

Mutational analysis of the *Tetrahymena* telomerase RNA: identification of residues affecting telomerase activity *in vitro*

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ABSTRACT

Telomere-specific repeat sequences are essential for chromosome end stability. Telomerase maintains telomere length by adding sequences *de novo* onto chromosome ends. The template domain of the telomerase RNA component dictates synthesis of species-specific telomeric repeats and other regions of the RNA have been suggested to be important for enzyme structure and/or catalysis. Using enzyme reconstituted *in vitro* with RNAs containing deletions or substitutions we identified nucleotides in the RNA component that are important for telomerase activity. Although many changes to conserved features in the RNA secondary structure did not abolish enzyme activity, levels of activity were often greatly reduced, suggesting that regions other than the template play a role in telomerase function. The template boundary was only altered by changes in stem II that affected the conserved region upstream of the template, not by changes in other regions, such as stems I, III and IV, consistent with a role of the conserved region in defining the 5' boundary of the template. Surprisingly, telomerase RNAs with substitutions or deletion of residues potentially abolishing the conserved pseudoknot structure had wild-type levels of telomerase activity. This suggests that this base pairing interaction may not be required for telomerase activity *per se* but may be conserved as a regulatory site for the enzyme *in vivo*.

INTRODUCTION

In *Tetrahymena* and yeast altering telomere sequences by expression of a mutated telomerase RNA template results in deregulation of telomere length and eventual cell death. Deletion of *TLC1*, the gene encoding the *Saccharomyces cerevisiae* telomerase RNA, results in telomere shortening and cell death. Thus telomerase is critical for telomere length maintenance (1–3). The RNA component of telomerase is best characterized in ciliates, where it has been cloned from 24 species (4; reviewed

in 5). The telomerase RNAs of different ciliate species range from 148 to 209 nt in length and contain short sequences of 9–15 nt that are complementary to the species-specific telomeric repeats. For example, *Tetrahymena* telomeres consist of TTGGGG repeats and the telomerase RNA contains the sequence 5'-CAACCCCAA-3'. With the exception of the template region and a conserved sequence adjacent to the template, the ciliate telomerase RNAs share little primary sequence identity (4,6,7).

To identify potential functional domains in the *Tetrahymena* telomerase RNA, a secondary structure model was derived from phylogenetic sequence comparison of telomerase RNAs from different tetrahymenine ciliates (8,9). A secondary structure for the *Tetrahymena* telomerase RNA was proposed that includes four conserved helices, numbered 5'→3' as helices I–IV (Fig. 1). In the proposed structure helix I involves long range base pairing, establishing the superstructure of the RNA, and is the most conserved of the helices. Helices II, III and IV are stem-loop structures. Conservation of stem-loop structures is often indicative of protein binding domains (10,11). Helix II is not conserved in all ciliates, however, upstream of the template there is a conserved sequence, 5'-(CU)GUCA-3', which plays a role in defining the 5' boundary of the template domain (7,9,12). An unstructured region is predicted between helices II and III and includes the template sequence. Nucleotides in the loop of helix III can base pair with a single-stranded region just upstream of the helix III stem, to form a pseudoknot (13). Pseudoknot structures are also often involved in the recognition and binding of proteins (14,15). Helix IV has the highest primary sequence conservation of the stem-loops. It was proposed that the conserved nucleotides of helix IV could be involved in alternative base pairing that might help mediate a conformational change during the translocation step of telomerase substrate elongation (8).

Chemical modification studies of the *Tetrahymena thermophila* and *Glaucoma chattoni* telomerase RNAs *in vivo* and *in vitro* support the phylogenetically derived secondary structure model, although some differences are seen between naked RNA and the RNP complex (16,17). Structural probing of the naked RNA of helix III suggests that this region is in an equilibrium favoring one of two helical structures. Conformational change between

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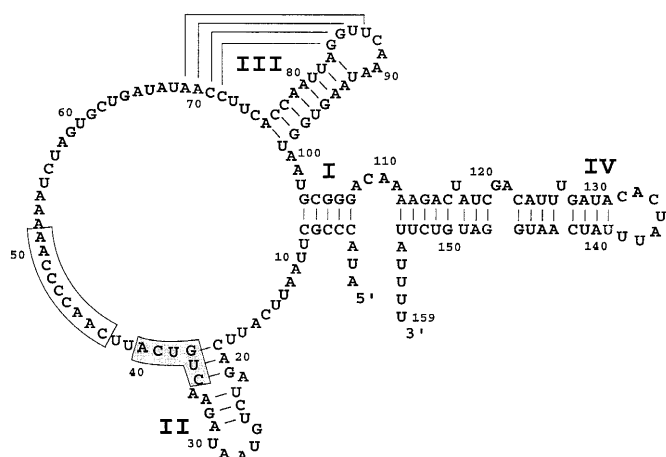


Figure 1. Diagram of the secondary structure of the *Tetrahymena* telomerase RNA, including the pseudoknot structure (brackets) (8,13). The telomerase RNA sequence is shown with functional domains, including the template and alignment regions 5'-CAACCCCAA-3' (residues 43–51, white box) and the upstream conserved region 5'-(CU)GUCA-3' (residues 35–40, shaded box), which regulates the 5' boundary of the template (7,12,20). The 159 nt *Tetrahymena* telomerase RNA is marked 5'→3' every 10 residues.

pseudoknot and hairpin states might be relevant to the enzymatic action of telomerase (7). A structurally conserved kink at the GA bulge in helix IV has been proposed to serve as a recognition motif for protein binding (16). Comparison of methylation patterns of protein-free telomerase RNA to that of RNA complexed with protein implicated protein binding for the hairpin loop of stem III adjacent to the pseudoknot, the GA bulge in stem IV and three sites around residues 15 and 16, 39 and 62 (17).

Telomerase RNAs from hypotrichous ciliates and *Paramecium* species can be folded into a conserved secondary structure similar to the derived *Tetrahymena* structure (4,6,7). Telomerase RNAs from human, mouse and the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* are longer and secondary structure predictions await phylogenetic comparisons and structural probing of the mammalian and yeast telomerase RNAs (2,3,18,19).

In *Tetrahymena* and human, *in vitro* reconstitution of telomerase activity from isolated protein and mutated RNA components has been instrumental in identification of functional domains of the telomerase RNA (12,20,21). To address the function of the conserved secondary structures of the telomerase RNA we analyzed the activity of *Tetrahymena* telomerase reconstituted *in vitro* with telomerase RNAs containing deletions or substitutions in specific secondary structures.

MATERIALS AND METHODS

Purification of oligonucleotides

Oligonucleotides were synthesized by Operon Technologies (Alameda, CA) or by the oligonucleotide facility at Cold Spring Harbor Laboratory. Oligonucleotides were purified as previously described (20) and concentrations were determined spectrophotometrically assuming 1 OD₂₆₀ unit equals 20 µg/ml.

Preparation of *Tetrahymena* telomerase

Tetrahymena telomerase was purified using a protocol modified from Collins *et al.* (21). *Tetrahymena* strains CU428 (kindly provided by Martin Gorovsky) were grown with shaking at 30°C to a density of 4.0×10^5 cells/ml in 2 × 36 l medium (2% proteose peptone, 0.2% yeast extract, 10 µM FeCl₃) supplemented with 250 µg/l both ampicillin and streptomycin. After harvesting the cells were resuspended in Dryls starvation medium (1.7 mM sodium citrate, 2.4 mM sodium phosphate, 2 mM CaCl₂) and incubated at 30°C for 18 h. Cells were harvested and resuspended in 2× the volume of the cell pellet of T2MG (20 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 10% glycerol) with 5 mM β-mercaptoethanol (β-me), 0.1 mM PMSF and protease inhibitors (0.25 µg/ml each leupeptin and pepstatin; Sigma). Lysis was enhanced by stirring the cells in this buffer at 4°C for 20 min in the presence of 0.2% NP-40 (Sigma). The lysed cells were centrifuged at 40 000 r.p.m. for 1 h in a Ti45 rotor (Beckman) at 4°C and the supernatant (S130 extract) collected and stored at –70°C after quick freezing in liquid nitrogen.

DEAE-purified telomerase was prepared as follows. S130 extract (255 ml, 1900 mg protein) was loaded onto a 150 ml ceramic hydroxyapatite (AIC) column equilibrated in T2MG with β-me and protease inhibitors as described above. Proteins were eluted with a 380 ml gradient to 0.2 M K₂HPO₄ in T2MG. The ceramic hydroxyapatite column was regenerated and equilibrated in T2MG and the second S130 fraction (225 ml, 2700 mg protein) loaded onto the column. Similarly, proteins were eluted with a 380 ml gradient to 0.2 M K₂HPO₄ in T2MG. Fractions containing maximal telomerase activity from the first ceramic hydroxyapatite column (580 mg) were loaded, after diluting 3-fold with T2MG, onto an 18 ml spermine-agarose (Sigma) column equilibrated in T2MG with 0.15 M potassium glutamate (Kglu). Proteins were eluted in T2MG with 0.65 M Kglu. Fractions containing maximal telomerase activity from the second ceramic hydroxyapatite column (95 mg) were loaded, after diluting 3-fold with T2MG, on a 3 ml spermine-agarose column as before. Proteins were eluted in T2MG with 0.65 M Kglu. Fractions with maximal telomerase activity from both spermine-agarose columns were pooled (60 ml, 48 mg) and loaded onto a 6 ml phenyl-Sepharose (Pharmacia) column equilibrated in T2MG with 0.6 M Kglu. Proteins were eluted in T2MG without salt and in T2MG with 1% Triton X-100. Fractions containing maximal telomerase activity in the no salt elution (45 ml, 6 mg) were loaded, after adjusting to no salt, onto a 2 ml DEAE-agarose (BioRad) column equilibrated in T2MG. Protein was eluted in T2MG with 0.4 M Kglu. The peak of telomerase was in fraction 3 (2 ml, 4 mg). For the experiments in this study fraction 4 was used (2 ml, 2.2 mg). DEAE-purified telomerase (1.1 mg protein/ml extract) was diluted 5-fold with T2MG before use in reconstitution reactions. The extract preparations remained active in elongation assays and in reconstitution assays for at least 24 months when stored at –70°C. Protein concentrations were determined by a Bradford assay with BioRad dye reagent.

Telomerase elongation activity assay

Telomerase assays were done as previously described (23). Briefly, 20 µl extract were added to 20 µl 2× reaction mix. The final concentrations of the components in the assay were 1× telomerase buffer (50 mM Tris-HCl, pH 8.5, 1 mM spermidine, 5 mM β-me and 50 mM KOAc), 800 nM primer oligonucleotide (0.2 µg),

100 μ M dTTP and 0.3125 μ M [α - 32 P]dGTP (1 μ l 800 Ci/mmol; NEN). In assays with telomerase reconstituted with certain mutated RNAs, reaction mixes contained 100 μ M both dTTP and dATP. In reactions with dideoxynucleotides ddTTP (100 μ M) replaced dTTP. Reaction times were 1 h at 30°C. Reactions were stopped by adding 100 μ l 21 mM EDTA, 10 mM Tris-HCl, pH 7.5, and 1.5 μ g/ml DNase-free RNase (Boehringer Mannheim). After phenol extraction 60 μ l 2.5 M NH_4OAc , 100 μ g/ml *Escherichia coli* tRNA (Sigma) and ethanol were added to the reactions and precipitated overnight at -20°C. Products were centrifuged, pellets dried and resuspended in formamide containing xylene cyanol. Samples were boiled, cooled on ice and loaded onto 8% polyacrylamide-7 M urea gels and electrophoresed at 1500 V for 2 h using 0.6 \times TBE buffer. Gels were dried, exposed to Fuji PhosphorImager screens for 16-24 h and then exposed to film (XAR5) for 10 days, unless otherwise indicated in the figures. Products were quantified by comparing the signal intensity for the first repeat in each lane using a BAS2000 PhosphorImager. The signal intensity in the no RNA lane was subtracted as background.

Micrococcal nuclease treatment and reconstitution assay conditions

DEAE-purified *Tetrahymena* telomerase extract was incubated with 0.1 U/ μ l extract micrococcal nuclease (MNase; Pharmacia) and 1 mM CaCl_2 for 10 min at 30°C. The MNase was inactivated by adding 2.5 mM EGTA. MNase was prepared as previously described (20). Under standard reconstitution conditions MNase-treated telomerase extract (~0.2 mg/ml protein) containing no detectable telomerase activity was incubated with 5 mM EDTA and 200 ng *in vitro* transcribed telomerase RNA per 20 μ l extract. Incubation was for 5 min at 37°C. One microliter of 200 mM MgCl_2 was added prior to assaying for elongation activity.

Site-directed mutagenesis

Using a methodology similar to that previously described (20) or using site-directed mutagenesis by overlap extension (24) plasmids containing mutated versions of the *Tetrahymena* telomerase RNA gene were constructed. Oligonucleotides with sequences corresponding to mutations at the desired positions in the RNA gene were synthesized and used in PCR, with pT7159 digested with *Eco*RI and *Hind*III as template (20) or pCG1 digested with *Hind*III as template (25).

For mutations at the 5'-end of the RNA, Δ 5'8, the oligonucleotide at the 3'-end was 3'T7PCR and the template used was pT7159. The sequence of the 3'T7PCR oligonucleotide was 5'-CAGTGAATTCGAGCTCGGTACCCGGGG-3', which includes an *Eco*RI restriction site. For mutations at the 5'-end of the RNA, including Δ 5'11, Δ 5'15, Δ 5'19, Δ 5'2525, Δ 5'36, sub4-8 and sub15-16, the oligonucleotide at the 3'-end was CG1*Bam*HI and the template used was pCG1. The sequence of CG1*Bam*HI was 5'-CCCCGGATCCAAAAAATAAGACATCCATTG-3', which includes a *Bam*HI restriction site. The sequences of the mutation-specific oligonucleotides were 5'-AGGGTCTAGATAATACGACTCACTATAGGGTAAATTCATTG-3' (d58), 5'-GGGGTCTAGATAATACGACTCACTATAGGGATTCAATCAGATCTGTAATAG-3' (d511), 5'-GGGGTCTAGATAATACGACTCACTATAGGGATTGATCTGTAATAGAACT-3' (d515), 5'-GGGGTCTAGATAATACGACTCACTATAGGGATCTGTAATAGAACTGTC-3' (d519), 5'-GGGGTCTAGATAATACGACTCACTATAGGGGTAATAGAACTGTCATTCAA-3' (d525), 5'-GGGGTCTAGATAATACGACTCACTATAGGGTCAATCAACCCCAAAATC-3' (d536), 5'-GGGGTCTAGATAATACGACTCACTATAGGGGATAAAACATTAATTCATCAGATCTGT-3' (S1AAACA), 5'-GGGTCTAGATAATACGACTCACTATAGGGGATACCCGCTTAATTGTTTCAGA-3' (1516GT) and include a *Xba*I restriction site.

For mutations at the 3'-end of the RNA, including t146, t138, t111 and t75, the 5' oligonucleotide used was 5'T7 and the template used was either pT7159 (t146) or pCG1 (t138, t111 and t75). The sequence of 5'T7 was 5'-GGGGTCTAGATAATACGACTCACTATAGGGGATACCCGCTTAATTCATTCA-3' and includes a *Xba*I restriction site. The sequences of the mutation-specific oligonucleotides were 5'-CCCCGAATTCATTGATAAATAGTGTA-3' (d313), 5'-CCCCGGATCCAATAGTGTATCAAATGTCTGA-3' (d321), 5'-CCCCGGATCCTTGTCCTCCGATTACCACTTA-3' (d348) and 5'-CCCCGGATCCGAAGGTTATATCAGCAC-TAG-3' (d384) and include an *Eco*RI restriction site (d313) or a *Bam*HI restriction site (d321, d348 and d384). PCR amplification was performed in a 100 μ l volume in the presence of 1 μ M each primer and 1 ng template DNA. Five units of Taq polymerase (Perkin-Elmer Cetus) were used in 1 \times PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl_2) and 250 μ M dNTPs. Reactions were cycled 30 times for 1 min at 94°C, 1 min at 46°C and 1 min at 72°C.

The PCR-amplified DNAs were cleaved with either *Xba*I and *Eco*RI and cloned into pT7159 cleaved with the same enzymes or *Xba*I and *Bam*HI and cloned into pUC119 cleaved with the same enzymes, using conventional cloning techniques (26). The resulting clones (listed below) contained the mutated versions of the telomerase RNA gene downstream of the T7 promoter, as confirmed by sequencing both strands of the inserted DNA by the dideoxy-mediated chain termination method as per the manufacturer's instructions (US Biochemical).

For the mutated genes constructed by overlap extension two fragments per mutated gene were initially generated using similar PCR conditions as described above. For all PCR reactions, except those noted below, the template used was pCG1 digested with *Hind*III. The equivalent of 0.1 μ l (~10 ng PCR product) of the two 100 μ l PCR reactions were then combined, in a second PCR, using the outermost 5' and 3' oligonucleotides used in the first two PCR reactions (generally 5'T7 and CG1*Bam*HI, except where noted below) to construct the desired mutated gene. The PCR-amplified DNAs were cleaved with *Xba*I and *Bam*HI and cloned into pUC119 cleaved with the same enzymes. The resulting clones were sequenced as described above.

For the mutation Δ 20-36, primers 5'T7 and d2036b were used with pT7159 digested with *Xba*I and *Bam*HI as template. Primers d2036c and CG1*Bam*HI were used to generate the second fragment. For sub26-29 the template used was pT7159 digested with *Hind*III and *Eco*RI. The primers used in the first step were 5'T7159*Xba*I and 26CATTb and 26CATTc and CG1*Bam*HI. In the second step primers 5'T7159*Xba*I and CG1*Bam*HI were used. For Δ 37-40 in the first step PCR primers 5'T7 and d3740b were used to generate one fragment and primers d3740c and CG1*Bam*HI were used to generate the second fragment. For sub 69-72 the oligonucleotides used were 5'T7 and 69TTGGb and 69TTGGc and CG1*Bam*HI. For Δ 76-99 primers 5'T7 and d7699b were used to generate one fragment and d7699c and CG1*Bam*HI were used to generate the second fragment. For sub84-87 primers 5'T7 and S38487b were used for one fragment

and S38487c and CG1*Bam*HI for the other. For sub89–90, primers 5'T7 and 89TTb and 89Ttc and CG1*Bam*HI were used. For the mutation Δ103–107, in the first step PCR, primers 5'T7 and d103107b were used for one fragment and primers d103107c and CG1*Bam*HI were used for the other fragment. For sub121–122 primers 5'T7 and 121CTb were used as well as 121CTc and CG1*Bam*HI. For sub133–136 the template used was pT7159 digested with *Hind*III and *Eco*RI. The primers were 5'T7159*Xba*I and 133TGATb and 133TGATc and 3'T7PCR. In the second step 5'T7159*Xba*I and 3'T7PCR were used. The sequences of the primers were 5'-GTTGAATGACGAATGAATTAAGCGGGT-AT-3' (d2036b), 5'-TAATTCATTCGTCATTCACCCCAA-ATC-3' (d2036c), 5'-GACAGTTCTAAATGAGATCTGA-AT-3' (26CATTb), 5'-ATTCAGATCTCATTTAGAACTGTC-3' (26CATTc), 5'-TGGGGTTGAAAGTTCTATTACAGATCTG-AA-3' (d3740b), 5'-TAATAGAACTTTCAACCCCAAAAAT-CTAGT-3' (d3740c), 5'-AATTGGTGAACCAATATCAGC-AC-3' (69TTGGb), 5'-GTGCTGATATTTGGTTCACCAATT-3' (69TTGGc), 5'-GTCCCGCATTGAAGGTTATATCAGCAC-TAG-3' (d7699b), 5'-TATAACCTTCAATGCGGGACAAAAG-ACTAT-3' (d7699c), 5'-CCACTTATTTGTTGGTAATTGGT-GA-3' (S38487b), 5'-TCACCAATTACCAACAAATAAGTGG-3' (S38487c), 5'-TTACCACTTATAAGAACCTAATT-3' (89TTb), 5'-AATTAGGTTCTTATAAGTGGTAA-3' (89Ttc), 5'-AGTCT-TTTGTATTACCACTTATTTGAACCT-3' (d103107b), 5'-AAGT-GGTAATACAAAAGACTATCGACATTT-3' (d103107c), 5'-TG-TATCAAATGAGGATAGTCTTT-3' (121CTb), 5'-AAAGAC-TATCCTCATTTGATACA-3' (121CTc), 5'-CCATTGATAAAAT-CAGTATCAAATG-3' (133TGATb), 5'-CATTTGATACTGATTT-TATC-AATGG-3' (133TGATc), 5'-GGGGTCTAGATAATACG-AC-3' (5'T7159*Xba*I).

Preparation of RNAs

RNAs used in reconstitution assays were *in vitro* transcribed with T7 RNA polymerase (Stratagene) using pT7159 and p5'8 digested with *Fok*I, pt111 and pt138 digested with *Bam*HI, pt146 digested with *Eco*RI, p4-8AAACA, pΔ5'11, pΔ5'15, p15-16GT, pΔ5'19, pΔ20-36, pΔ5'25, p26-29CATT, pΔ5'36, pΔ36-40, p69-72TTGG, pt75, pΔ76-99, p84-87CCAA, p89-90TT, pΔ103-107, p121-122CT and p133-136TGAT digested with *Xba*I and *Bam*HI as templates. p38-40AGT was prepared as previously described (12). Standard *in vitro* transcription reaction conditions recommended by the T7 RNA polymerase manufacturer were used. The transcription reactions were treated with 3 U/μg DNA RNase-free DNase (Pharmacia) for 10 min. The RNA concentrations were determined by fluorimetry. The integrity and size of the RNAs were determined by Northern analysis or staining with ethidium bromide.

RESULTS

Deletional analysis of the 5'-end of telomerase RNA

To study the effect of RNA mutations on telomerase activity we analyzed both the efficiency and the pattern of elongation products produced by the enzyme *in vitro*. *Tetrahymena* telomerase can processively elongate telomeric substrates generating a distinct 6 nt banding pattern or periodicity (23,27). *Tetrahymena* telomerase activity can be reconstituted with wild-type and mutated telomerase RNA (12,20). To address the function of telomerase RNA residues at the 5'-end of the telomerase RNA, including stems I

Table 1.

Mutant RNAs ^a	Structure or sequence targeted by mutation	Specific nucleotide changes	% Telomerase activity ^b	Cleavage activity ^c
wild-type	none	none	100 (10)	+
sub4-8	stem I	CCCGC to AAACA	35.7 ± 17.8 (3)	+
Δ5'8 (Δstem I)	stem I	none	<10* (4)	+
Δ5'11	stem I	none	19.3 ± 5.0 (3)	+
Δ5'15	stem I	none	0 (2)	n.a.
sub15-16	nucleotides 15-16	CA to GU	38.2 ± 8.4 (2)	+
Δ5'19	stem I	none	0 (3)	n.a.
Δ20-36 (Δstem II)	stem II	none	25.1 ± 23.8 (2)	+
Δ5'25	stem I-II	none	0 (3)	n.a.
sub26-29	stem II	GUAA to CAUU	140.6 ± 52.7 (4)	+
Δ5'36	stem I-II	none	0 (3)	n.a.
Δ37-40	conserved region (stem II)	none	12.6 ± 11.9 (2)	+
sub69-72 (subψ)	pseudoknot (ψ)	AACC to UUGG	52.6 ± 27.0 (4)	+
Δt75 (Δstem III-IV)	stem III-IV	none	<10 (5)	n.d.
Δ76-99 (Δstem III)	stem III	none	63.5 ± 42.2 (5)	+
sub84-87 (subψ)	pseudoknot (ψ)	GGUU to CCAA	123.8 ± 48.2 (4)	+
sub89-90	stem III loop	AA to UU	114.8 ± 46.8 (4)	+
Δ103-107 (Δstem I)	stem I	none	34.3 ± 8.3 (3)	+
Δt111 (Δstem IV)	stem IV	none	<10 (3)	+
sub121-122	stem IV (GA bulge)	GA to CU	92.6 ± 35.6 (4)	+
sub133-136	stem IV loop	ACUA to UGAU	14.3 ± 10.9 (2)	+
t138	stem IV	none	<10 (2)	+
t146	stem IV	none	<10 (6)	+

^aNomenclature of the mutant *Tetrahymena* telomerase RNAs: for example Δ5'8 is a deletion of residues 1–8 from the 5'-end of the RNA; sub, substitution t, truncation.

^bTelomerase activity of *Tetrahymena* telomerase reconstituted with the mutant RNAs relative to reconstitution with wild-type RNA indicated as a percentage ± SD, with the number of times assayed and quantitated in parentheses.

^cTelomerase activity of *Tetrahymena* telomerase reconstituted with the mutant RNAs or wild-type assayed using the oligonucleotide d(G₃T₂G)₃.

*For some mutants with weak activity signal intensities were between 1 and 10%: after subtraction background values were often zero. However, these mutants clearly generated telomerase repeats, so activity was scored as <10%.

n.a., no telomerase activity.

n.d., not determinable because activity was too weak.

and II, terminal deletions of a specific number of residues were constructed (Δ5'8, Δ5'11, Δ5'15, Δ5'19, Δ5'25, Δ5'36). All *Tetrahymena* telomerase RNA mutations constructed and discussed in this paper are listed in Table 1. The numbers in the mutation names refer to the starting position of the alteration within the 159 nt *Tetrahymena* telomerase RNA. Assays were performed *in vitro* using telomerase reconstituted with a fixed amount (200 ng) of these mutated RNAs and the products were compared with reconstituted wild-type activity (Fig. 2). Products were quantified by comparing the signal intensity for the first repeat in each lane using a BAS2000 PhosphorImager (Table 1 and Fig. 6). Comparison of the signal intensity for all repeats gave similar results, however, because of the high background in certain lanes, quantification of the signal intensity for the first repeat was more reproducible. The signal intensity in the no RNA lane was subtracted as background. For some mutated telomerases with weak activity signal intensities were between 1 and 10% of the signal intensity of the elongation products of telomerase reconstituted with the wild-type telomerase RNA. After subtraction of the background, values were often zero. This is due to the fact that there were differing background signals in the different reactions. However, these mutated enzymes clearly generated telomerase repeats, so activity was scored as <10%. The activity reconstituted by each mutated enzyme was variable. However, within each experiment the mutated telomerases gave the same relative activity levels compared with each other and with wild-type (see Figs 2–6 and data not shown).

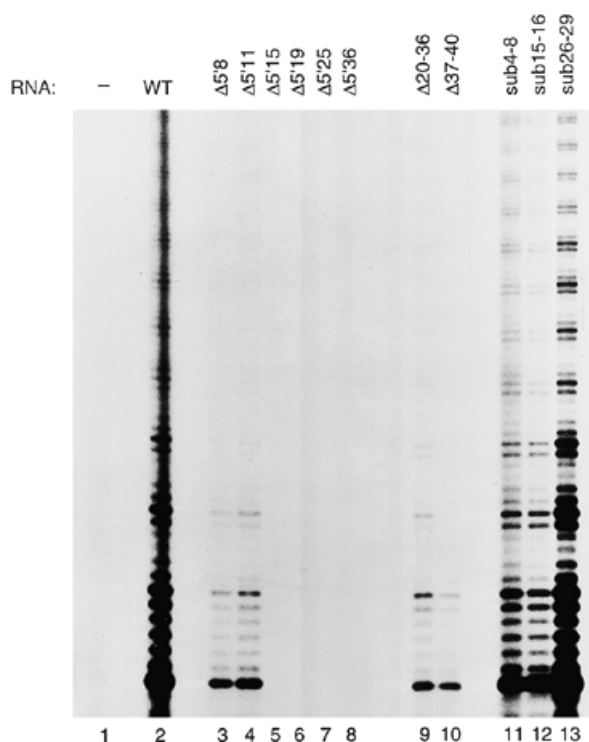


Figure 2. Reconstitution of telomerase activity of telomerase RNAs with altered 5' sequences. Elongation products of telomerase reconstituted with the indicated mutated RNAs and assayed in the presence of [α - 32 P]dGTP and dTTP and the telomeric primer d(GGGGTT)₃. The RNAs used were no RNA (lane 1), WT (lane 2), Δ 5'8 (lane 3), Δ 5'11 (lane 4), Δ 5'15 (lane 5), Δ 5'19 (lane 6), Δ 5'25 (lane 7), Δ 5'36 (lane 8), Δ 20-36 (lane 9), Δ 37-40 (lane 10), sub4-8 (lane 11), sub15-16 (lane 12) and sub26-29 (lane 13).

Terminal deletions of eight and 11 residues at the 5'-end of the RNA decreased reconstituted activity to <20% of wild-type reconstituted activity (Table 1). Deletion of eight residues reproducibly resulted in less activity than deletion of 11 residues. Deletion of 15-36 residues abolished telomerase activity *in vitro*, suggesting that some or all residues between 11 and 15 may be important for activity (Fig. 2). To specifically address the role of the paired bases of stem I residues 4-8 were mutated from CCCGC to AAACA (sub4-8, sub refers to substitution). In most of the *Tetrahymena* telomerase RNA substitutions made and discussed in this paper purines were replaced by pyrimidines and pyrimidines by purines (see Materials and Methods). Deletion of stem I (Δ 5'8) reduced activity to <10% and substitution of residues 4-8 of stem I (sub4-8) reduced activity to 35% of the level from enzyme reconstituted with wild-type RNA (Fig. 2). Also, to address the role of the paired bases of stem I residues 103-107 were deleted (Δ 103-107). Consistent with substitution of residues 4-8 in stem I, deletion of residues 103-107 decreased activity to 34% of reconstituted wild-type activity (Fig. 3). These changes did not abolish activity, suggesting that the long range base pairing may be important but not essential for telomerase function.

Methylation analysis implicated residues 15 and 16 in protein binding or protein-induced RNA structure formation (17). To determine whether such interactions might be sequence specific, C15-A16 were changed to G15-U16 (sub15-16). This substitution

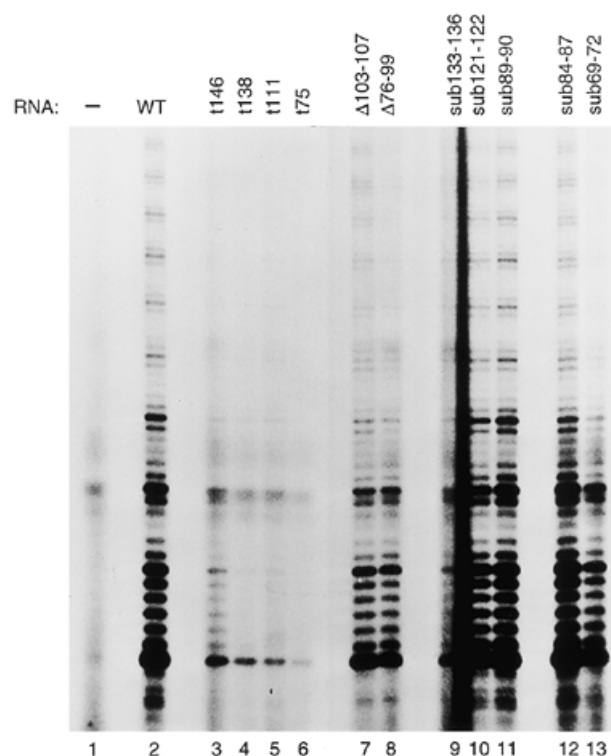


Figure 3. Reconstitution of telomerase activity of telomerase RNAs with altered 3' sequences. Elongation products of telomerase reconstituted with no RNA (lane 1), WT (lane 2), t146 (lane 3), t138 (lane 4), t111 (lane 5), t75 (lane 6), Δ 103-107 (lane 7), Δ 76-99 (lane 8), sub133-136 (lane 9), sub121-122 (lane 10), sub89-90 (lane 11), sub84-87 (lane 12) and sub69-72 (lane 13). Elongation reactions were carried out in the presence of [α - 32 P]dGTP and dTTP and the telomeric primer d(GGGGTT)₃.

decreased telomerase activity to 38% of wild-type, suggesting that these residues play a role in telomerase function but that the specific nucleotides may not be essential for activity *in vitro* (Fig. 2).

To test the role of stem II and the adjacent conserved region the entire stem (Δ 20-36) and, independently, residues 37-40 were deleted. Deletions of stem II and the conserved region decreased activity to 25 and 13% respectively, further supporting the role of this region in telomerase template function (Fig. 2; 7,12). To determine whether the loop of stem II was involved in sequence-specific interactions, residues 26-29 were mutated from GUAA to CAUU (sub26-29). Substitution of these residues in the loop of stem II did not decrease telomerase activity (Fig. 2). In fact, telomerase activity of this mutated enzyme was increased compared with telomerase activity of telomerase reconstituted with wild-type telomerase RNA.

Deletional analysis of the 3'-end of telomerase RNA

To address the function of telomerase RNA residues at the 3'-end of the RNA, including stems III and IV, terminal deletions of a specific number of residues at the 3'-end were constructed (t146, t138, t111 and t75, t refers to truncation). To address the role of the residues in the loop of stem IV residues 133-136 were changed from ACUA to UGAU (sub133-136). Telomerase assays were performed with these mutated RNAs and the level of products were compared with reconstituted wild-type telomerase

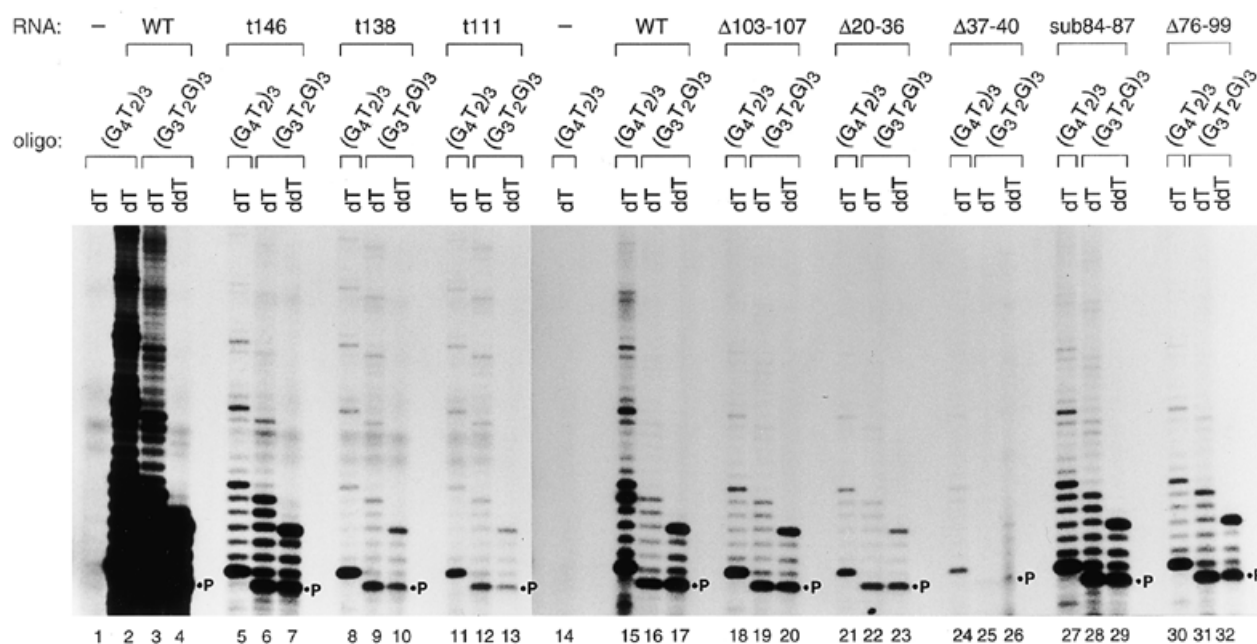


Figure 4. Reconstitution of telomerase cleavage activity of mutated telomerase RNAs. Telomerase was reconstituted with no RNA (lanes 1, 14), WT (lanes 2–4, 15–17), t146 (lanes 5–7), t138 (lanes 8–10) or t111 (lanes 11–13) telomerase RNA. Telomerase was reconstituted with $\Delta 103$ –107 (lanes 18–20), $\Delta 20$ –36 (lanes 21–23), $\Delta 37$ –40 (lanes 24–26), sub84–87 (lanes 27–29) or $\Delta 76$ –99 (lanes 30–32). Elongation reactions were performed using primer d(GGGTTG)₃ or d(GGGTTG)₃ as indicated in the presence of [α -³²P]dGTP and dTTP or [α -³²P]dGTP and ddTTP. Labeled input primer indicative of cleavage activity is indicated by P. The gels were exposed to film for 11 (lanes 1–13) or 4 days (lanes 14–32).

activity levels (Fig. 3). Deletions of as few as 13 residues at the 3'-end (t146), as large as the entire stem IV (t111) and substitution of residues in the loop region of stem IV (sub133–136) decreased activity to <15% of wild-type, indicating that stem IV is a functionally important structure (Table 1 and Fig. 3). It has been proposed that the structurally conserved kink introduced by the GA bulge in helix IV (residues 121 and 122) may serve as a recognition motif for protein binding (16). To test whether such an interaction might be sequence specific the GA residues were changed to CU (sub121–122). This substitution of the GA bulge did not decrease telomerase activity (Fig. 3).

To examine the role of stem III the entire stem, including residues 84–87, proposed to form a pseudoknot structure, was deleted ($\Delta 76$ –99). Surprisingly, this deletion retained 63% of the activity of wild-type reconstituted enzyme, while deletion of stems I, III and IV (t75) almost completely abolished activity (Fig. 3). Methylation protection analysis implicated residues 89 and 90 in protein binding or protein-induced RNA structure formation (17). When these two positions were changed from adenine to uracil residues (sub89–90) there was no decrease, but a slight increase, in the level of enzyme activity compared with wild-type. In addition, two changes were made in stem III that were predicted to disrupt pseudoknot formation (69–72 and 84–87) and/or protein interaction (69–72) (17). The substitution of GGUU for CCAA (sub84–87) should abolish base pairing interaction of the pseudoknot, yet did not decrease activity from wild-type levels. In fact, activity was increased. The other sequences involved in pseudoknot formation have also been proposed to interact with a protein component based on methylation protection (17). Substitution of residues 69–72 (sub69–72) decreased activity to 53% (Fig. 3). The effects of

these changes are consistent with 63% activity levels on complete deletion of stem III ($\Delta 76$ –99) (Table 1) and indicate that the pseudoknot may not be essential for processive elongation by telomerase *in vitro*.

Role of specific secondary structures in telomerase-mediated cleavage activity

Tetrahymena telomerase possesses a specific nucleolytic activity that can remove the terminal dG from primers containing the telomeric repeat d(GGGTTG)_n at the 3'-end (28). The G residue at the 3'-end of these primers can align with the C residue at the extreme 5'-end of the RNA template, where cleavage is thought to occur. During cleavage the 3'-terminal G residue is removed and [α -³²P]dGTP is added, generating a labeled primer-sized product. The primer specificity of the telomerase cleavage reaction is reconstituted with synthetic telomerase RNA and cleavage activity can be abolished by specific mutations in the template and the upstream conserved region of the telomerase RNA (12,20).

To determine whether regions outside the template and the upstream conserved regions play a role in cleavage, cleavage activity was tested using telomerase reconstituted with RNAs mutated in all of the stem-loop structures (Fig. 4 and Table 1). All mutated telomerases tested which had high enough levels of telomerase to test for cleavage had cleavage activity. The primer-sized product that results from cleavage is indicated with a P in Figure 4. The intensity of the cleavage product varied widely between mutated telomerases and appeared to parallel the levels of telomerase activity. The level of reconstituted activity was similar using two different telomeric primers, d(GGGTTG)₃ and d(GGGTTG)₃ (Fig. 4).

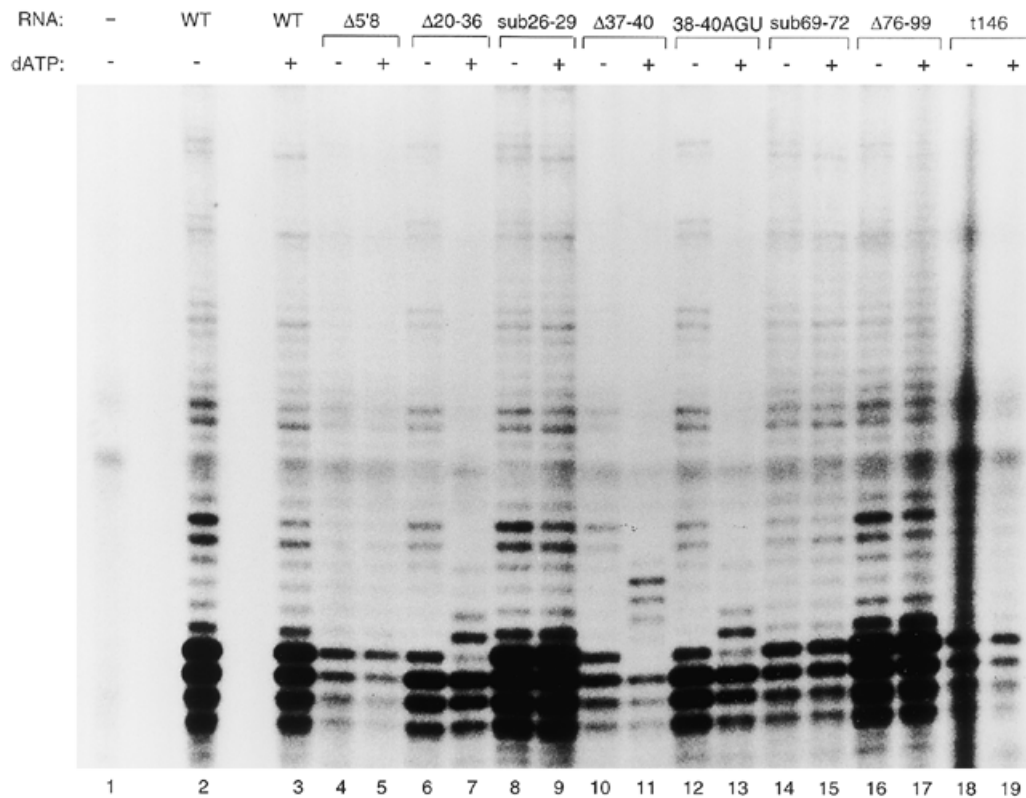


Figure 5. Sequences in stem II and the upstream conserved region, not stems I, III or IV, define the 5' template boundary. Telomerase was reconstituted with no RNA (lane 1), WT (lanes 2 and 3), $\Delta 5'8$ (lanes 4 and 5), $\Delta 20-36$ (lanes 6 and 7), sub26-29 (lanes 8 and 9), $\Delta 37-40$ (lanes 10 and 11), 38-40AGU (lanes 12 and 13), sub69-72 (lanes 14 and 15), $\Delta 76-99$ (lanes 16 and 17) or t146 (lanes 18 and 19). Elongation reactions were performed using primer d(GTTGGG)₃ with [α -³²P]dGTP and dTTP or with [α -³²P]dGTP, dTTP and dATP as indicated in the figure.

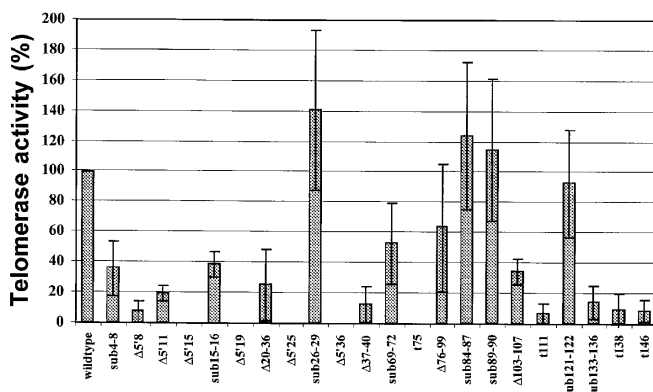


Figure 6. Bar graph representation of the telomerase activity reconstituted by the different mutated RNAs compared with the telomerase activity reconstituted by wild-type telomerase RNA. The error bars represent the standard deviations.

DideoxyTTP was used to determine how many residues were added onto the primer substrates. Because the sequence at the 3'-end of telomeric substrates specifies the first nucleotide added in elongation reactions with telomerase (20,23) the site of termination can be predicted. For all mutated RNAs tested, as for wild-type RNA, ddTTP incorporation occurred four residues from the 3'-end of the primer d(GGGTTG)₃ (Fig. 4). The ability of all the mutated telomerase RNAs tested to reconstitute telomerase cleavage activity

and incorporate ddTTP at the same position from the 3'-end of the primers used suggests that none of the mutations affect alignment of the cleavage substrate with the template.

Stem II and the conserved region, not stems I, III or IV, regulate the 5' template boundary

In wild-type *Tetrahymena* telomerase RNA there are two U residues that separate the template domain from the upstream conserved region (Fig. 1). Unlike wild-type telomerase, telomerase reconstituted with a mutated RNA in the upstream conserved region (38-40AGU) will copy past the template domain and incorporate dATP into elongation products. This results in a change in the pattern of the elongation products, compared with the pattern of elongation products generated in the absence of dATP (12). The incorporation of dATP into elongation products occurs due to utilization of the U residues 5' of the template domain as template residues. To determine whether sequences or structures other than the conserved region play a role in regulation of the 5' template boundary, telomerase enzymes reconstituted with RNAs mutated in stems I-IV were assayed in the presence or absence of dATP. The elongation products were compared with the elongation products generated by telomerase reconstituted with wild-type telomerase RNA and 38-40AGU (Fig. 5). Only telomerase reconstituted with 38-40AGU and mutated RNAs affecting the conserved domain, $\Delta 20-36$ and $\Delta 37-40$, had altered patterns of elongation products in the presence of dATP (Fig. 5).

Thus only stem II and, more specifically, the conserved sequence associated with this stem appear to regulate the 5' boundary of the template. These results clearly demonstrate the role of these residues in template function.

DISCUSSION

We identified residues in the *Tetrahymena* telomerase RNA that are important for enzyme activity, either directly in catalysis or indirectly by mediating proper RNA structure and binding to protein components. When residues at the 5'-terminus and in stems I, II and IV were deleted or changed, activity decreased to 15–35% of wild-type levels. These data support the importance of these structures in protein binding and/or RNA interactions for telomerase activity. Interestingly, mutations affecting the potential pseudoknot structure of the telomerase RNA, deletions of the entire stem III and a substitution of the GA bulge in stem IV did not significantly affect activity.

Many of the mutated RNAs described in this paper were in structures predicted to have critical roles in telomerase function. Yet many were able to reconstitute telomerase activity to wild-type levels. Although it is possible that pieces of endogenous *Tetrahymena* telomerase RNA remain following micrococcal nuclease digestion and that these could complement the mutated telomerase RNAs in an intermolecular manner, we think this is unlikely. No undigested RNA was detectable after Northern analysis of micrococcal nuclease-treated extract. In addition, no activity was detected in the control reaction without added telomerase RNA. Thus the reconstituted activity is likely to be due to addition of synthetic RNA and not to complementation of synthetic RNA by undegraded portions of the RNA (data not shown).

All of the mutated RNAs described in this paper were tested for reconstitution of telomerase activity. It is likely that some of the effects of the RNA mutations on telomerase activity are indirect, via defects in binding, and it will be important to determine the binding affinities of these RNAs to recombinant telomerase protein components. Moreover, during transcription, the mutated RNAs may undergo differential RNA folding and alternative base pairing, which may affect the positioning or spacing between critical regions of the RNA. Secondary structure analysis of these mutated RNAs will be required to assess the different roles of RNA folding, binding and catalysis.

Deletion of >11 residues at the 5'-end of the telomerase RNA completely abolished activity, suggesting that important sequence or structure interactions occur in this region. Substitution of residues 15 and 16, however, only reduced levels of reconstituted activity to 38% of wild-type, suggesting that protein contacts at these residues, predicted by structural probing (17), are not sequence dependent and are not the only important residues in the 5'-region.

The long range base pairing interactions in stem I do not appear to be essential for telomerase function. Of the three mutated RNAs that affect the stem I base pairing, two, $\Delta 103$ –107 and sub4–8, decreased activity to ~35% of wild-type levels. The fact that the $\Delta 5'8$ mutated RNA had very low levels of activity might be due to additional residues that are deleted in this mutated RNA besides stem I residues. However, the $\Delta 5'11$ mutated RNA has more activity than $\Delta 5'8$, suggesting a negative role for residues 9–11. Conservation of the long range base pairing of stem I in all of the ciliate telomerase RNAs strongly supports the role of this structure in telomerase function. Perhaps this region is involved in RNA folding or RNA assembly. The protein components of

telomerase may bind the ends of the RNA and bridge this long range interaction, so that it forms even in the absence of base pairing.

Although the conserved sequence upstream of the template plays an essential role in telomerase function, stem II appears not to be required. Deletion of residues in stem II ($\Delta 20$ –36) reduces activity to 25% of wild-type. It is possible that this mutated RNA forms alternative structures that compensate for deletion of stem II. Although activity decreases only slightly more when four residues adjacent to stem II are deleted ($\Delta 37$ –40), this mutated RNA also affects template boundary determination (12). Initially 6 nt (residues 35–40) were identified that were conserved upstream of the template. The later identification of additional ciliate telomerase RNAs indicated that only four residues are absolutely conserved (8,9). The placement of the template and the upstream conserved sequence is conserved among all ciliate telomerase RNAs identified to date, including *Paramecium* (4). However, stem II is not conserved in all ciliates, suggesting that it may not play an essential role in telomerase function. Consistent with this, substitution of residues in the loop region of stem II (sub26–29) did not decrease telomerase activity. Thus no significant interactions in loop II appear to be required for telomerase activity *in vitro*.

All deletions and substitutions affecting stem IV, except for a substitution of the GA bulge at positions 121 and 122, decreased telomerase activity to <10–15% of wild-type. This is consistent with the high sequence conservation of stem IV. This stem has also been suggested to mediate a conformational change involving alternative base pairing with stem II during enzyme translocation (8). However, stem II does not appear to be essential for telomerase activity *in vitro*, suggesting that any alternative base pairing with this region may not be required for translocation.

Mutations in the conserved GA bulge bases in helix IV did not affect telomerase activity, indicating that sequence-specific contacts to this region are not essential. In *Paramecium* species and the hypotrichs helix IV is more extended than in tetrahymenine ciliates and the conserved GA bulge that is a distinct feature of *Tetrahymena* telomerase RNAs is not apparent (4,7). Structural probing experiments in *Tetrahymena* suggested that protein interactions are likely occurring at the GA bulge (16,17). Our finding that substitution of the GA bulge (sub121–122) does not affect telomerase activity suggests that if interactions are occurring at these residues, they may not be sequence dependent. These results also indicate that any interactions which occur here may not be required for catalysis, cleavage or translocation. Perhaps this region interacts with regulatory proteins that are not purified as a part of the telomerase core enzyme. However, *in vivo* both a deletion of the GA bulge (Δ GA) and a double mutated telomerase RNA deleted for the GA residues and with a substitution at position 44 in the template (44G) result in an increased rate of cell death and shortened telomeres, indicating an important role for these residues *in vivo* (29).

The pseudoknot formed by stem III is conserved in all ciliate telomerase RNAs. Surprisingly, mutations predicted to abolish base pairing in this region did not abolish telomerase activity. It is possible that these mutated RNAs form alternative structures that perform functions similar to the pseudoknot. Of all the deletions and substitutions affecting stem III and the pseudoknot structure only two mutations decreased activity. These were a complete deletion of stem III, which reduced activity to 63%, and a substitution of residues 69–72, predicted to be involved in both pseudoknot structure and protein interactions, which reduced

activity to 53%. Pseudoknot structures are often involved in recognition and binding of proteins (14,15). The existence of the pseudoknot has been verified experimentally by structural probing and it has been suggested to serve as a conformational switch during synthesis of telomeric DNA (7,16,17). Conservation of the pseudoknot structure in all of the ciliate telomerase RNAs indicates that it plays a role in telomerase function (4). Again, like the GA bulge region in helix IV, this conserved function might not be part of the telomerase enzyme as defined by the *in vitro* telomerase assay. There may be *in vivo* regulators of telomerase that interact with a conserved structure on the enzyme. Interestingly, one mutation (sub84–87) predicted to affect pseudoknot structure formation had increased telomerase activity. Residues 84–87 may normally be involved in interactions which negatively regulate telomerase activity. Similarly, one mutation in stem III (sub89–90) previously predicted to be involved in protein binding or protein-induced RNA structure formation (17) had increased telomerase activity, suggesting a possible inhibitory function of these residues on telomerase activity.

The structure and sequence of telomerase RNA has been proposed to play a role in active site function. A detailed analysis of the role of the template residues in telomerase function suggests that a three-way interaction exists between the DNA product, the telomerase RNA template region and the telomerase proteins to promote active site function (30). The *in vitro* data presented in this paper provide additional support that base-specific interactions involving telomerase RNA residues in and adjacent to the template are important for catalysis by telomerase. The ability of all the mutated telomerase RNAs tested to reconstitute telomerase cleavage activity, albeit to different levels, and incorporate ddTTP at the same position from the 3'-end of the primers used suggests that none of the mutated RNAs affect alignment of the cleavage substrate with the template. Recently telomerase activity of a telomerase RNP consisting of *Tetrahymena* proteins and *Glaucoma* telomerase RNA was assayed (31). The telomerase RNAs from these two species are quite different outside the 23 bases containing the template. This hybrid enzyme had aberrant cleavage activity, suggesting that interactions outside the template domain, perhaps in the secondary structures, affect function at the active site (31).

Our data suggest that stem III, the pseudoknot and stem IV, previously proposed to be involved in translocation at the active site, are not essential *in vitro* for substrate alignment, cleavage, translocation and realignment of the product DNA. Moreover, stems I, III and IV play no role in dictating the template boundary and, therefore, the sequence synthesized by telomerase at the active site. Only the upstream conserved region and stem II, presumably because the conserved region is in part contained within stem II, are responsible for regulating addition of nucleotides onto substrates present at the active site. A detailed analysis of similar mutations *in vivo* and structural probing of mutated RNAs within the telomerase RNP will be required to fully understand the roles of the secondary structures of the telomerase RNA in telomerase function.

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