binding interactions and optimal template positioning during telomerase assembly and/or telomere synthesis.

The TERT RT domain

The catalytic domain of TERT is the most characterized region of the protein and contains seven evolutionarily conserved RT motifs (see Figures 1A and 2C) essential for enzymatic activity (19,20,30,31,33,51,76–78). The TERT RT domain is organized into two subdomains that resemble the 'fingers' and 'palm' subdomains of prototypical RT enzymes (Figure 2C) (30,31,79). These regions are connected by a loop that contains the conserved 'primer grip' region of motif E (42). Molecular models predict that the loop makes direct contacts with the RNA-DNA hybrid and the 3'-end of the ssDNA, suggesting that the primer grip region could be involved in positioning the 3'-end within the enzyme active site (Figure 2B) (42). This model is supported by biochemical studies of yeast and hTERT that have identified an important role for this region in ssDNA-binding and processive telomere synthesis *in vitro* and *in vivo* (80,81). Further evidence for contacts between the TERT primer grip and DNA primer have been provided by structural studies of *T. castaneum* TERT in complex with an RNA-DNA hairpin designed to resemble the putative telomerase RNA template and telomeric DNA (55).

One unique structural feature of the telomerase RT domain is a large insertion between motifs A and B', called the 'insertion in fingers' domain (IFD) (Figure 1A) (30,31). The IFD is comprised of two antiparallel α -helices that are sandwiched between the fingers and palm subdomains (Figure 2C) (42). The crystal structure reveals that the IFD is engaged in intramolecular protein-protein interactions that organize and stabilize this region of the RT domain (42). Importantly, the IFD makes extensive contacts with an α -helix that is implicated in making direct contacts with the backbone of the incoming DNA primer (42). Mutations that alter the structural integrity of the IFD are expected to indirectly compromise protein-DNA and RNA-DNA interactions, thus impairing enzyme activity. Consistent with this, alanine substitutions of moderately conserved residues in the IFD of Est2p (yeast TERT) were found to impair telomerase activity and processivity *in vitro* and prevented telomere maintenance *in vivo* (52).

The TERT active site contains three invariant aspartic acids, one of which resides in motif A and the other two within motif C (Figure 2D) (30,31). These residues are conserved in RTs and form a catalytic triad that participates directly in nucleotide addition *via* a two-metal ion mechanism (30,31). Alanine substitution of these amino acids abrogates telomerase activity (19,33,64,76,77). Conserved residues from motifs 1, 2, A, B', C and D form the nucleotide-binding pocket, located at the interface between the palm and fingers subdomains (Figure 2D) (42). Two conserved surface-exposed

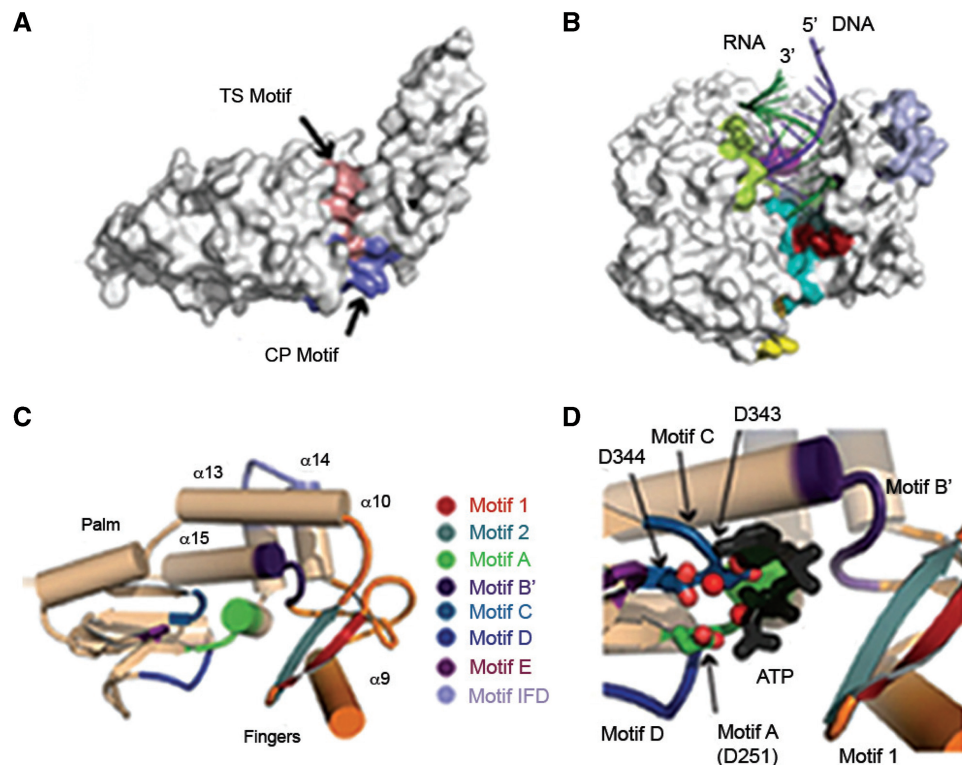


Figure 2. Molecular models of *T. thermophila* (A) and *T. castaneum* (B–D) TERT. (A) Surface representation of the isolated TRBD from *T. thermophila* TERT, reprinted with permission from ref. (56). This cartoon shows the two asymmetric lobes that comprise the TRBD. Amino acids that form the TS motif are shown in pink and those that comprise the CP motif are indicated in blue. (B) Model of the protein suggested to be *T. castaneum* TERT (surface representation) in complex with the telomerase RNA subunit (dark green) and single-stranded telomeric DNA (dark purple), reprinted with permission from ref. (42). The hallmark motifs of the RT domain are illustrated as follows: motif 1 (red), 2 (grey), A (green), B' (dark purple), C (blue), D (dark blue), E (magenta) and IFD (light blue). The TRBD comprises residues from the CP (yellow) and TS (cyan) motifs. Structural elements of the TERT C-terminal extension (light green) are predicted to stabilize and orientate the TERT–RNA–DNA complex. (C) Domain folds of the predicted *T. castaneum* TERT RT domain, colored as in (B) and reprinted with permission from ref. (42). Elements that form the palm domain are colored in tan and those that form the fingers domain are shown in orange. (D) The active site and nucleotide-binding pocket of *T. castaneum* TERT, reprinted with permission from ref. (42). This figure shows the predicted location of three invariant aspartic acids (D251, D343 and D344) that form the catalytic triad of the active site in complex with a modeled nucleotide of ATP (black stick). The RT motifs are colored as in (B).

residues from motifs A and C (tyrosine and valine, respectively) form a hydrophobic patch that is predicted to bind the base of the nucleotide substrate. This interaction would facilitate nucleotide positioning in the active site for co-ordination with a metal ion and the 3'-end of the DNA primer (42). This model is supported by biochemical studies of ciliate, yeast and hTERT that have implicated these residues in nucleotide insertion rate, polymerase fidelity and enzyme processivity (78,80,82).

Sequence alignment of the TERT RT domain revealed a novel telomerase-specific motif (motif 3) between motifs 2 and A (Figure 1A) (83,84). Interestingly, although the predicted secondary-structure of motif 3 is evolutionarily conserved, the primary sequence is only conserved in vertebrate and ciliate telomerases. This suggests that motif 3 may regulate species-specific aspects of telomerase biochemistry, such as repeat addition processivity (84). Alanine substitution screening of this motif in hTERT identified mutations that either abrogated telomerase activity or caused a significant increase in the rate or processivity of telomere synthesis *in vitro* (84). These biochemical characterizations have provided new insight into

the telomerase reaction mechanism by identifying specific amino acids in hTERT that independently regulate two critical aspects of telomere synthesis. An important area of future research will be to elucidate how mutations in motif 3 affect the biogenesis, activity and regulation of telomerase.

The TERT C-terminal extension

In contrast to the N-terminus and RT domain, the C-terminus of TERT shows only weak sequence conservation suggesting that it may have species-specific functions or that different amino acid sequences have evolved to fold into similar structural domains. The C-terminal extension of TERT adopts a novel protein fold, although structural comparison of TERT with the closely related HIV RT indicates that this region represents the thumb domain of telomerase (42). The C-terminus is a helical bundle that contains several surface-exposed loops that are thought to contribute to the formation and stabilization of an RNA–DNA heteroduplex in the enzyme active site (Figure 2B) (42,55). Mutations in this region affect the nucleotide

and repeat addition processivity of human and yeast telomerase, telomere length maintenance in human cells and the subcellular localization of hTERT (49,53,54,80,85). Furthermore, it was shown that the addition of an epitope tag to the C-terminus of hTERT abolishes telomere elongation *in vivo*, suggesting that the conformation of this region is important for hTERT function (86). Interestingly, the C-terminal extension is essential for telomerase activity in *T. thermophila* and humans, but is dispensable for telomerase activity in yeast (58,62,63,65) and is completely absent in other organisms (40). These observations further suggest that the C-terminal extension has species-specific roles *in vivo*. Additional structure–function studies are needed to understand how this region contributes to the biochemical and molecular properties of telomerase from different organisms. In this regard, the *in vitro* and *in vivo* characterization of disease-associated hTERT mutants will likely provide invaluable insight into these aspects of human telomerase (87,88).

THE TELOMERASE RNA SUBUNIT

A unique feature of telomerase is that the RNA template for DNA synthesis is an integral component of the holoenzyme. Although a detailed discussion of the RNA subunit is beyond the scope of this review, it is necessary to comment on some of the key properties of the vertebrate TR. The reader is referred to Theimer and Feigon (89) for a comprehensive review of the structure and function of TR subunits from various organisms.

Phylogenetic comparative analysis of vertebrate TR predicts three conserved domains: (i) the pseudoknot/template core domain; (ii) the CR4/CR5 domain (for conserved regions 4 and 5, respectively); and (iii) a box H/ACA domain (90). The core domain is essential for telomerase activity *in vitro* and *in vivo* (89). This region contains the template for telomere addition, the 5'- and 3'-boundary elements that prevent the incorporation of non-template nucleotides, a putative TERT binding site and a conserved pseudoknot structure (90,91). Human telomerase activity can be reconstituted *in vitro* by co-expressing the pseudoknot/template domain and the CR4/CR5 domain in the presence of hTERT (92,93). The TR domains work together to mediate TR-TERT interactions, nucleotide and repeat addition processivity, and enzyme fidelity (89). The 3'-end of vertebrate TR contains two conserved motifs, the box H and ACA elements (box H/ACA domain), which serve as binding sites for proteins involved in RNA processing, stability and subcellular localization (89). The box H/ACA domain is essential for TR stability, processing, nuclear localization and telomerase activity *in vivo* (89,94,95). Mutations in the gene encoding TR have been linked to the multi-system disorder called dyskeratosis congenita, highlighting the clinical importance of this molecule. Furthermore, the TR gene is amplified in several human cancers (96–98), thus making it a potential target for therapeutic inhibitors of telomerase activity in cancer cells (99).

THE TELOMERASE REACTION CYCLE

Telomerase has the unique ability to catalyze multiple rounds of template copying and add hundreds of nucleotides to the same DNA primer. This contrasts with prototypical RTs, which copy a relatively large RNA genome into a single molecule of complementary DNA. As illustrated in Figure 3 and discussed in the following sections, the telomerase reaction cycle can be divided into three basic steps: (i) primer recognition and binding; (ii) synthesis of the first telomeric repeat; and (iii) translocation and realignment of the new DNA 3'-end to initiate the next round of telomere synthesis.

During telomere elongation, the RNA template is reverse transcribed using canonical Watson–Crick base-pairing to specify the telomeric sequence. Telomere synthesis proceeds by the sequential addition of deoxynucleotide triphosphates (dNTPs) to the free 3'-hydroxyl group of the telomeric ssDNA primer. The telomeric ssDNA overhang is believed to be the natural primer for telomerase-mediated DNA synthesis *in vivo*. However, telomerase can elongate almost any G-rich linear ssDNA primer that contains a free 3'-end *in vitro* (16, 100). Parallel intermolecular G-quadruplex substrates are also efficient substrates for ciliate telomerase *in vitro* (101–103).

Telomerase incorporates consecutive dNTPs without dissociating from the primer by a reaction known as nucleotide addition processivity (38). During telomere elongation, the RNA–DNA hybrid is kept at a constant length of seven to eight base pairs by melting bonds at the distal end of the template as new bonds are formed at the proximal end (48). When the 5'-template boundary element is reached, a translocation step repositions the new DNA 3'-end within the template for a second round of telomere synthesis. The ability of telomerase to catalyze more than one round of DNA synthesis while bound to the same telomeric primer is referred to as repeat addition processivity. As discussed below, interactions between the 5'-end of the primer and regions of TERT outside the RT domain are required for repeat addition processivity and have been termed 'anchor sites' to emphasize their predicted role in anchoring TERT to the DNA primer during telomere synthesis (38).

Repeat addition processivity

Repeat addition processivity was first observed with *T. thermophila* telomerase and subsequently with human telomerase (104,105). Many rodent and fungal telomerases, however, are relatively non-processive and catalyze short elongation products *in vitro* and *in vivo* (106–108). Furthermore, genetic studies with mutant *T. thermophila* telomerase RNAs indicate that telomerase has limited processivity *in vivo* (109,110). These studies raise questions regarding the significance of repeat addition processivity *in vivo*. Nonetheless, biochemical and genetic studies indicate that processive repeat addition is a biologically relevant property of telomerase. First, telomerase-specific residues that mediate processivity have been located in the RT domain (52,78,84), the N-terminal extension (71,74,111–113) and the C-terminal extension (53).

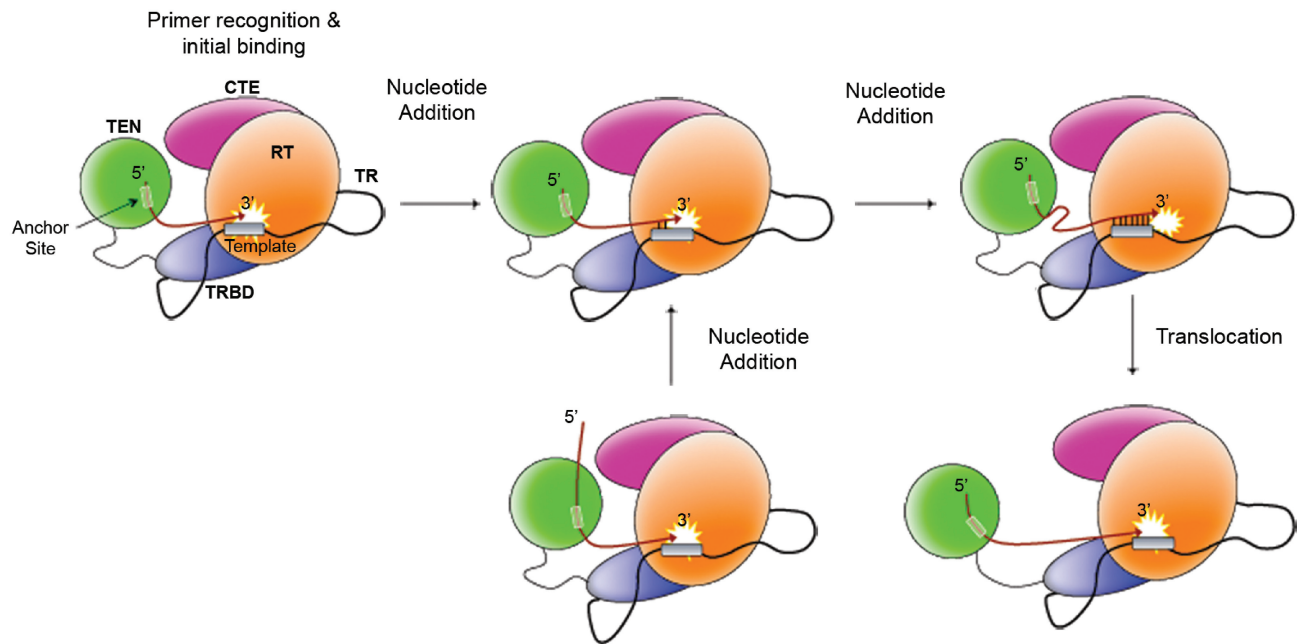


Figure 3. Schematic diagram of the telomerase reaction cycle. This figure summarizes the fundamental steps of the telomerase reaction cycle: initial primer recognition and binding, nucleotide addition and translocation. The telomerase RNA (TR) subunit is represented by a black line (not drawn to scale). The TR supplies the template (grey rectangle) for telomere synthesis. DNA synthesis is catalyzed by the TERT. TERT contains a TEN domain (pink sphere), a TRBD (purple oval), RT domain (blue sphere) and CTE (green oval). The TEN domain contains a unique ssDNA-binding region, called the telomerase anchor site (transparent rectangle). The red line represents telomeric ssDNA. Telomerase binds the telomeric ssDNA such that the 3'-end is aligned with the TR template in the active site (white star) and the 5'-end is positioned within the telomerase anchor site. Telomerase reverse transcribes the template region, 1 nt at a time (nucleotide addition), until reaching the 5'-template boundary element (top right). At this point, a translocation step repositions the new DNA 3'-end within the template for a second round of telomere synthesis (bottom). Conformational changes within the telomerase holoenzyme are believed to facilitate nucleotide and repeat addition processivity.

Mutations in these regions in human, ciliate or yeast TERT selectively alters repeat addition processivity *in vitro* and causes defects in telomere length maintenance *in vivo* (52,69,74,84,111–113). Second, these regions are important for telomerase-dependent extension of partially or completely nontelomeric primers, which provides indirect evidence for a role in primer binding (74,111–113). Third, mutations that specifically increase repeat addition processivity *in vitro* and cause telomere over-elongation *in vivo* have been identified in ciliate and yeast TERT (71,78). Finally, *in vivo* analyses of budding yeast telomerase show that repeat addition processivity is significantly increased at extremely short telomeres, suggesting that this unique feature of telomerase may enable cells to rapidly elongate critically short telomeres (114,115). This model predicts that the extent of processive repeat addition depends upon telomere length and thus, could partially explain the variance observed between telomerase repeat addition processivity *in vitro* and *in vivo*. Further studies will be required to completely understand this unique aspect of telomerase biochemistry.

Telomerase anchor sites

A long-standing notion in telomerase biochemistry is that the enzyme contains DNA-binding regions outside the RT domain that are required for processive telomere elongation. The existence of one or more telomerase

'anchor sites' was precipitated by studies demonstrating that ciliate and human telomerase could elongate non-telomeric G-rich ssDNA primers *in vitro* (16,17,105,116). Furthermore, telomerase was shown to add telomeric repeats to chromosome breaks that contained non-telomeric DNA during *in vivo* chromosome healing (110,117). It was subsequently shown that primers containing almost any sequence of ssDNA could be extended *in vitro* if telomeric repeats were added to the 5'-end (100,118). These observations suggested that hybridization between the DNA 3'-end and RNA template was not required for primer elongation. This hypothesis was further supported by biochemical studies of human and yeast telomerase, which showed that the enzyme/primer complex was mainly stabilized by contacts with the catalytic protein subunit (119,120).

The first direct evidence for a telomerase anchor region was provided by photo-cross-linking studies performed with *E. aediculatus* telomerase (121). Specifically, a TERT-sized protein was cross-linked to the 5'-end of photo-reactive ssDNA primers that were aligned in the enzyme active site (121). The most efficient protein–DNA cross-links were established with bases 20–22 nt upstream of the primer 3'-end and required the telomerase RNA subunit. More recent studies have shown that human and *T. thermophila* TERT can interact sequence-specifically with telomeric ssDNA in the absence of TR (75,81). These studies used biotinylated ssDNA primers to

investigate TERT–DNA interactions *in vitro*. One important finding was that a fragment of hTERT encompassing amino acids 1–300 bound telomeric ssDNA as efficiently as the full-length protein, providing the first physical evidence for an anchor site in the N-terminus of telomerase (81). An equally important finding was that the *T. thermophila* TERT (tTERT) C-terminus exhibited telomeric ssDNA-binding activity *in vitro* (75). Furthermore, it has been recently shown that a purified human TEN domain spanning amino acids 1–200 directly interacts with telomeric DNA and this interaction is dependent on the length and register of the telomeric repeat (73). These studies indicate that TERT contains multiple anchor sites, which are believed to co-ordinate and regulate various stages of primer binding and extension. Importantly, some studies indicate that TERT–DNA interactions are enhanced by the telomerase RNA subunit, suggesting that TR has an important role in regulating anchor site interactions (57,75,111,121).

Genetic and biochemical studies with ciliate, yeast and mammalian telomerase suggest that the anchor site may be comprised of a template-proximal and a template-distal component (57,71,74,81,105,112,116,120,122–125). In this model, both regions contribute to telomere binding and elongation, but only relatively long primers (≥ 20 nt) extend into the template-distal anchor region (47). The existence of two separate anchor sites indicates a tripartite mode of DNA-binding: (i) the primer 3'-end hybridizes with the RNA template in the active site; (ii) the primer nucleotides adjacent to the template-hybridizing region interact with a template-proximal anchor site; and (iii) the primer nucleotides at the 5'-end interact with the template-distal anchor site (123).

The template-proximal telomerase anchor region has been physically and functionally mapped to the N-terminus of ciliate, yeast and hTERT (38,71,72,74,75,81). The crystal structure of the tTERT TEN domain revealed a previously-unrecognized ssDNA-binding groove on the surface of this domain. Photo-cross-linking studies showed that the tTERT TEN domain possessed telomere-specific ssDNA-binding activity *in vitro* (57,72). Mutagenesis of key residues thought to be involved in ssDNA-binding, such as an invariant glutamine (tTERT Q168), significantly reduced the interaction between tTERT and telomeric ssDNA, and impaired enzyme activity *in vitro* (57,72). Furthermore, mutation of the corresponding residue in yeast Est2p (Q146A) caused severe growth defects and telomere loss *in vivo* (71). Most recently, hTERT Q169 was shown to be important for the structure of the human telomerase N-terminal extension, primer binding and nucleotide incorporation *in vitro*, as well as telomere length maintenance *in vivo* (70,73). Interestingly, Q169 appears to be particularly important for incorporating the second nucleotide during telomere extension *in vitro* (70). Collectively, the above studies indicate that this glutamine residue forms part of an evolutionarily conserved anchor site that facilitates primer recognition and orientation in the active site for telomere synthesis.

Telomerase structural mobility

DNA polymerases undergo a series of conformational changes during DNA synthesis (79,126). Structural mobility confers polymerases with the ability to discriminate against nucleotide misinsertion and incorporate multiple nucleotides without dissociating from the DNA primer. With respect to telomerase, it has been long proposed that conformational changes mediate the transition between telomere synthesis initiation, elongation and translocation (104).

Recent work with *T. thermophila* telomerase has provided new insights into the structural mobility of the telomerase anchor site (74). The crystal structure of the tTERT TEN domain revealed that an evolutionarily-conserved leucine (L14), in close proximity of Q168, was involved in forming one edge of the ssDNA-binding groove (57). The branched, hydrophobic side-chain of leucine can theoretically engage in intra- or inter-molecular protein–protein interactions. Structure–function studies of tTERT identified an essential role for L14 in the repeat addition processivity of ciliate telomerase *in vitro* (74). Importantly, L14 mutants displayed normal nucleotide addition processivity, thus identifying a specific role for L14 in the telomerase translocation step (74). An attractive model has been proposed in which L14 represents an intramolecular ‘door latch’, which holds the TEN domain in close proximity of the catalytic site and facilitates processive nucleotide addition (Figure 3) (74). After synthesizing a complete telomeric repeat, a conformational change occurs within telomerase that releases the door latch (‘open’ state) and allows the new DNA 3'-end to be repositioned within the RNA template and alignment regions for the next round of telomere synthesis. This model is supported by cross-linking studies indicating that the tTERT TEN domain is displaced relative to the catalytic site during telomere synthesis *in vitro* (72). A second prediction of this model is that the 5'-end of the elongated DNA slides through the ssDNA-binding groove on the surface of the TEN domain (Figure 3). This movement is believed to signal telomerase to re-establish the intramolecular protein–protein interaction (‘closed’ state) and initiate the next round of telomere synthesis (74).

Processivity factors

In addition to anchor sites located within telomerase itself, accumulating evidence suggests that telomerase-associated proteins facilitate the enzyme's repeat addition processivity *in vivo*. For example, the TPP1–POT1 heterodimer stimulates the activity and processivity of human telomerase *in vitro* (127–129). TPP1 has been shown to interact with hTERT *in vitro* (128) and mutation of a conserved glycine residue in the hTERT TEN domain (G100) was shown to suppress the stimulatory effect of TPP1–POT1 on human telomerase processivity *in vitro* (129). These results are important because they identify a physical link between the human shelterin complex and telomerase, and provide new insight into the mechanism of processive telomere synthesis. One interpretation of these observations is that

telomere-bound TPP1–POT1 interacts with hTERT to transfer the telomeric ssDNA 3'-end to telomerase for subsequent elongation. It will be interesting to elucidate whether the interaction between hTERT and TPP1 is direct or indirect (i.e. mediated by the telomeric ssDNA primer) and to investigate the conformational changes that occur in hTERT upon binding the TPP1–POT1 and TPP1–POT1–ssDNA complexes. Identification of the TERT binding site in TPP1 should also help clarify the relationships between telomerase, shelterin and telomeric ssDNA.

How else might telomere-associated proteins contribute to telomerase activity and repeat addition processivity *in vivo*? It is possible that ssDNA-binding proteins, such as RPA or POT1, coat the elongating telomere as it slides through the TEN domain. This protein–DNA interaction could stabilize and protect the otherwise naked ssDNA and facilitate primer movement through telomerase. In this model, telomere-binding proteins fulfill the template-distal anchor site function. Another possibility is that one or more telomerase-associated proteins mediate anchor site function *in vivo*. In this regard, a recent study of *T. thermophila* telomerase identified an RPA1-like DNA-binding protein p82 in stable association with the endogenous holoenzyme (130). Interestingly, p82 exhibits telomere sequence-specific ssDNA-binding activity and confers ciliate telomerase with high levels of repeat addition processivity *in vitro* (130,131). Recent observations suggest that the function of p82 may be to bind the telomeric primer and stabilize its association with telomerase as well as to suppress the formation of DNA structures that could impede processive telomere synthesis (131). It will be important to determine if repeat addition processivity is regulated by similar proteins and mechanisms in other organisms. To this end, it will be useful to devise purification strategies that can be used to characterize the composition of the telomerase holoenzyme at various stages of the cell cycle and in different organisms and cell types. This would provide new insight into specific factors that regulate telomerase biogenesis and activity *in vivo*.

TELOMERASE, TELOMERES AND HUMAN DISEASE

Many studies have illustrated the molecular complexity of telomerase and telomere biology by implicating these processes in a broad spectrum of human diseases, ranging from cancer to premature aging disorders. This area has been the focus of several excellent reviews (132–134), and therefore, we will only briefly summarize the relationships between telomerase and human disease to emphasize the clinical importance of understanding telomerase biochemistry.

Telomerase, chromosome instability and cancer

Telomerase is active in germ line and stem cells but repressed in most somatic cells and tissues, which is achieved through various transcriptional and post-transcriptional mechanisms (32,135). In the absence of telomerase

activity, telomeres shorten during each round of cell division until they reach a critically short-length threshold (136–138). This triggers a p53 and pRB-dependent DNA damage response and induces a non-proliferative state called replicative senescence, which is an important barrier to tumorigenesis (139–142). Cells with defective p53 and pRB pathways can bypass this barrier and undergo additional 20–30 cell divisions (143,144). During this extended period of proliferation, telomeres continue to shorten and eventually undergo chromosome end-to-end fusions (i.e. the breakage-fusion-bridge cycle), which promotes genome instability by generating loss of heterozygosity or the amplification of genetic loci (132). This usually triggers a second proliferative blockade called crisis, which is characterized by massive genome instability and apoptosis (145). However, rare clones (~1 in 10⁷ human cells) can emerge from crisis by up-regulating telomerase or homologous recombination-based telomere maintenance mechanisms called alternative lengthening of telomeres (ALT), all of which stabilize critically short telomeres and permit further rounds of cell division (146). Telomerase activation is the most common pathway for cellular immortalization and transformation; at least 85% of human cancer cells constitutively express the telomerase catalytic subunit and utilize telomerase-dependent telomere elongation to attain unlimited growth potential (147). Thus, telomerase is an attractive target for the development of anti-cancer therapeutics that specifically target cancer versus healthy cells (99). However, some mammalian tumors and immortalized cell lines lack telomerase activity and use ALT pathways for telomere maintenance (146). A better understanding of these non-telomerase telomere-maintenance pathways could lead to novel anti-cancer therapies.

Diseases associated with telomerase deficiency

The first disease-associated mutations in human telomerase were identified in patients afflicted with a rare, multi-system disorder called dyskeratosis congenita [see Walne (148) for a historical review of dyskeratosis congenita]. The clinical manifestations of dyskeratosis congenita generally appear during childhood and include a monocutaneous triad of abnormal skin pigmentation, nail dystrophy and oral leukoplakia. These symptoms are accompanied by a spectrum of other somatic abnormalities, such as developmental delay, premature hair loss and organ failure. Bone marrow failure is the principal cause of premature mortality. More recently, telomerase mutations have been detected in the context of aplastic anemia (149–151), Hoyeraal–Hreidarsson syndrome (152–155), idiopathic pulmonary fibrosis (156,157) and liver disease (158). Aplastic anemia is a hematological disorder characterized by reduced red blood cell counts, bone marrow failure, and liver and lung disease. Hoyeraal–Hreidarsson syndrome is a multisystem disorder characterized by bone marrow failure, immunodeficiency and severe growth retardation. Idiopathic pulmonary fibrosis is a chronic, progressive, and fatal disease that is defined by irreversible lung fibrosis. The unifying molecular characteristic of these

diseases is that patients harbor telomeres that are significantly shorter than age-matched control subjects (133). This indicates that mutations in the telomerase holoenzyme compromise its ability to maintain telomere length, which is thought to impair stem cell function and limit the renewal capacity of highly proliferative cells. Indeed, inherited forms of dyskeratosis congenita exhibit disease anticipation, whereby successive generations exhibit earlier disease onset and present with more severe phenotypes (159–161). The mechanism of disease anticipation is thought to be caused by the inheritance of short telomeres that continue to shorten at an accelerated rate in the afflicted offspring. Furthermore, patients afflicted with dyskeratosis congenita are predisposed to developing carcinomas, lymphomas and leukemias. This is believed to result from the accelerated rate of telomere attrition, which promotes genetic instability and tumor formation.

Mutations have been detected in five subunits of the human telomerase holoenzyme (TERT, TR, dyskerin, NHP2 and NOP10) and one shelterin protein (TIN2) [the reader is referred to the telomerase database (<http://telomerase.asu.edu/diseases.html>) for a current list of disease-linked mutations]. Dyskerin, NHP2 and NOP10 are essential for the biogenesis of human telomerase and disease-linked mutant proteins have been shown to compromise enzyme stability (162–165). Similarly, mutations in hTR can either impair its stability or disrupt the catalytic activity of human telomerase (88,159,166–168). hTERT mutants exhibit catalytic defects *in vitro* and fail to maintain telomere length in human cells (84,87,88,150,161). Importantly, telomere maintenance defects observed in cultivated human cells can be rescued by the ectopic expression of wild-type hTR or hTERT, providing direct evidence that these mutations impair telomerase-mediated telomere maintenance (150,163,169). However, a comprehensive study that describes how these mutations influence the biochemical and cellular properties of human telomerase is not yet available. One possibility that has not been explored is that some of these mutations interfere with enzyme activity by disrupting critical protein–protein interactions within the holoenzyme. It will be interesting to isolate human telomerase from healthy and diseased cells and compare the composition of the holoenzyme in these different cellular contexts. These experiments will provide novel insights into the mechanism of telomerase regulation in healthy and diseased human cells.

CONCLUSIONS

Telomerase has emerged as a central player in several devastating human disorders, including various forms of cancer, bone marrow failure and pulmonary fibrosis. This emphasizes the need for a detailed understanding of telomerase structure and function. High-resolution crystal structures of ciliate and insect TERTs provide much-needed insight into the intricate details of this unique RT. An important future challenge will be to determine the crystal structure of TERT in complex with TR and integral protein components of the telomerase

holoenzyme. This structural information will form the basis for biochemical and molecular studies that could provide important insights into telomerase assembly and regulation. An equally important challenge will be to solve the crystal structure of the full-length TERT from an organism with well-characterized enzyme activity, especially in light of the controversy regarding the identity of *T. castaneum* TERT. Finally, structure–function studies of disease-associated TERT mutants will be particularly informative since these mutations have abrogated amino acids that are required for telomerase function *in vivo*. The information obtained from these studies may ultimately translate into novel therapeutic strategies that improve the diagnosis, treatment and management of human diseases associated with telomerase dysfunction.

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