Structural basis of template-boundary definition in Tetrahymena telomerase

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Telomerase is required to maintain repetitive G-rich telomeric DNA sequences at chromosome ends. To do so, the telomerase reverse transcriptase (TERT) subunit iteratively uses a small region of the integral telomerase RNA (TER) as a template. An essential feature of telomerase catalysis is the strict definition of the template boundary to determine the precise TER nucleotides to be reverse transcribed by TERT. We report the 3-Å crystal structure of the Tetrahymena TERT RNA-binding domain (tTRBD) bound to the template boundary element (TBE) of TER. tTRBD is wedged into the base of the TBE RNA stem-loop, and each of the flanking RNA strands wraps around opposite sides of the protein domain. The structure illustrates how the tTRBD establishes the template boundary by positioning the TBE at the correct distance from the TERT active site to prohibit copying of nontemplate nucleotides.

Telomeres are essential nucleoprotein structures that help maintain genome integrity by differentiating natural chromosome ends from sites of DNA damage. In most cells, a progressive shortening of telomere length with each round of cell division provides a molecular signal for cell aging and regulates entry into permanent cell-growth arrest or apoptosis. In contrast, cells with a high proliferative capacity (e.g., stem cells, inflammatory cells and other self-renewing tissues) maintain telomere length through the enzymatic action of the specialized reverse transcriptase telomerase. The discovery of loss-of-function telomerase mutations in patients with diseases of the hematopoietic system (e.g., dyskeratosis congenita and aplastic anemia) has illustrated the requirement for telomerase in highly proliferative tissues. However, aberrant activation of telomerase is deleterious, providing a mechanism for ~90% of human cancers to occur.

The minimal requirements for in vitro reconstitution of the active telomerase ribonucleoprotein (RNP) include a TERT and the TERT protein. The telomerase RNP directs de novo DNA synthesis at chromosome ends through a unique reverse-transcription mechanism wherein an internal region of the telomerase RNA serves as the template (Fig. 1c). During telomere-repeat synthesis, telomerase binds a single-stranded DNA substrate through Watson-Crick base-pairing with the RNA template and through additional TERT-DNA interactions termed ‘anchor sites’. Next, TERT catalyzes the synthesis of a telomere DNA repeat according to the sequence specified by the RNA template. Completion of a telomere repeat initiates telomerase RNP translocation, which repositions the TERT active site, telomerase RNA and DNA substrate to the original DNA primer-alignment configuration to allow for further rounds of repeat addition. The precise definition of a region of TER that may access the TERT active site and template the synthesis of a telomere DNA repeat is a hallmark of telomerase function; however, the precise structural details of how this template boundary is defined have not been characterized.

TERTs from diverse organisms are highly conserved and share a common domain organization including the essential N-terminal domain (TEN), RNA-binding domain (RBD), reverse transcriptase domain (RT) and C-terminal extension (CTE) (Fig. 1a). The contribution of individual TERT domains to telomerase assembly and catalytic activity has been studied by in vitro deletion and mutagenesis as well as by single-molecule fluorescence resonance energy transfer. The TEN domain makes contacts with both DNA and RNA and is essential for telomerase processivity. RBD binds TER through several conserved regions known as the T motif, CP motif and ciliate-specific CP2 motif (Supplementary Figs. 1 and 2). RBDs are far more divergent with respect to both size and sequence, but they maintain a conserved organization of multiple RNA elements including a TBE, the template region, an RNA pseudoknot domain and a distal RNA stem-loop (Fig. 1b). In telomerase from the model organism Tetrahymena thermophila, the base of stem-loop II and its flanking single-stranded RNA compose the TBE of TER and have been proposed to interact with the T, CP and CP2 motifs within the TERT RBD. Consistently with this notion, sequence mutations within the TBE or each of the conserved TERT RBD motifs result in a loss of protein-RNA binding affinity as well as template readthrough defects, as assayed in direct primer extension assays in vitro.

The previously reported high-resolution structure of the tTRBD domain has revealed the organization of the T and CP motifs, which together form a putative RNA-binding pocket. However, this tTRBD structure did not include RNA and lacked the CP2 motif, which is...
essential for high-affinity RNA interactions\textsuperscript{21}. The more recently reported structures of TRBDs from diverse organisms have indicated that this domain shares a common overall folding topology\textsuperscript{24-26}. Moreover, the structure of the Tribolium castaneum (tc) TERT bound to a model RNA-DNA hybrid in the active site has revealed the orientation of the RNA template with respect to the tcTRBD and supports the model in which a high-affinity RNA interaction in this region of TERT establishes the template boundary\textsuperscript{11}. The first vertebrate TRBD structure was reported for the fugu fish Takifugu rubripes (tr) protein, and it has revealed the position of a conserved TFLY motif near the putative RNA-binding region of trTRBD\textsuperscript{25}. The TFLY motif was proposed to serve a similar role to that of the ciliate-specific CP2 motif in promoting a high-affinity RNA interaction; however, the lack of RNA in the structure precluded a detailed description of the TRBD-RNA interaction network.

We set out to structurally determine the basis of template-boundary definition in Tetrahymena telomerase. Here, we report the X-ray crystal structure of tTRBD bound to its cognate TBE RNA fragment. The structure highlights how the T, CP and CP2 motifs within the tTRBD cooperate to mediate interactions with the base of stem-loop II in Tetrahymena TERT. The structure of the tTRBD in complex with the TBE RNA explains the results of previously reported biochemical mutagenesis experiments\textsuperscript{17,18,20-22}, provides new insights into the mechanism of telomerase catalysis and places new constraints on the organization of the rest of the telomerase RNA subunit within the telomerase RNP complex.

RESULTS

Structure of TERT RBD bound to the TBE RNA

To determine the molecular mechanism of template-boundary definition in Tetrahymena telomerase, we sought to develop protein and RNA constructs amenable to high-resolution structure determination. The previously reported structure of the tTRBD does not include the cognate telomerase RNA or the ciliate-specific CP2 peptide\textsuperscript{25}. Prior mutagenesis experiments have established the importance of the CP2 motif for protein-RNA interactions that direct template-boundary definition\textsuperscript{20-22}, and subsequent site-directed hydroxyl-radical probing have shown that the CP2 peptide is in spatial proximity to the TER TBE located at the base of stem-loop II\textsuperscript{23}. Beginning with a tTRBD fragment (amino acids (aa) 195–516) that contains multiple sites of RNA interaction including the CP2 motif\textsuperscript{14,21}, we made a set of truncation mutants to identify a highly soluble tTRBD domain (aa 217–516) that retains sequences required for specific high-affinity interaction with TER (Fig. 1a).

We optimized the TER stem-loop II construct by replacing the native A22-A34 bulge with a canonical U22-A34 base pair (Fig. 1b). This base substitution occurs naturally in several closely related ciliate TERs\textsuperscript{27} and does not affect Tetrahymena telomerase function in vitro and in vivo assays\textsuperscript{26,28}. Indeed, full-length TER containing the A22U substitution supported wild-type levels of binding to tTRBD (Supplementary Fig. 3a) as well as catalytic activity and template-boundary definition (Supplementary Fig. 3b). To improve

| Table 1 Data collection and refinement statistics |
|-----------------------------------------------|
| **Data collection**                             |
| Space group                                    | P2$_1$2$_1$2$_1$ |
| Cell dimensions                                | 58.53, 117.77, 131.18 |
| a, b, c (Å)                                    | 90, 90, 90 |
| Resolution (Å)                                 | 41.51 (3.00)* |
| Rmerge                                         | 0.231 (0.969) |
| I / σI                                        | 7.8 (2.1) |
| Completeness (%)                               | 99.5 (99.3) |
| CC$_{1/2}$                                     | 0.990 (0.720) |
| Redundancy                                     | 6.8 (6.8) |
| **Refinement**                                 |
| Resolution (Å)                                 | 41.51-3.00 |
| No. reflections                                | 18,649 |
| Rwork / Rfree                                  | 0.239 / 0.290 |
| No. atoms                                      | 4,793 |
| Protein                                        | 4,767 |
| Ion                                            | 2 |
| Water                                          | 24 |
| B factors                                      | 62.90 |
| Protein                                        | 65.50 |
| Ion                                            | 43.80 |
| r.m.s. deviations                              | 0.003 |
| Bond lengths (Å)                               | 0.82 |

*One crystal was used.

*Values in parenthesis are for highest-resolution shell.
the folding efficiency of the isolated RNA stem construct, we separated stem-loop II into two short oligonucleotides (Fig. 1b). The tTRBD protein and the optimized TER TBE RNA were mixed together at a 1:1.5 stoichiometry to yield a monodispersed protein–RNA complex, as determined by size-exclusion chromatography (Supplementary Fig. 3c). We crystallized the tTRBD–TBE complex and solved the structure to 3-Å resolution by molecular replacement, using the previously reported tTRBD structure as a search model23 (Table 1). The calculated electron density map permitted unambiguous modeling of most of the protein–RNA complex, with the exception of a small region of the tTRBD protein and several RNA nucleotides that are probably disordered in the crystal. The tTRBD–TBE complex crystallized in a 1:1 stoichiometry with two protein–RNA complexes (A and B) per asymmetric unit (Supplementary Fig. 4a). Complexes A and B showed a high degree of overall similarity and an r.m.s. deviation of 0.98 Å2; however, regions of the protein-RNA binding interface in complex B were close to crystal-packing contacts (Supplementary Fig. 4b,c) and were substantially more disordered than in complex A, in which this same interface was far from any lattice contacts. We therefore focused our structural analyses on the more ordered complex A.

In the structure, the tTRBD is wedged into the base of the TER TBE RNA stem-loop II, thus diverting the paths of the 5’- and 3’-flanking strands in opposite directions (Fig. 2a). All protein-RNA contacts occur at the base of stem-loop II, and the distal region of stem-loop II (corresponding to the location of the native stem-loop II) extrudes away from the protein interface. This arrangement is consistent with results from TER-mutagenesis experiments and site-directed hydroxyl-radical probing, which have demonstrated that the base of stem-loop II interacts with the tTRBD21, as well as with the observation that the distal region of stem-loop II is permissive to both sequence and structure modifications20,28. The three conserved sequence motifs within tTRBD (CP2, CP and T) compose the core RNA-binding platform (Fig. 2a,b). Surprisingly, the majority of the protein-RNA contacts are not sequence specific with respect to TER, with a few notable exceptions (described below). Instead, the base of the TER TBE is bound through an extensive network of polar contacts between the three conserved sequence motifs within tTRBD and the sugar-phosphate backbone of the RNA.

The CP and T motifs position the CP2 motif for RNA binding
The previously reported structure of the tTRBD (aa 254–519) has revealed the organization of the conserved CP and T motifs23. Together, these regions of tTRBD form an electropositive groove that was previously hypothesized to represent a site of RNA interaction. However, our structure indicates that a major function of the T-CP pocket is to bind and orient the CP2 peptide (Fig. 3a); this peptide is essential for RNA binding and was lacking in the previously crystallized construct23. The position of the CP2 peptide is fixed by a network of polar interactions between conserved amino acid side chains as well as by a number of backbone contacts. For example, the side chain of Arg226 in CP2 makes several hydrogen-bonding interactions with the backbone of Phe408, Met411, Lys412 and Glu415, which together form a pocket that anchors the N-terminal region of the CP2 peptide within the tTRBD (Fig. 3b). The hydroxyl group of Tyr231 in CP2 is hydrogen-bonded to the guanidinium and carboxylate groups of Arg413 and Glu480, respectively (Fig. 3c). These polar contacts between Tyr231 and other regions of the tTRBD induce a nearly 90° kink in the protein backbone, thus redirecting the path of the CP2 peptide toward the base of the TBE RNA. The side chain of Arg473 in the T motif makes hydrogen bonds with the backbone of CP2 residues Phe230 and Gln228 (Fig. 3d). In addition, the N1 position of the T-motif residue Trp496 indole group is hydrogen-bonded with the carboxamide group of Asn233 in CP2. Finally, the terminal amino groups of two conserved lysines within the T motif (Lys493 and Lys497) make polar backbone contacts with residues Cys232 and Asn233 within the CP2 peptide (Fig. 3e). Thus, our structure of the tTRBD bound to the TBE RNA stem demonstrates that many of the conserved residues in the T motif are indirectly required for RNA binding and function to position the CP2 peptide in a geometry that is favorable for making RNA contacts.

Figure 3 Protein-protein interactions between conserved tTERT RBD motifs. (a) Cartoon representation highlighting CP2 peptide (purple) interactions. Coloring scheme is as in Figure 2a. (b–e) Interactions outlined by red dashed boxes in a. (b) Arg226 makes polar contacts with the core of RBD. (c) Tyr231 interacts with the T-motif residue Glu480. (d) Gln228 and Phe230 make polar contacts with the T-motif residue Arg473. (e) Asn233 interacts with T-motif residues Trp496 and Lys497 as well as with CP2-peptide residue Tyr231.
Figure 4 Protein-RNA interactions within the tTRBD–TBE complex. (a) Simulated annealing omit map of the TER TBE RNA at 2.0σ contour level, superimposed on a cartoon model of the tTRBD–TBE structure. (b) Simulated annealing omit maps of the U38-U18 and G37-C19 base pairs, contoured at 1.5σ. A side view of the stacked U38-U18 and G37-C19 base pairs is shown at bottom. (c) Schematic diagram of tTRBD–TER TBE interactions. The TBE RNA nucleotides are cyan, and the rest of the RNA is black. tTRBD residues from CP2-, CP- and T-motif residues are purple, orange and blue, respectively. Arrows indicate sites of polar interactions. (d) Interaction of Arg237 with nucleotides U38 and G37 at the base of the TBE RNA stem, and interaction of His234 with U38. (e) Detailed view of polar contacts. The TBE RNA nucleotide C15 fits into a polar pocket between the CP and T motifs. C15 makes polar contacts with T-motif residue Arg492 and CP-motif residues Asn324, Lys332 and Lys328.

tTRBD-TBE interactions define the template boundary

The TBE RNA stem forms an A-form helix (Fig. 4a), which is consistent with an earlier NMR structure of a model Tetrahymena stem-loop II sequence lacking the entire TBE region. The crystal structure presented here reveals two critical RNA base pairs located at the interface between the tTRBD and the TBE RNA: a canonical Watson-Crick base pair between C19 and G37 and a previously unidentified wobble pair between U18 and U38 (Fig. 4b and Supplementary Fig. 5). The observation of these base-pairing interactions and their central role in mediating RNA binding explains the high degree of sequence conservation observed for these nucleotide positions among ciliate telomerase RNAs. Conserved residues from the CP2, CP and T motifs all contribute to the tTRBD surface that binds the TBE RNA (Fig. 4c). Within the CP2 peptide, the guanidinium group of Arg237 extends into the major groove of the TBE RNA stem, making hydrogen-bonding contacts with the Hoogsteen face of G37 and with the carbonyl group of U38, while the imidazole group of His234 makes a stabilizing polar contact to the carbonyl of U38 (Fig. 4c,d).

In addition to interactions with the helical portion of the TBE RNA, the tTRBD makes a large number of polar contacts between the 5’-flanking single-stranded RNA and conserved T- and CP-motif side chains. Specifically, the Watson-Crick face of RNA nucleotide C15 makes hydrogen bonds with the side chains of Arg492 (T motif) and Asn324 (CP motif), and the 2’-hydroxyl group of the C15 ribose moiety contacts the terminal amino group of Lys332 (CP motif) (Fig. 4c,e). The side chains of several additional CP-motif residues (Lys328, Tyr337, Glu338 and Lys341) make polar contacts with the RNA phosphate backbone (Fig. 4c,e). With respect to the 3’ single-stranded RNA flanking the base of the TBE RNA stem, only a single nucleotide (C39) could be unambiguously fit into the electron density. Although nucleotides A40–U42 and U13 are distant from any lattice contacts, they are probably disordered in the crystal and therefore could not be fit into the electron density map. Several

Figure 5 Conservation and function of observed interactions in the tTRBD–TBE complex. (a) Conserved residues in the CP2 and T motifs that are important for RNA binding and/or telomerase function. Boxed residues represent CP2 residues that are conserved across ciliates and T-motif residues that are conserved across species. (b) Conserved RNA residues that are critical for RBD binding. Boxed nucleotides are conserved across ciliates and have previously been demonstrated to be important for RBD binding. (c) CP2-TFLY alignment. The CP2 motif of Tetrahymena was aligned manually to the TFLY motif of vertebrates. Invariant residues are orange, and residues with conserved chemical properties are red. A segment of the conserved T-motif alignment is also shown. (d) Structural alignment of tTRBD, iTRBD and oTRBD. The iTRBD CP2 motif and T motif are purple and blue, respectively. iTRBD (PDB 4LMO (ref. 25)) and oTRBD (PDB 4O26 (ref. 26)) are dark gray and light gray, respectively. (e) Structural alignment of the iTRBD CP2 motif and the oTRBD TFLY motif. Color scheme is as in d.
tTRBD residues make polar contacts with the phosphate backbone in the 3′ single-stranded region, including the guanidinium group of Arg473 (T motif). Thus, our structure reveals the sequence- and structure-specific interactions between the CP2-, CP- and T-motif residues that mediate the protein-RNA interactions governing template-boundary definition.

**Conservation and function of tTRBD-TBE interactions**

Many of the residues that participate in orienting the CP2 peptide, or that interact directly at the tTRBD-TBE binding interface (Fig. 5a,b), are highly conserved among ciliate TRBDs (Supplementary Fig. 2). The positions of these amino acids in our structure are in accord with this sequence conservation, as well as with the observation that alanine substitution at each of these sites can induce defects in RNA binding and/or telomerase activity. Notably, the T-motif residues Glu480 and Arg492 are invariant among TRBDs from ciliates, yeasts and vertebrates (Supplementary Fig. 1). The side chain of Arg492 in the T motif makes base-specific contacts with C15 in the TBE RNA (Fig. 5b), thus raising the possibility that this residue may be directly involved in RNA binding interactions in TRBDs from other organisms. A second invariant T-motif residue, Glu480, is hydrogen-bonded to the side chain of Tyr231 in CP2 (Fig. 5a).

Interestingly, the recently reported structure of the trTRBD identified the TFLY motif, which is conserved among vertebrate TRBDs. Comparison of the sequences of the CP2- and TFLY motifs suggests that certain residues are conserved (Fig. 5c). In order to ascertain whether the ciliate-specific CP2 motif is structurally homologous to the vertebrate TFLY motif, we aligned our structure with the two available vertebrate TRBD structures from *T. rubripes* and the closely related medaka fish *Oryzias latipes* (ol) (Fig. 5c). Indeed, in all three TRBD structures, the invariant T-motif glutamate side chain is well aligned, and Tyr321 from the olTRBD TFLY motif overlaps with Tyr231 from the trTRBD CP2 motif (Fig. 5d). In contrast, the corresponding tyrosine side chain from trTRBD (Tyr305) is substantially displaced. This alternative positioning of the TFLY motif in the trTRBD structure is probably because this region is directly involved in making crystal-packing contacts. Further comparison of the trTRBD CP2 motif and the olTRBD TFLY-motif structures suggests additional regions of homology. For example, the side chains of Phe230 in trTRBD and Phe319 in olTRBD are in proximity in the structural alignment. Last, two conserved basic trTRBD CP2 residues (His324 and Arg237) that compose a sequence-specific RNA-binding patch are also present within the olTRBD (Arg324 and His327), albeit with an inverted sequence order (Fig. 5e). Together, the results of our structural analysis suggest that the ciliate-specific CP2 motif is structurally homologous to the vertebrate TFLY motif, which may serve a similar function to mediate sequence-specific protein-RNA interactions.

**A molecular model of template-boundary definition**

In order to further understand the mechanism of template-boundary definition in telomerase, we aligned the tTRBD–TBE structure with the complete TERT structure obtained from the flour beetle *T. castaneum* and containing a model RNA-DNA hairpin bound in the active site. The resulting model demonstrates the relative positions of the TBE and the template RNA within the telomerase complex (Fig. 6a). In this model, residue C39 is positioned ~17 Å from the nearest template RNA residue, roughly corresponding to the contour length of the three RNA nucleotides not present in the structural model. Previous work has demonstrated that the TBE is a site of protein-RNA interaction that is critical for preventing entry of nontemplate residues into the TERT active site. The structural model that we propose directly demonstrates the mechanism by which this is accomplished. The location of the tTRBD–TBE complex is precisely positioned to prevent entry of the nontemplate residue U42 into the active site of the enzyme, thereby establishing the template boundary.

**DISCUSSION**

We report the structure of the tTRBD in complex with the TBE of TERT. The position of stem-loop II with respect to TERT observed in our high-resolution structure is consistent with the proposed location of this same RNA element in a structural model derived from a recent cryo-EM *Tetrahymena* telomerase holoenzyme structure. The most salient features of the tTRBD–TBE structure are the interactions between three conserved sequence motifs (CP2, CP and T) and the manner in which these motifs cooperate to position the TBE RNA with respect to tTRBD. Previously reported biochemical experiments have supported a model in which a high-affinity interaction between tTRBD and the TBE RNA establishes template-boundary definition by limiting the amount of RNA that can access the active site of the TERT RT domain. More recent bulk biochemical and single-molecule experiments have demonstrated that the
RNA-template region of TER exists in either a compressed or extended conformation, depending on the stage of the telomere repeat-synthesis reaction. This finding supports the notion that the tTERT RT domain exerts a stretching force on the RNA template during telomere DNA synthesis and that a point of protein-RNA contact must resist this pulling force to establish the template boundary. In our structure of tTRBD bound to the TBE RNA, the protein domain is wedged against the base of the TBE RNA stem and makes several sequence-specific contacts in the RNA major groove (Fig. 6a).

This arrangement is well suited to provide mechanical stability against the reverse transcriptase pulling force (Fig. 6b), because both protein-RNA and RNA-RNA contacts would need to be disrupted to permit more RNA to enter the reverse transcriptase active site.

Further structural analysis is required to determine whether the mechanism of template-boundary definition revealed by the tTRBD–TBE structure is a general feature found in telomerases from other organisms. The P1 stem found in vertebrate telomerase RNAs has been proposed to serve a functionally analogous role to that of the Tetrahymena TER stem-loop II. However, more recent experiments have demonstrated that the sequence of the vertebrate telomerase RNA template can itself govern the location of the telomeric anchor-site function. A r t i c l e s

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank under accession code 5C9H.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
L.I.J., R.M.A., A.O., C.L., S.M.R. and M.D.S. designed the experiments. L.I.J., R.M.A., S.M.R. and M.D.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Formation of the RBD–TBE complex. Histidine-tagged RBD (217–516) was expressed in Escherichia coli BL21-CodonPlus (DE3)-RP competent cells (Agilent) and purified by nickel-exchange chromatography. The RBD was further purified by size-exclusion chromatography (Superdex 200 column). The TBE RNA construct was prepared by annealing two individual strands, each containing one side of stem II (Fig. 1b) (strand A, 5′ UUCAUUCAGUUCU 3′; strand B, 5′ UAGAACUGUCAUU 3′). To anneal the construct, the two strands were heated to 95 °C for 3 min in annealing buffer (500 mM NaCl and 20 mM Tris-HCl, pH 7.8) and then allowed to slowly cool to room temperature. Then MgCl₂ and DTT were each added to a final concentration of 1 mM. The tTRBD protein was mixed with the annealed TBE RNA at a stoichiometry of 1:1.5 in high-salt buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.8, 1 mM MgCl₂ and 1 mM DTT). This mixture was then dialyzed overnight (~16 h) into low-salt buffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.8, 1 mM MgCl₂ and 1 mM DTT). Formation of protein–RNA complex was analyzed with diagnostic sizing chromatography on a Superdex 200 column (Supplementary Fig. 3c) before crystallization trays were set up.

Protein crystallization and data collection. tTRBD–TBE RNA crystals that diffracted to 3 Å appeared overnight and grew to a final size of ~10–20 µm after 4–5 d. Crystals were grown by the sitting-drop vapor-diffusion method with an ARI GRYPHON in Hampton Research Natrix HT condition A5 (200 mM KCl, 10 mM MgCl₂, 50 mM MES monohydrate, pH 5.6, and 4% PEG 8000) at a 3:1 ratio of sample to screen. Crystals were transferred into cryoprotectant solution consisting of 20% ethylene glycol, 160 mM KCl, 8 mM MgCl₂, 40 mM MES monohydrate, pH 5.6, and 3.2% PEG 8000 at 4 °C. Crystals were harvested by flash freezing in liquid nitrogen. X-ray data were collected at ALS beamline 501 at a wavelength of 1 Å and a temperature of 100 K. The data were processed with MOSFLM. Crystals were in the P_{21}2_12_1 space group, and there were two tTRBD–TBE complexes in the asymmetric unit.

Structure determination and refinement. Phases were calculated in CCP4 by molecular replacement with the previously solved Tetrahymena RBD structure (PDB 2R4G (ref. 23)) as the initial search model. Model building was performed in Coot and refined in PHENIX. The structure was refined to good stereochemistry, with 93% of residues in the most-favored region of the Ramachandran plot, 6% in the allowed region and 1% outliers.

Electrophoretic mobility shift assay (EMSA). WT TER and TER A22U were transcribed in vitro with T7 RNA polymerase and PCR templates. The RNA was gel purified, treated with CIP and DNase and ethanol precipitated. The RNA was then end-labeled with radiolabeled ATP. RNA was heated to 60 °C and allowed to cool to room temperature. RDB was diluted into binding buffer containing 10% glycerol, 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl₂ and 1 mM DTT. Each binding reaction contained the indicated amount of tTRBD plus 0.4 nM end-labeled RNA in binding buffer containing 10% glycerol, 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA, 0.1 mg/ml yeast tRNA and 0.1 lU RNasin (Promega). Reactions were incubated for 20 min at 30 °C. Samples were run on a 5% native acrylamide gel (37.5:1 acrylamide/ bisacrylamide, 4% glycerol and 0.5× TBE) and run for 3 h at 4 °C at 200 V. The gel was dried and imaged overnight and then analyzed by phosphorimager.

Primer-extension assay. Telomerase for in vitro primer-extension assays was prepared in RRL (Promega) as described previously13. 2 µL of the RRL telomerase reconstitution reaction was added to 2 µM DNA primer (GGGGTT)₃, 100 µM dTTP, 9 µM dGTP and 1 µM [α-32P]dGTP, in a final volume of 15 µL in a buffer containing 50 mM Tris, pH 8.0, 1.25 mM MgCl₂ and 10% glycerol. To test for template-boundary defects, reactions were performed in the presence or absence 100 µM dATP. Reactions were incubated for 1 h at 30 °C and then phenol/chloroform extracted and ethanol precipitated. Extension products were resolved on a 12% PAGE DNA sequencing gel and imaged with a Typhoon scanner with a phosphorimaging screen.