Articles

A Stem−Loop of *Tetrahymena* Telomerase RNA Distant from the Template Potentiates RNA Folding and Telomerase Activity†

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ABSTRACT: The ribonucleoprotein enzyme telomerase adds telomeric repeats to the ends of linear chromosomes. The *Tetrahymena* telomerase reverse transcriptase (TERT) protein and the telomerase RNA can be reconstituted into an active complex in vitro in rabbit reticulocyte lysates. We have probed the structure of the telomerase RNA in the reconstituted complex with RNases T1 and V1. Upon TERT binding to the RNA, sites of both protection and enhancement of cleavage were observed, suggesting potential protein-binding sites and conformational changes in the RNA. Especially prominent was a large region of RNase V1 protection in stem−loop IV. A number of loop IV mutants still bound TERT but showed drastic decreases in the level of telomerase activity and the loss of protein-dependent folding of the pseudoknot region of the telomerase RNA. The telomerase activity defect and the misfolding of the pseudoknot were partially separable, leading to the proposal of two functions for stem−loop IV: to aid in the folding of the pseudoknot and to function more directly in the active site of telomerase. Thus an RNA element far from the template makes a major contribution to *Tetrahymena* telomerase enzyme activity.

The ends of eukaryotic linear chromosomes are capped by DNA−protein complexes called telomeres. Telomeres confer protection from degradation and chromosomal fusion as well as providing a means for the complete replication of the chromosome by telomerase. Telomerase is a ribonucleoprotein particle that uses an intrinsic RNA subunit as a template for the addition of G-rich repetitive sequences to the end of the chromosome (*1*, *2*). The minimal functional telomerase complex in vitro consists of the telomerase reverse transcriptase (TERT)[1] protein and the telomerase RNA subunit (*3*−*5*). The TERT protein was first identified in the hypotrichous ciliate *Euplotes aediculatus* and the yeast *Saccharomyces cerevisiae* (*6*), and homologues have since been identified in other ciliates (*5*, *7*), fission yeast (*8*), mammals (*8*−*14*), and plants (*15*).

Other accessory proteins found in the telomerase complex appear to be far less conserved and vary between organisms (*16*−*20*). *Tetrahymena thermophila* telomerase, the enzyme studied in this paper, is associated with two additional proteins, p80 and p95 (*21*). Although the genes encoding these proteins are not essential for cell viability, loss of p80 or p95 in vivo results in telomere lengthening (*22*). Not yet identified in *Tetrahymena* is a homologue of p43 found in *Euplotes* telomerase (*23*); both the *Tetrahymena* and *Euplotes* telomerase RNAs are transcribed by RNA polymerase III, so it might be expected that the p43 La-motif protein would be found in both.

The telomerase RNA subunit, first identified from the holotrichous ciliate *T. thermophila* (*24*), has been cloned from a variety of organisms including other ciliates (*25*−*29*), yeast (*30*, *31*), and mammals (*32*−*35*). The secondary structures of the ciliate and mammalian telomerase RNAs have been determined by phylogenetic sequence analysis (*26*−*28*, *35*) and have common features suggesting conservation of the overall telomerase RNA function.

The *T. thermophila* telomerase RNA has four stems (I, II, III, and IV) that are numbered from the 5′ end to the 3′ end (Figure 1). Stem I is a long-range base-pairing interaction that consists entirely of G-C base pairs. Although conserved throughout ciliates and in most mammals, stem I is not critical for in vitro telomerase function (*4*, *36*−*38*). Stem II is present in the telomerase RNA from most holotrichous ciliates but not in those from hypotrichous ciliates. Portions of this stem have been shown to be necessary for in vitro telomerase activity and TERT binding to telomerase RNA in a reconstituted *T. thermophila* system (*39*). Stems IIIa and IIIb form a pseudoknot structure present in all telomerase RNAs where the secondary structures have been determined (*27*, *28*, *35*, *40*). The pseudoknot is essential for the assembly of the *T. thermophila* telomerase complex in vivo (*41*). However, this region of the RNA is not required for binding to TERT or for telomerase activity in in vitro reconstituted systems (*36*, *39*). The structure of stem IV is well conserved

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[1] Abbreviations: TERT, telomerase reverse transcriptase; WT, wild type; RNase, ribonuclease; CMCT, *N*-cyclohexyl-*N*′-[2-(*N*-methyl-4-morpholino)ethyl]carbodiimide *p*-toluenesulfonate.

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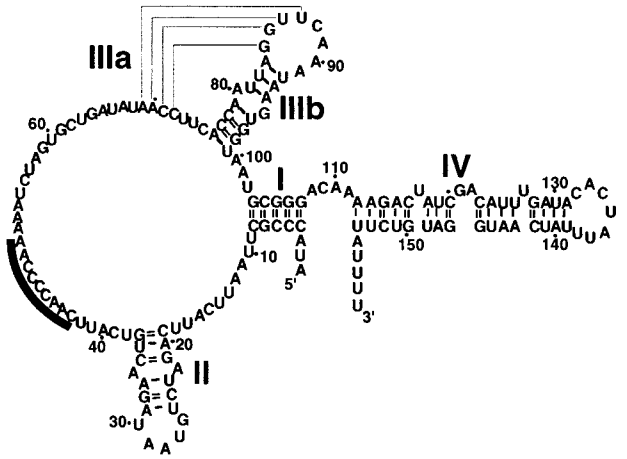

FIGURE 1: *T. thermophila* telomerase RNA secondary structure as described previously (*28*). The helices are numbered 5′ to 3′, and the template is indicated by the black line.

among *Tetrahymena* species but diverges in the hypotrichous ciliates. Deletion of stem IV in *T. thermophila* telomerase RNA reduces in vitro telomerase activity to less than 10% of the wild-type (WT) telomerase activity (*36*, *39*).

Although the secondary structure of the *T. thermophila* RNA has been well defined, the protein-binding site of TERT has only begun to be defined recently (*39*, *42*), and the tertiary fold of the RNA in the mature complex has not been elucidated. In vivo chemical probing of the RNA has shown that a majority of the RNA is protected, suggesting multiple potential sites of protein binding (*43*). Interpretation of these in vivo results is difficult because the RNA is likely bound by a number of proteins including TERT, p80, p95, and perhaps a p43 homologue. The reticulocyte lysate based reconstitution system (*5*, *44*) allows a more detailed analysis of TERT binding sites and the effect of protein binding on the conformation of the RNA in the context of a better defined protein−RNA complex. However, the reconstituted TERT−RNA complex may contain other non-*Tetrahymena* proteins from the reticulocyte lysate. Notably, the human telomerase complex reconstituted from reticulocyte lysates has been shown to contain the chaperone proteins Hsp90 and p23 (*45*), and *Tetrahymena* telomerase may also require these or other proteins in the reticulocyte lysate for complex formation (*39*).

In the present study we used RNase T1 and RNase V1 footprinting to examine the structure of the active *T. thermophila* telomerase RNA−TERT complex assembled in a reticulocyte lysate in vitro transcription−translation system. On the basis of these results, we made and analyzed a series of deletions, block mutations, and point mutations in the telomerase RNA. Our results support a complicated assembly of the telomerase enzyme and reveal the unexpected involvement of an RNA element distant from the template in the formation of the active site.

MATERIALS AND METHODS

Plasmid Construction. Plasmids encoding mutant telomerase RNAs were constructed using PCR mutagenesis (*46*) with pTETteloHH as a template as previously described (*43*). HH designates that a 5′ hammerhead ribozyme was incorporated; self-cleavage of the hammerhead gives a uniform 5′ terminus. The PCR products were cloned into pUC19 or pTETteloHH and sequenced to confirm the mutation and lack of second site mutations.

Transcription of Telomerase RNA. Telomerase RNA was transcribed from pTETteloHH or mutant plasmids that had been digested with the restriction enzyme *Ear*I which cuts at the 3′ end of the telomerase RNA. *Ear*I-digested plasmid (25 μg) was transcribed in a 1 mL reaction containing 40 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 1 mM each NTP, and 50 μL of T7 RNA polymerase (1 mg/mL) at 37 °C for 2 h. Cleavage of the hammerhead ribozyme was enhanced by addition of 10 μL of 1 M $MgCl_2$ and incubation at 45 °C for an additional 20 min. After ethanol precipitation, the RNA (in formamide loading buffer) was then gel purified on a 4% polyacrylamide gel (29:1 acrylamide/bisacrylamide) containing 7 M urea and 1 × TBE (0.1 M Tris base, 0.083 M boric acid, and 1 mM EDTA) and recovered by a crush and soak method. The concentration of RNA was determined by measuring the absorbance at 260 nm.

Reticulocyte Lysate Translation of TERT. *T. thermophila* TERT with N-terminal T7 and His-6 tags was translated using a rabbit reticulocyte lysate in vitro transcription−translation system (TNT Coupled Reticulocyte Lysate kit, Promega) as described (*44*). A 50 μL reaction contained 1 μg of pET28a-TERT DNA (*44*), 0−1 pmol of telomerase RNA as indicated, and kit components as specified by the manufacturer. The reaction was incubated for 60 min at 30 °C.

Enzymatic Probing of the Telomerase Complex. Gel-purified telomerase RNA was 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and then re-gel purified. Approximately 1 pmol of ^{32}P-labeled RNA was used in a standard 100 μL reticulocyte lysate reaction (+TERT) or a 100 μL reaction lacking pET28a-TERT (−TERT). Immediately following translation, the reticulocyte lysate reaction was placed on ice, and 20 μL was diluted 1:5 into 1× TEL buffer (50 mM Tris−acetate, pH 8.0, 1.25 mM $MgCl_2$, 5 mM DTT) to a final volume of 100 μL. To initiate the RNase reaction, 0.01 unit of RNase T1 (Calbiochem) or 0.2 unit of RNase V1 (Pharmacia) was added, and the reaction was placed at 30 °C for 15 min. To stop the reaction 100 μL of TES (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.2% SDS) and 200 μL of phenol/chloroform/isoamyl alcohol were added, and the reaction was vortexed. The supernatant from the phenol extraction was extracted with chloroform/isoamyl alcohol (24:1) and ethanol precipitated. Half the dissolved product was counted using a scintillation counter, and the samples were adjusted to be within 5% of the same counts per million. Samples were then run on an 8% polyacrylamide/7 M urea/15 TBE sequencing gel. Gels were visualized and quantitated using a Phosphorimager and Imagequant software (Molecular Dynamics).

Telomerase Activity Assay. Telomerase assays were done by a procedure modified from that described previously (*44*). Telomerase RNA (0.02 or 0.2 pmol) was added to 10 μL of the reticulocyte lysate in vitro transcription−translation reaction after TERT translation and incubated at 30 °C for 10 min to allow complex formation. The telomerase assay was initiated by adjusting the reaction to 1× TEL buffer, 1 μM primer [$(G_4T_2)_3$], 100 μM TTP, and 10 μM [α-^{32}P]dGTP at 80 Ci/mmol for a final volume of 20 μL. The reaction was incubated for 60 min at 30 °C and stopped by addition

of 130 μL of TES and 150 μL of phenol/chloroform/isoamyl alcohol. The aqueous layer was ethanol precipitated and dissolved in 10 μL of 0.5× formamide loading buffer. A ^{32}P-radiolabeled 100-mer DNA oligonucleotide (5000 cpm) was added before phenol extraction as a recovery and loading control. Half of the product was resolved on an 8% polyacrylamide/7 M urea sequencing gel.

Immunoprecipitation Binding Assay. The relative amounts of RNA bound to TERT were measured by co-immunoprecipitation of telomerase RNA with the T7-tagged TERT. Immunoprecipitations were performed essentially as described (*44*). The reticulocyte lysate translation mixture (40 μL) containing TERT translated in the presence of [^{35}S]-methionine was added to 40 μL of blocking buffer (20 mM Tris−acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM DTT, 100 mM potassium glutamate, 0.5 mM lysozyme, 0.5 mg/mL bovine serum albumin, 0.05 mg/mL glycogen, and 0.1 mg/mL yeast RNA) and centrifuged in a microcentrifuge for 10 min to remove particulates. The supernatant was added to 20 μL of blocked beads and incubated with agitation at 4 °C for 2 h. The beads were then washed 3 times on ice with 150 μL of Wash 300 buffer (20 mM Tris−acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM DTT, 300 mM potassium glutamate), once in Wash 100 buffer (20 mM Tris−acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM DTT, 100 mM potassium glutamate), and twice in TMG (10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 10% glycerol). The beads were resuspended in 18 μL of TMG. To normalize for the amount of protein immunoprecipitated in each sample, 3 μL of resuspended beads was boiled in Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.005% bromophenol blue, 20% glycerol, 0.72 mM β-mercaptoethanol) and analyzed by 4−20% SDS−PAGE (Novex). The dried gel was visualized and quantitated using a Phosphorimager.

Beads containing an equal amount of immunoprecipitated protein or 10 μL of the reticulocyte lysate reaction were assayed for telomerase RNA. A 5′ end-labeled DNA oligonucleotide was added to the beads after immunoprecipitation as a recovery and loading control. To digest the proteins, 100 μL of ProK solution (10 mM Tris-HCl, pH 7.5, 7.5 mM EDTA, 0.6% SDS, 0.05 mg/mL proteinase K) was added to the beads or reticulocyte lysate. The mixture was incubated at 37 °C for 30 min and then phenol/chloroform extracted and ethanol precipitated. The pellet was dissolved in 0.5× formamide loading buffer and run on a 4% polyacrylamide/7 M urea gel. The nucleic acids were transferred to Hybond N+ membrane (Amersham) and probed with a 5′ end-labeled telomerase RNA specific oligonucleotide in Church buffer (*47*). The membrane was visualized and quantitated using a Phosphorimager.

Analysis of the Dissociation Rate Constant (k_{off}). The dissociation rate constant of the TERT−telomerase RNA complex was measured using a pulse−chase assay. WT or mutant telomerase RNA was 5′ end-labeled using [γ-^{32}P]-ATP and T4 polynucleotide kinase and separated from free nucleotides using a G-25 Sephadex spin column for RNA (Boehringer Mannheim). The RNA was diluted to approximately 10^6 cpm/pmol, and 9 pmol of RNA was used per 450 μL of the reticulocyte lysate reaction. TERT was translated using [^{35}S]methionine in the presence of the ^{32}P-labeled telomerase RNA. To begin the chase, 630 pmol of cold WT telomerase RNA (70-fold excess) was added to 450 μL of the reticulocyte lysate translation mixture at 30 °C. Samples (50 μL) were taken out at 0, 15, 30, 50, 90, 150, and 250 min and added to 50 μL of Block buffer and 15 μL of T7-tag antibody−agarose beads that had been washed and blocked as described (*48*). The beads were agitated for 2 h at 4 °C and then washed three times with Wash 300 buffer and once with Wash 100 buffer. The washed beads were then resuspended in 12 μL of TMG. The 1:1 slurry was then mixed with Laemmli loading buffer and electrophoresed on a 4−20% gradient SDS−PAGE (Novex). After being dried, the gel was visualized on the Phosphorimager. A second exposure was taken with a piece of X-ray film between the gel and screen to shield the ^{35}S signal. The amount of TERT protein was quantitated using the first exposure, and the amount of RNA was visualized from the second exposure. The RNA signal was divided by the protein signal, and the ratio was normalized to a value of 1 for the zero time point. The normalized RNA values were plotted versus time and fit to a single-exponential equation ($y = ae^{-kt} + b$, where $k = k_{off}$).

Cross-Linking of Telomeric Primers to the Telomerase Complex. Cross-linking experiments followed procedures modified from Hammond et al. (*49*). We first immunoprecipitated the Flag-tagged TERT in complex with WT RNA, mutant RNA, or no RNA. The Flag-tagged construct was constructed by substituting the sequence encoding His-6 and T7 tags from pET28a-TERT with the sequence encoding a Flag tag (gift from Karen Goodrich). Nonspecific competitor RNA (100 pmol, 5′AGC CAC TAT CGA CTA CGC GAT CAT 3′) was prebound to 10 μL of the immobilized TERT complex in 1× TEL buffer for 10 min at 30 °C. A 5-iodouracil, ^{32}P-end-labeled DNA primer, I(1,7,13,19)TET13 (5 pmol, 5′ ^{I}UTG GGG ^{I}UTG GGG ^{I}UTG GGG ^{I}UT 3′), was then added to the beads and incubated at 10 min at 30 °C in a final volume of 20 μL. The complex was cross-linked using UV light (312 nm) for 10 min on a cold, parafilm-covered aluminum block in a UV Stratalinker (Stratagene). The entire reaction was then boiled in Laemmli loading buffer and run on a 4−20% SDS−PAGE (Novex).

Extension of Cross-Linked Primers. Cross-linking of cold I(1,7,13,19)TET13 was done as described above using telomerase immobilized on Flag antibody beads. Telomerase extension was initiated by addition of [α-^{32}P]dGTP at 80 Ci/mmol to a final concentration of 10 μM in 1× TEL buffer. The primer was extended for 1 h at 30 °C. The reaction was stopped by addition of 10 μL of Stop buffer (250 mM Tris-HCl, pH 6.8, 12% SDS) and heating to 95 °C for 5 min to denature the proteins and release from the beads. The reaction products were separated from free nucleotides using a Micro Biospin 6 chromatography column (Bio-Rad). The reaction was then boiled in Laemmli loading buffer and analyzed by 4−20% SDS−PAGE (Novex).

RESULTS

Footprinting of the Telomerase RNA−TERT Complex. The nuclease cleavage patterns of telomerase RNA alone were compared to those of the same RNA bound to TERT in in vitro translation reactions. Telomerase complexes formed in reticulocyte lysates were digested with either RNase T1,

which cuts 3′ of single-stranded guanines, or RNase V1, which cuts double-stranded or stacked RNA without sequence specificity (reviewed in ref *50*). A number of other RNases and chemical probes, including RNase CL3, RNase U2, RNase T2, dimethyl sulfate, and CMCT, were also tried but were not sufficiently reactive in reticulocyte lysates.

The RNase T1 and RNase V1 cleavage patterns were highly reproducible and showed distinct changes upon addition of reticulocyte lysate (Figure 2A, compare lanes 5 and 6) and again upon TERT binding (Figure 2A, compare lanes 4 and 5). The data can be more easily visualized by plotting the signal intensity versus distance of migration. By superimposing the +TERT and −TERT data, nucleotide positions that display either protection or enhancement of cleavage upon protein binding can be visualized (Figure 2B,C). This comparison allows the detection of large-scale conformational changes of the RNA upon TERT binding, as well as the determination of regions of the RNA that are protected upon protein binding.

In the absence of protein (blue), the region of stem II close to the loop appeared to be partially unfolded. Nucleotides G21, G32, and G37 were all cleaved to a significant extent by RNase T1 in the absence of TERT protein, indicating a significant amount of single-stranded character (Figure 2A,B,D). A lack of pairing of the RNA in this region was also indicated by the lack of RNase V1 cleavage at nucleotides 22−24 (Figures 2A,C,D). However, upon TERT binding to the telomerase RNA (red), nucleotides G21, G32, and G37 were all protected from RNase T1 cleavage (Figure 2B), and nucleotides 22−24 showed enhanced RNase V1 cleavage (Figure 2C). Both results indicated an increase in pairing and folding of stem II upon protein binding (Figure 2D,E). Interestingly, under the same buffer conditions but without the addition of reticulocyte lysate, stem II appeared to be well formed even in the absence of TERT; nucleotides G21, G32, and G37 were protected from digestion by RNase T1 (Figure 2A; compare lanes 5 and 6). Thus, the folding of this helix may not be protein dependent under all conditions. We do not know whether the relatively unpaired conformation in reticulocyte lysates or the more fully paired conformation in simple buffer conditions is more representative of the state of the unbound RNA in vivo.

Although the region of the telomerase RNA between stems II and III (including the template) is depicted as single stranded in the phylogeny-based secondary structure diagram, this region has been proposed to be involved in a stacking or base-pairing interaction (*51*). Consistent with previously reported data (*51*), we detect cleavage by RNase V1 in the single-stranded region between stems II and III on naked RNA (Figure 2A,C). Upon protein binding, the cleavage by RNase V1 was enhanced, perhaps indicating stabilization of the structure in this region by bound protein (Figure 2A,B,E). Upon protein binding there was also a small but reproducible decrease in RNase T1 digestion at G59 and G61, again consistent with a decrease in single-stranded character.

The pseudoknot, consisting of stems IIIa and IIIb, also showed structural stabilization upon protein binding. Our data in the absence of protein (Figure 2) were consistent with those of Bhattacharyya and Blackburn (*51*), who previously showed that this region was only partially base paired in the absence of protein. Upon protein binding, we now find that the pseudoknot region shows an enhancement in RNase V1 cleavage and a protection of cleavage from RNase T1, suggesting that the complete folding of this region is TERT dependent.

Stems I and IV were efficiently cleaved by RNase V1 in the absence of protein and were not cleaved efficiently by RNase T1, suggesting that these regions fold into stable helices in the absence of TERT [Figure 2A−D (*51*)]. Upon protein binding, stem IV was protected from both RNase V1 and RNase T1 digestion, suggesting that the protein is shielding the RNA from cleavage either directly through protein binding or indirectly by a conformational change in the RNA. One exception to this protection is nucleotide G114, which was cleaved by RNase T1 in both the presence and absence of protein. This nucleotide is near the end of stem IV, and its cleavage may indicate some local unfolding.

Mutations in Stem IV Have a Large Effect on Telomerase Activity. Because the footprinting data implicated stem IV as being particularly protected upon binding of TERT to telomerase RNA, mutants were constructed to test directly for protein binding to stem IV and to assay the contribution of stem IV to telomerase activity. Mutant ΔIV has a complete deletion of stem IV (nucleotides 107−159). Additional mutations were made in two regions of stem IV that are highly conserved among *Tetrahymena* telomerase RNAs (*28*): the GA bulge and the loop that terminates stem IV (loop IV). The GA bulge (nucleotides 121−122) was altered by substitution (GA to UU) or by deletion (ΔGA). A final set of mutations tested the importance of loop IV by single base changes (C132G, A133U, U135A, A136U, U137A, and U138A) or by a substitution of the seven nucleotide loop with a GAAA tetraloop (L4GAAA).

The ability of TERT to bind the mutant telomerase RNAs was measured by the efficiency with which the telomerase RNA co-immunoprecipitated with N-terminally T7-tagged TERT. This co-immunoprecipitation binding assay showed that the binding of telomerase RNA to TERT is not significantly affected by mutations in stem IV. Approximately equal amounts of WT, GA to UU, and L4GAAA RNAs were co-immunoprecipitated with TERT, while deletion of the bulged nucleotides (ΔGA) reduced the amount of RNA co-immunoprecipitated approximately 2-fold (Figure 3A, lanes 1 and 3−5). Additionally, none of the single nucleotide substitutions in loop IV decreased the amount of RNA co-immunoprecipitated (data not shown). Even a complete deletion of stem IV resulted in only a 2-fold decrease in RNA binding to TERT (Figure 3A, lane 2), suggesting that the protection seen in the RNase V1 footprinting assay was not because stem IV is the predominant site of protein binding. As a control for the specificity of binding, mutation of four nucleotides (15−18) identified by Lai et al. (*42*) as being critical for TERT binding also eliminated most of the binding in our assay (Figure 3A, lane 9).

To further assess the importance and function of both stem IV and loop IV, the activity of the mutant RNAs in a telomerase primer extension assay was measured. The ΔIV mutant RNA reconstituted with TERT showed only a small amount of residual activity (Figure 3B, lanes 3 and 4), consistent with previous studies (*36*, *39*). Deletion of the GA bulge reduced telomerase activity to less than 10% of WT activity (Figure 3B, lanes 7 and 8). In contrast, mutating the GA bulge to a UU bulge had very little effect on telomerase

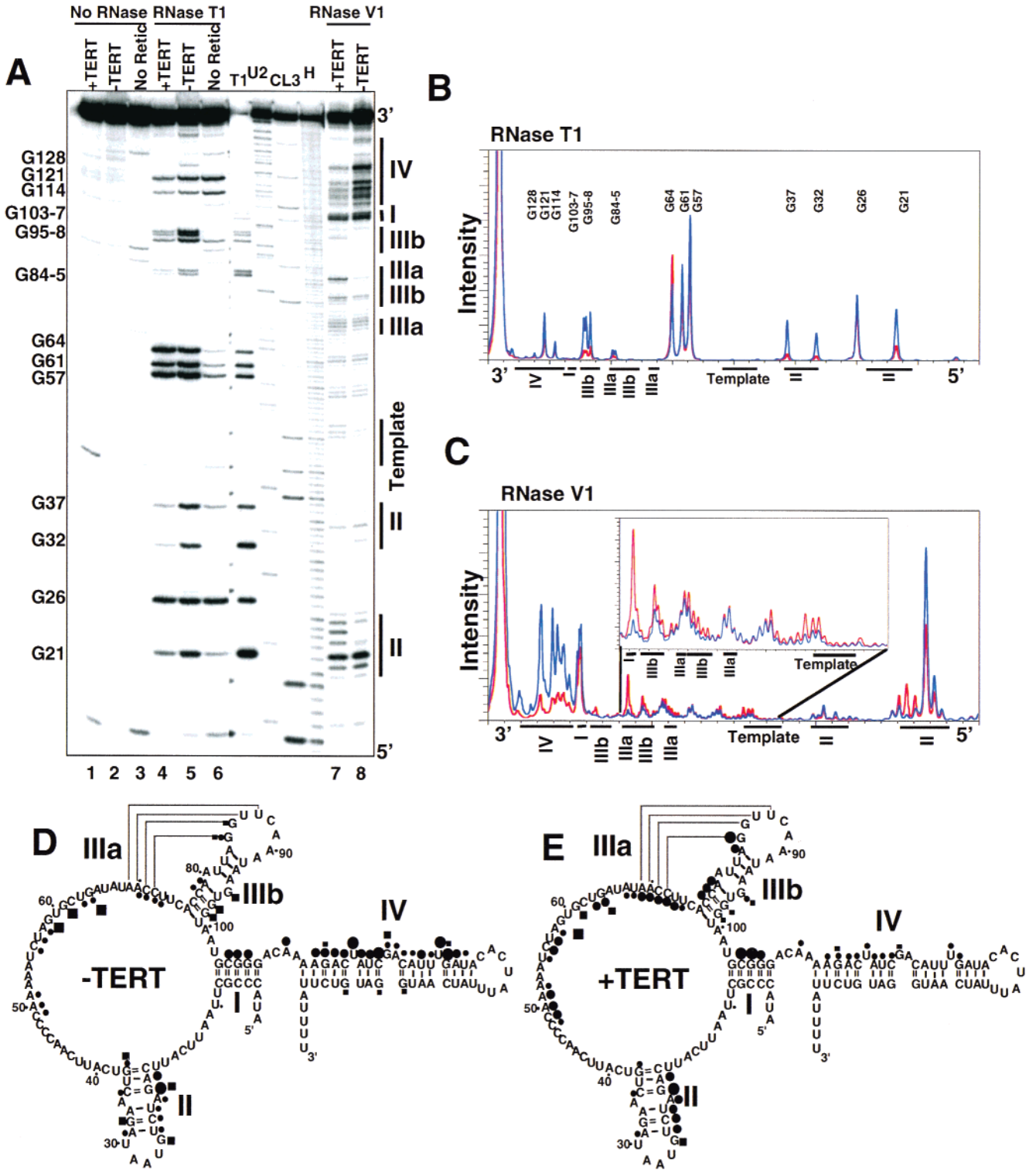

FIGURE 2: Enzymatic footprinting of the *T. thermophila* telomerase complex reconstituted in reticulocyte lysates. +TERT: TERT was translated in reticulocyte lysates in the presence of 5′ ^{32}P-end-labeled telomerase RNA. −TERT: the end-labeled RNA was treated identically except no pET28a-TERT plasmid was included in the translation. Following translation, the telomerase complex was incubated with the indicated RNase. (A) Samples were run on an 8% polyacrylamide sequencing gel. Locations of all guanines are indicated to the left of the gel, and the location of secondary structure elements and the template as described in Figure 1 are indicated to the right. T1, U2, and CL3: denatured telomerase RNA cleaved with RNase T1 (G's), RNase U2 (A's), and RNase CL3 (C's) to serve as internal sequence markers. H: hydroxide ladder to mark every nucleotide. No retic indicates samples where the reticulocyte lysate was absent from the in vitro translation reaction. (B) Quantitation of the RNase T1 data. Signal intensity in Phosphorimager units is plotted versus distance of migration for the +TERT sample (red) and the −TERT sample (blue). The nucleotide identities are indicated above and the positions of the secondary structure elements are indicated below the graph. (C) Quantitation of the RNase V1 samples. As above, the signal intensity is plotted for the +TERT (red) and −TERT (blue) samples. The region of the gel corresponding to the template and pseudoknot is magnified for clarity. (D) −TERT and (E) +TERT results are superimposed on the secondary structure model of *T. thermophila* telomerase RNA. Squares (■) indicate cleavage by RNase T1, and circles (●) indicate cleavage by RNase V1. The size of the symbol correlates with the relative intensity of the peak.

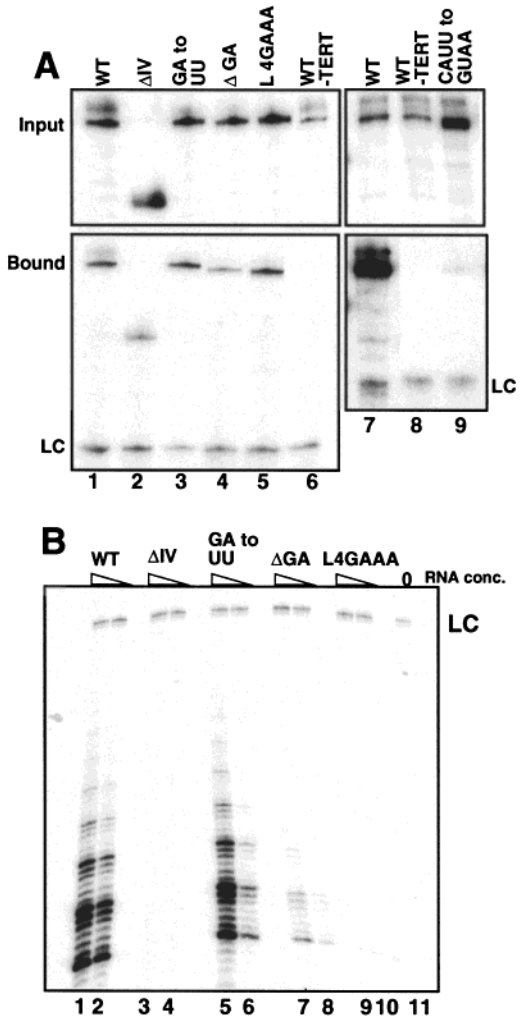

FIGURE 3: TERT binding and telomerase activity of stem IV telomerase RNA mutants. (A) T7-tagged TERT was expressed in reticulocyte lysate in the presence of the indicated telomerase RNA. An aliquot of this reaction was retained as the input. The remaining reaction was immunoprecipitated with an antibody to the T7 tag on the TERT protein. The RNA from the input sample and from an aliquot of the beads normalized for the amount of bound TERT (see Materials and Methods) was isolated, run on a polyacrylamide gel, and Northern blotted with a telomerase RNA specific probe. LC: a labeled 55-mer DNA oligonucleotide added to the immunoprecipitation beads to serve as a recovery and loading control. (B) TERT was expressed in reticulocyte lysates in the absence of telomerase RNA. The indicated RNA was added to final concentrations of 1 or 10 nM, and the resulting telomerase complex was assayed for the ability to extend a telomeric primer $(G_4T_2)_3$. LC: an end-labeled 100-mer DNA oligonucleotide used as a control for recovery and loading.

activity (Figure 3B, lanes 5 and 6). This conserved GA bulge has been proposed to induce a bend in the RNA helix (*51*). Our results would then suggest that the ability of the helix to bend, and not the specific identity of the bulged nucleotides, is critical for telomerase function.

In contrast, loop IV was critical for telomerase activity and was very sensitive to single base changes. When loop IV was mutated to a GAAA tetraloop, a stable loop common in many RNAs (*52*), activity was reduced to a very low level (<1% of WT) similar to that resulting from deletion of the entire helix (Figure 3B, compare lanes 9 and 10 with lanes 3 and 4). The base sequence is highly conserved among the *Tetrahymena* species (Figure 4A). Mutants C132G, U137A,

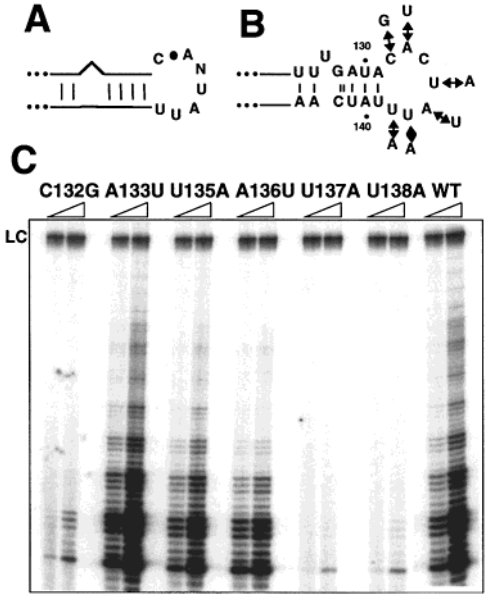

FIGURE 4: Telomerase activity of point mutants in loop IV. (A) The conservation of individual positions in loop IV are indicated here (*28*). Key: capital letters, conserved in all known *Tetrahymena* telomerase RNAs; N, less than 70% conserved; ●, an additional nucleotide present in less than 50% of the *Tetrahymena* telomerase RNAs. (B) Locations of individual point mutations made in loop IV. (C) TERT was expressed in reticulocyte lysates in the absence of telomerase RNA. The indicated RNA was added to final concentrations of 1 or 10 nM and assayed for the ability to extend a telomeric primer $(G_4T_2)_3$. LC: an end-labeled 100-mer DNA oligonucleotide added as a control for recovery and loading.

and U138A each had less than 2% of WT activity (Figure 4B,C). Mutants U135A and A136U were intermediate, both having less than 50% of WT activity (Figure 4). The remaining two mutants, A133U and C134G, retained WT activity (Figure 4 and A. Zaug, T. Bryan, and T. R. Cech, personal communication). Interestingly, the nucleotides that lie closer to the paired region had the strongest phenotype when mutated.

Mutations in Loop IV with Low Activity Affect Pseudoknot Folding. One potential reason for the large effect of loop IV mutations on the activity of telomerase was that mutations in loop IV affect the overall conformation of the complex. To examine this possibility, we used the RNase V1 footprinting assay to examine changes in the protection pattern of the loop IV RNA mutants compared to WT RNA, both alone and as RNA−TERT complexes. When the loop was mutated to a GAAA tetraloop, very little change was seen in the cleavage pattern of the RNA alone [Figure 5, panels A (lanes 3 and 4) and B]. Small differences in the cleavage pattern were observed in the loop that had been mutated, as one might expect. However, comparison of the protection patterns from the WT and L4GAAA mutants bound to TERT showed a distinct difference in the RNase V1 protection pattern in the pseudoknot region of the RNA [Figure 5, panels A (compare lanes 1 and 2) and C]. The most dramatic difference in the cleavage pattern occurred at nucleotide G84 in stem IIIa, which was cleaved to a significant extent in the WT RNA but not in the L4GAAA mutant (Figure 5C). Stem IIIb in the L4GAAA RNA also failed to give the enhancement in cleavage upon protein binding that was seen in WT RNA (Figure 5C). Since the L4GAAA mutant was competent to bind TERT (as assayed by immunoprecipita-

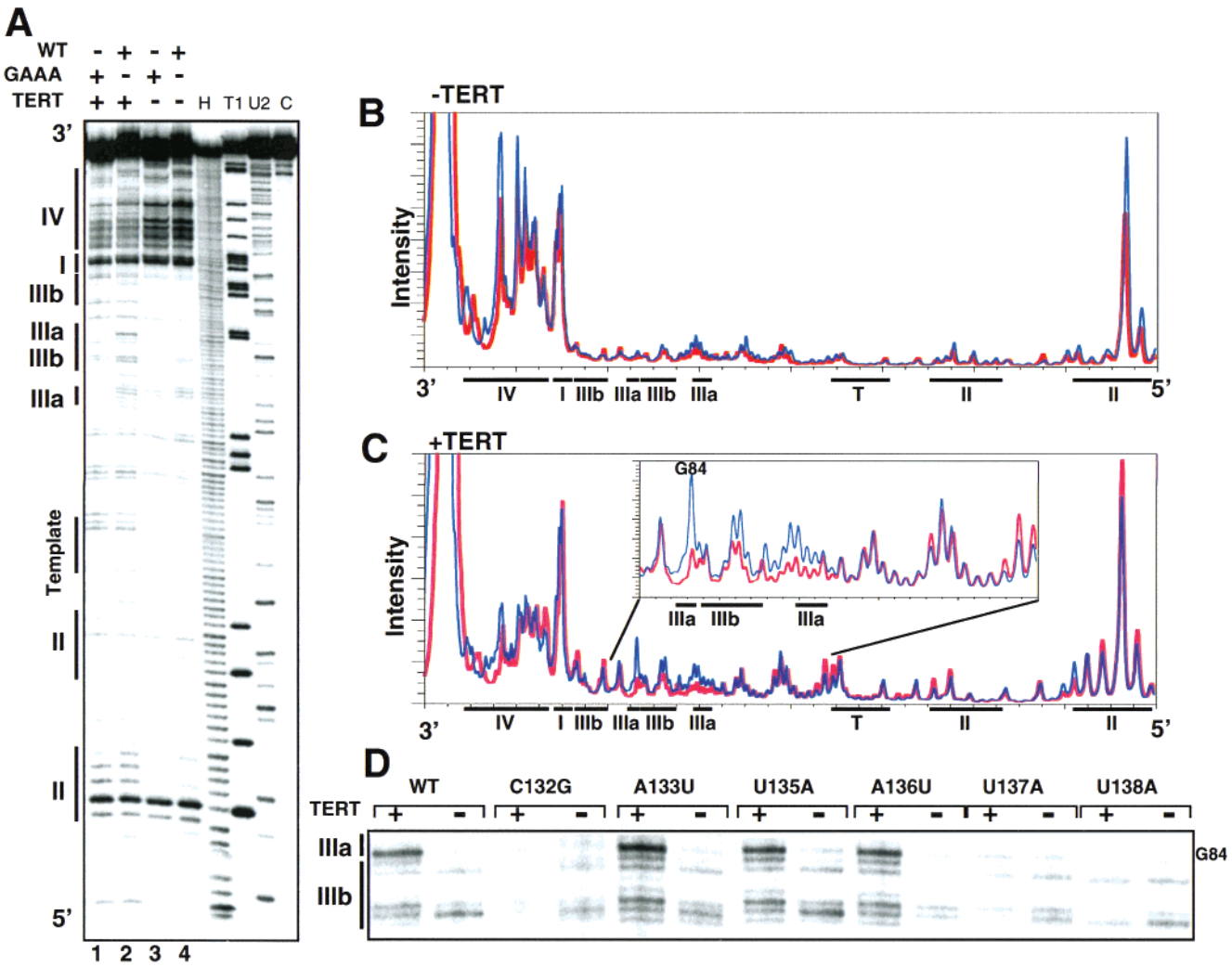

FIGURE 5: RNase V1 footprinting of loop IV RNA mutants. (A) Phosphorimager scan of an 8% polyacrylamide gel showing the RNase V1 cleavage pattern for both WT and L4GAAA (GAAA) telomerase RNA in the presence and absence of TERT. Positions of the RNA stems and the template are indicated to the left of the gel. The positions of all the G's and A's were identified by cleavage of the denatured RNA by RNase T1 and U2, respectively. The hydroxide cleavage ladder (H) provides molecular weight markers that allow identification of the remaining nucleotides. A control lane (C) lacks any added nuclease. (B) Quantitation of the −TERT lanes from (A) is shown with WT in blue and the L4GAAA in red. The 5′ and 3′ ends of the RNA are indicated at the bottom of the graph as well as features of the RNA secondary structure; T, template. (C) Quantitation of the +TERT lanes from (A) with WT in blue and L4GAAA in red. (D) Phosphorimager scan of an 8% polyacrylamide gel showing the RNase V1 cleavage pattern at stem IIIa (nucleotides 84−87) and stem IIIb (nucleotides 92−99) for single point mutants in loop IV in both the presence and absence of TERT.

tion, Figure 3A), these differences in the cleavage pattern suggest that the pseudoknot region of the L4GAAA RNA was not induced to base pair upon protein binding. Other changes in the RNA cleavage pattern that occur upon protein binding still occurred in the L4GAAA mutant; these include enhancement of cleavage in stem II and in the single-stranded region between stems II and III and also protection of stem IV (Figure 5C). Thus, mutation of loop IV to a GAAA tetraloop perturbs the protein-induced structure of the RNA in the pseudoknot while maintaining binding of the RNA to the protein.

The conformations of telomerase RNAs containing point mutations in loop IV were also probed using RNase V1 footprinting. C132G, U137A, and U138A mutant RNAs, all of which had less than 2% of WT telomerase activity, showed an absence of the TERT-specific enhancement in stem III cleavage as illustrated by the loss of the most prominent enhancement at position G84 (Figure 5D). In contrast, loop IV mutations with intermediate telomerase activity (U135A, A136U) and WT telomerase activity (A133U) all retained a WT cleavage pattern (Figure 5D). Therefore, loss of protein-induced base pairing in the pseudoknot correlated with the loss of telomerase activity.

Separation of the Contributions of Loop IV and the Pseudoknot. The correlation between the effect of loop IV mutations on telomerase activity and on misfolding of the pseudoknot region suggested that the defect of the loop IV mutant might be entirely mediated through pseudoknot formation. However, previous work had shown that deletion of the pseudoknot reduced but did not eliminate telomerase activity (*39*). One can imagine that disorganization of the pseudoknot region may have a larger effect on telomerase activity than a simple deletion. However, it is also possible that loop IV has another critical function in the active telomerase complex. We predicted that if the disorganization of the pseudoknot region was the major cause of the telomerase activity defect, then the activity of an RNA containing both the pseudoknot deletion (ΔIII, deletion of

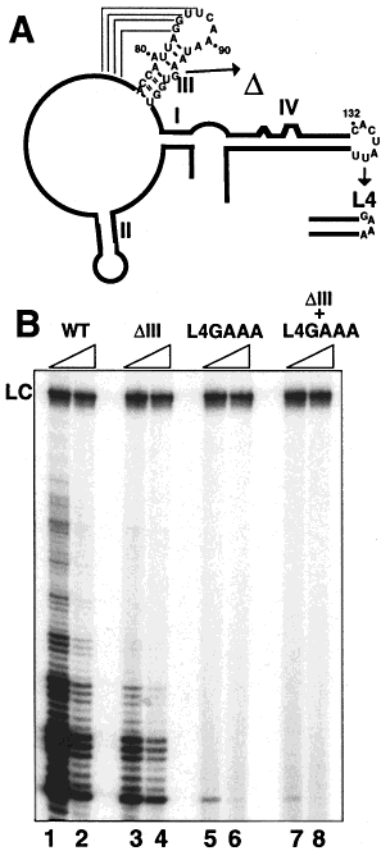

FIGURE 6: Contribution of stem III and loop IV to telomerase activity. (A) Diagram of *T. thermophila* telomerase RNA showing the position of the ΔIII and L4GAAA mutations. (B) Telomerase activity assay with the indicated RNA added at concentrations of 1 or 10 nM after TERT translation. LC: an end-labeled 100-mer oligonucleotide added as a precipitation and loading control.

nucleotides 76−99) and the loop IV mutation (L4GAAA) should be similar to that of ΔIII. In contrast, if loop IV is involved in more than promoting folding of the pseudoknot region, the double mutant RNA would be predicted to behave similarly to the L4GAAA single mutant. As shown in Figure 6B, the telomerase activity of the ΔIII/L4GAAA double mutant was reduced to an extent similar to that of the L4GAAA single mutant (Figure 6B, compare lanes 7 and 8 with lanes 5 and 6), while the ΔIII mutant showed a much less dramatic reduction (Figure 6B, lanes 3 and 4). These results suggest that loop IV may have a function or functions beyond facilitation of pseudoknot formation.

Loop IV Mutations Do Not Affect Primer or Nucleotide Binding. To explore other possible functions of loop IV, we assessed the contribution of loop IV to primer and nucleotide binding. If the loop IV mutations decreased the ability of the complex to bind the substrate nucleotides dGTP and TTP, we predicted that increasing the concentration of those nucleotides would partially rescue the telomerase activity defect. However, increasing nucleotide concentration did not disproportionately increase activity of the mutant RNAs (data not shown). We conclude that nucleotide binding is not significantly affected by mutations in loop IV.

To look at the effect of loop IV mutations on telomeric primer DNA binding, we applied a protein−DNA cross-linking assay similar to that used by Hammond et al. (*49*). In this assay a telomeric primer containing 5-iodouracil in place of thymine at four positions, I(1,7,13,19)TET13, is

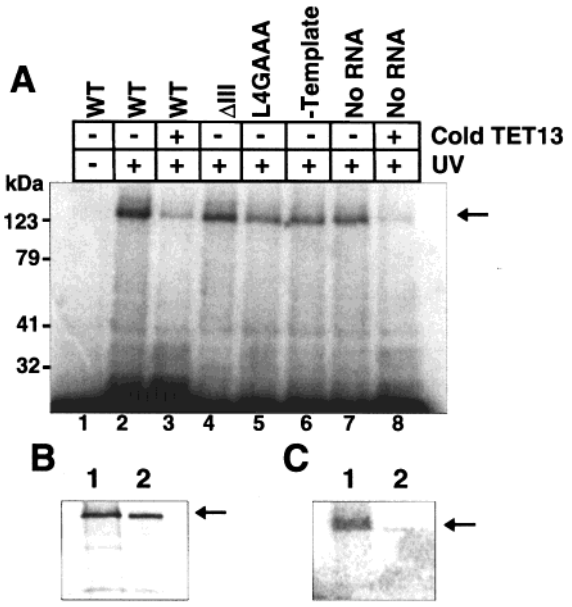

FIGURE 7: Cross-linking of a telomeric primer to TERT. (A) Reticulocyte lysate expressed, Flag-tagged TERT and the indicated telomerase RNAs were immunoprecipitated on Flag antibody beads. ^{32}P-labeled I(1,7,13,19)TET13 DNA primer was prebound to telomerase in the presence of a 20-fold excess of cold nonspecific competitor. The complex was then irradiated with 312 nm light to initiate cross-linking, and the resulting products were separated by SDS−PAGE. An arrow indicates the cross-linked product. Lane 1: immunoprecipitated telomerase complex in the absence of UV irradiation. Lanes 3 and 8: cold TET13 as a specific competitor added prior to addition of I(1,7,13,19)TET13 primer. (B, C) Extension of cross-linked primer by telomerase. Immunoprecipitated WT telomerase complex with [^{35}S]methionine-labeled TERT was cross-linked as above to the unlabeled I(1,7,13,19)TET13 primer. [α-^{32}P]dGTP was added to initiate extension of the primer by telomerase, and the resulting products were separated by SDS−PAGE. (B) The bands seen represent both the ^{35}S-labeled TERT protein and any primer that has been cross-linked to TERT. Lane 1: WT telomerase complex cross-linked to I(1,7,13,19)TET13 primer and TERT and incubated in the presence of [α-^{32}P]dGTP under extension conditions. Lane 2: WT telomerase complex in the absence of primer cross-linking and extension. (C) Same gel as in (B) but exposed to the Phosphorimager plate through film in order to block a majority of the ^{35}S signal. The remaining signal in lane 1 represents cross-linked primer that has been extended by telomerase and is therefore incorporated [α-^{32}P]dGTP.

prebound to the telomerase complex; upon irradiation with 312 nm light, cross-links are formed to nearby aromatic amino acids in TERT. We confirmed that cross-linking of the telomeric primer was dependent on exposure to UV light and could be inhibited by addition of an excess of unlabeled TET13 primer (Figure 7A, lanes 1−3). Additionally, the cross-link was not competed when an excess of nontelomeric oligonucleotide was added prior to addition of I(1,7,13,19)-TET13, which indicates that this is a specific cross-link to TERT. We found that this cross-link was not dependent on the presence of stem III, loop IV, or the template portion of telomerase RNA (Figure 7A, lanes 4−6). Indeed, no telomerase RNA at all was required for efficient cross-linking of the primer to TERT (Figure 7A, lane 7), and the extent of primer cross-linking in the presence of different unlabeled competitor primers was independent of the presence of telomerase RNA (data not shown). These results indicate that telomeric primers are bound specifically by TERT and that mutations in the RNA do not significantly affect the ability of TERT to bind telomeric primers.

To confirm that the cross-linked product reflects an active conformation, we tested the ability of the cross-linked primer to be extended by telomerase. Following cross-linking of a cold 5-iodouracil-substituted telomeric primer to the wild-type telomerase complex, [α-^{32}P]dGTP was added, and the ability of the telomerase to incorporate the label into the cross-linked primer was assayed. Because the labeled telomeric primer is covalently attached to TERT, the ^{35}S-labeled TERT and the ^{32}P-labeled oligonucleotide comigrate on the gel (Figure 7B, lane 1). By placing film over the gel, it was possible to largely eliminate detection of the ^{35}S signal (Figure 7, compare panel B, lane 2, with panel C, lane 2). This treatment allowed clear visualization of the ^{32}P that was incorporated into the cross-linked primer by telomerase (Figure 7C, lane 1) and confirmed that the primer is cross-linked to telomerase in an active conformation.

Stem III and IV Mutations Have Small Effects on TERT Dissociation. The co-immunoprecipitation assays used to measure binding of the telomerase RNA to TERT are not quantitative, so we used a pulse−chase method to measure the dissociation rate constant (k_{off}) of the RNA−TERT complex. A telomerase complex with [^{35}S]methionine-labeled TERT and ^{32}P-labeled telomerase RNA was formed in reticulocyte lysates and then chased with an excess of unlabeled WT telomerase RNA. The amount of labeled telomerase RNA still bound to TERT was measured as a function of time by immunoprecipitation of the TERT−telomerase RNA complex. Figure 8 shows a representative experiment, and the calculated k_{off} values are given as ranges of values obtained in two or more experiments. WT RNA had the slowest dissociation rate at 0.024−0.030 min^{-1} (Figure 8, panels A and E). Deletion of stem III resulted in an approximately 2-fold increase in k_{off} with a value of 0.058−0.061 min^{-1} (Figure 8, panels B and E). This difference in k_{off} between WT and the ΔIII RNAs was not seen in the simple co-immunoprecipitation binding assay (data not shown and ref *39*). Fourfold or higher increases in k_{off} were seen upon mutation of stem IV. The ΔIV RNA and the L4GAAA mutants gave dissociation rate constants of ≥0.11−0.12 min^{-1} and ≥0.11−0.15 min^{-1}, respectively. These rates are lower estimates because at the first time point (15 min) the dissociation was largely complete. Thus, while the ΔIV and L4GAAA mutants still bind TERT, the complex is somewhat destabilized.

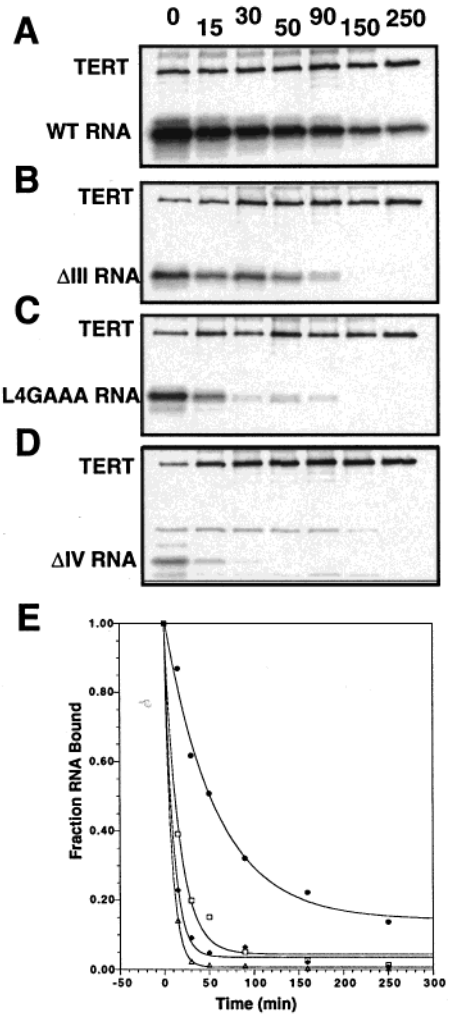

FIGURE 8: Determination of the dissociation rate constant for the complex of TERT with WT and mutant telomerase RNAs. The telomerase complex was formed in reticulocyte lysates with ^{35}S-labeled TERT and ^{32}P-labeled telomerase RNA. A 70-fold excess of cold WT telomerase RNA was then added to the reticulocyte lysate. Aliquots of the reaction were removed at the designated times (minutes), the telomerase complex was immunoprecipitated, and TERT was separated from bound RNA by SDS gel electrophoresis. Panels: (A) WT RNA, (B) ΔIII RNA, (C) L4GAAA RNA, and (D) ΔIV RNA. Additional bands in (D) are protein degradation products. The locations of TERT protein and telomerase RNA are labeled on each gel. (E) The amount of ^{32}P-labeled RNA in each lane was normalized to the amount of protein immunoprecipitated to control for differences in the immunoprecipitation efficiency and loading. To do this, the signal in each gel was quantitated by Phosphorimager, and the ratio of the TERT signal to bound RNA at 0 min was normalized to 1 as described in Materials and Methods. The fraction RNA bound was plotted versus the time following addition of cold telomerase RNA, and the data were fit to a single exponential: (●) WT RNA; (□) ΔIII RNA; (◆) L4GAAA RNA; (△) ΔIV RNA

DISCUSSION

Previous studies have shown that telomerase RNA acts as a template for telomeric DNA synthesis and as a scaffold for protein binding. In addition, regions of the *T. thermophila* and *Kluveromyces lactis* telomerase RNAs have been found to specify the template boundary for telomeric DNA synthesis (*53*, *54*) and to contribute in other ways to enzymatic function (*39*, *55*). We have identified an additional, functionally critical RNA element in *Tetrahymena* telomerase: stem−loop IV of the telomerase RNA. Loop IV is distant from the template region of the RNA and does not contribute significantly to TERT binding, yet mutations of the loop have dramatic effects on telomerase activity. These loop mutations result in misfolding of the pseudoknot region of the RNA, suggesting that stem−loop IV may play an important role in the organization of the telomerase complex. In addition, this RNA element may contribute directly to active site function.

Effect of Stem−Loop IV Mutations. Footprinting of the TERT−telomerase RNA complex showed one region of the RNA, stem IV, with strong protection from RNase V1. The remaining regions of the RNA showed either no change or enhancement of RNase V1 cleavage upon TERT binding. Protection of RNA from a nuclease upon addition of a protein can be interpreted as either a site of protein binding or a protein-induced RNA conformational change. However, it

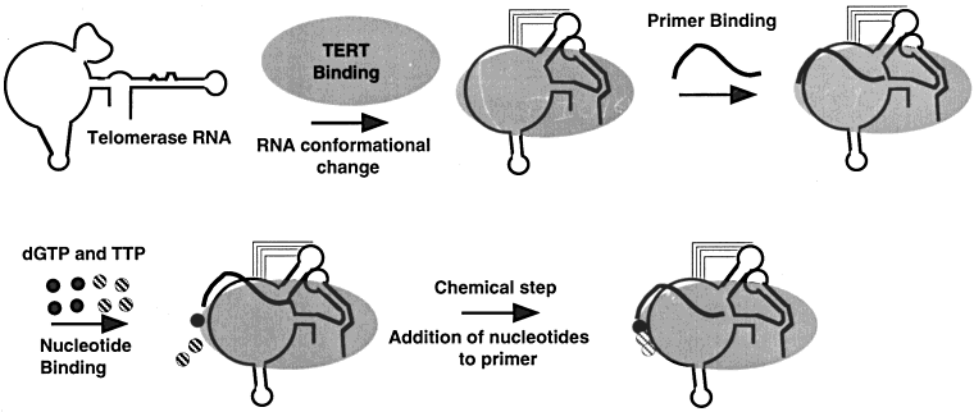

FIGURE 9: Schematic representation of telomerase complex assembly and activity. The telomerase RNA is bound by TERT, causing conformational changes in the telomerase RNA including stabilization of the stem II helix and the structure between stems II and III. The pseudoknot region folds into the phylogenetically predicted structure in a loop IV dependent manner. The particular interaction of loop IV shown here is not supported by data and is shown only to illustrate the type of interaction that may be possible. The complex binds primer at the anchor site in TERT and also base pairs with the template in the telomerase RNA. Substrate nucleotides are bound and then added to the primer in the chemical step of the reaction. We propose that loop IV plays an important role either directly or indirectly in this chemical step.

is difficult to distinguish between these two possible interpretations on the basis of footprinting experiments alone. Deletion of stem IV had only a 2-fold effect on binding of TERT in a co-immunoprecipitation binding assay and an approximately 4-fold effect on the rate of protein dissociation. Since the stem did not contribute much to protein binding, it seems unlikely that the protection seen in this region of the RNA results from direct protein binding; more likely it results from a protein-induced RNA conformational change.

Although stem−loop IV did not have a large effect on TERT binding, its deletion conferred a loss of telomerase activity. We further dissected stem−loop IV by making mutations in sequence elements that are conserved throughout the *Tetrahymena* telomerase RNAs (*28*). Loss of activity upon deletion of the conserved GA bulge in stem IV suggested that the flexibility or bending of the stem may be important for telomerase activity. The increase in the rigidity of this helix may prevent correct positioning of loop IV which, we have shown, has an important role in telomerase function. In fact, most of the telomerase activity effect of the stem IV deletion can be attributed to loop IV alone.

How could loop IV affect telomerase activity in such a manner? A clue came from looking at the RNA structure in mutants of loop IV. In loop IV mutants that have severe telomerase activity defects (including L4GAAA, C132G, U137A, and U138A), the pseudoknot no longer folds upon protein binding as occurs with WT RNA. Loop IV appears to have an important function in correctly organizing the pseudoknot region of the RNA. We have been unable to determine through cross-linking or further mutagenesis if loop IV is directly interacting with the pseudoknot. Another possibility is that loop IV is involved in correctly positioning the protein to direct folding of the pseudoknot. It will be interesting to see how the protein and loop IV work together to promote the correct conformation of this region of the RNA.

We propose that loop IV has at least one other function in the telomerase enzyme beyond promoting pseudoknot formation. Two single nucleotide substitutions in loop IV that have intermediate levels of telomerase activity support this hypothesis. These mutants have a telomerase activity defect but do not appear, within the levels of detection of the assay, to disrupt the folding of the pseudoknot. A stem II/loop IV double mutant also supports a second function of loop IV. The double mutant has telomerase activity levels more severe than the stem III mutant and similar to the loop IV single mutant. This result supports the hypothesis that loop IV has at least two separable functions: to promote the correct conformation of telomerase RNA and to function in the active site of telomerase.

Where Does TERT Bind to Telomerase RNA? Our data do not identify where TERT is binding to the telomerase RNA, because the only large protection of the RNA seen in the footprinting assay proved to be in a region not critical for binding. However, the RNA protection patterns described here can be used to exclude some portions of the RNA as likely sites of protein binding. Regions of the RNA that display TERT-specific enhancement of RNase V1 are unlikely to be binding sites for TERT, because the RNA must be accessible in order to be cleaved. These regions include the loop-proximal region of stem II, the single-stranded region between the template and pseudoknot, and the pseudoknot itself. In stem II, only nucleotides 20−24 are cleaved by RNase V1 probing. Upon protein binding, nucleotides 22−24 show an enhancement in RNase V1 cleavage, and nucleotides 20−21 show no reproducible change in cleavage pattern. This pattern is inconsistent with protein binding to the helix and instead suggests that protein binding at a different site may stabilize this helix. Licht and Collins (*39*) have shown that mutations in stem II can have large effects on TERT binding and telomerase activity in a similar reconstitution system. These phenotypes may indirectly result from disruption of stem II since we have no footprinting evidence that stem II is binding directly to TERT.

The pseudoknot region has been a candidate for a protein-binding site, based on protections seen by footprinting of the native telomerase complex in vivo with dimethyl sulfate (*43*). In addition, Gilley and Blackburn (*41*) have shown that disruption of stem IIIa disrupts the stable assembly of active telomerase in vivo. In contrast, in vitro telomerase activity and binding of telomerase RNA to TERT are not dependent

on the presence of the pseudoknot (*36*, *39*). Furthermore, the presence of in vitro telomerase activity in the absence of stem III and the observation that RNase V1 cleavage in this region is actually enhanced upon protein binding now provide convincing evidence that the pseudoknot region is not a binding site of TERT.

Taken together, these observations leave only a small number of single-stranded regions of the RNA as potential sites of protein binding: RNA 5′ of stem II (for which we have no footprinting data), between stem II and the template, between stem IIIb and stem I, and a number of one or two nucleotide stretches throughout the RNA that were not effectively probed by the nucleases used in this study. Recent studies from the laboratory of K. Collins have identified one of these regions—the nucleotides 5′ of stem II [CAUU (nucleotides 15−18)]—as critical for binding of TERT to the RNA (*39*, *42*). Their finding is confirmed in Figure 3A here.

TERT Alone Is Sufficient for Specific Telomeric Primer Binding. Primer binding was measured by assessing the degree of cross-linking of a 5-iodouracil-labeled primer to telomerase containing WT or mutant RNA. The telomerase enzyme has been hypothesized to have at least two primer binding sites: one in the RNA (the template) and a second in TERT (the anchor site) (*49*, *56*, *57*). The primer cross-linked to the TERT protein in a sequence-specific manner, regardless of the presence or absence of the telomerase RNA. Consistent with this finding, mutations in the RNA also had no effect on the ability of telomeric primer to cross-link to TERT. Indeed, mutants completely lacking the template nucleotides (and therefore expected to lack any ability to base pair with the primer) also showed no decrease in primer binding. These results provide further evidence for sequence-specific primer binding in the anchor site of TERT and suggest that, at least in vitro, the RNA supplies a minimal contribution to primer binding in *T. thermophila*.

The Role of Stem−Loop IV in Telomerase Function. The ability of telomerase to achieve catalytic function in vitro can be divided into at least four steps (Figure 9). By studying telomerase reconstituted in reticulocyte lysates, the effect of loop IV mutations on each of these steps was assessed. The first step is complex formation. Telomerase activity requires that TERT protein bind to the telomerase RNA and induce a conformational change presumably in which the pseudoknot completely forms. The next steps are binding of the telomeric DNA primer and binding of nucleotides in the active site. Last, nucleotides are added to the primer in the chemical step of the reaction. As discussed earlier, mutations in loop IV clearly affect the first step in which TERT binds to the RNA and induces specific conformational changes, but this effect cannot entirely explain the severity of the loop IV mutants. Because increasing either dGTP or TTP concentration in the telomerase reaction did not rescue activity of the C132G or A136U loop IV mutants, it is unlikely that a decrease in nucleotide affinity is responsible for the decrease in activity. The mutants are also competent to bind telomeric DNA primers; indeed, no RNA was required for specific primer binding to TERT.

On the basis of these results, we propose a model for the contribution of loop IV to the action of telomerase. Clearly, integrity of loop IV is required to facilitate large-scale changes in RNA conformation upon protein binding, especially in the pseudoknot region. In addition, we have also provided evidence for a second important function unrelated to the conformation of the pseudoknot which may be a direct effect of loop IV on the active site of telomerase. *K. lactis* telomerase RNA also contains two regions, one a stem−loop, that have been proposed to be directly involved in active site function (*55*); it is possible that the *K. lactis* and *T. thermophila* stem−loops are serving similar functions in their respective telomerases. In both cases it remains to be seen if there is a direct role of the RNA element in catalysis or if it is more indirectly involved in the organization of a functional active site.

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