A critical stem–loop structure in the CR4–CR5 domain of mammalian telomerase RNA

Jiunn-Liang Chen, Kay Keyer Opperman and Carol W. Greider*

Department of Molecular Biology and Genetics, 725 North Wolfe Street, Hunterian #617, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

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ABSTRACT

Telomerase is an enzyme that maintains telomere length by adding telomeric sequence repeats onto chromosome ends. The telomerase ribonucleoprotein complex consists of two essential components, a reverse transcriptase and an RNA molecule that provides the template for telomeric repeat synthesis. A common secondary structure of vertebrate telomerase RNA has been proposed based on a phylogenetic comparative analysis of 35 sequences. Here we report the identification of an additional essential base-paired region in the CR4–CR5 domain of mammalian telomerase RNA, termed P6.1. Mouse telomerase RNAs with mutations that disrupted base pairings in the P6.1 helix were unable to reconstitute telomerase activity in vivo. In contrast, an RNA mutant with compensatory mutations that restored base pairings in the P6.1 helix restored telomerase activity. In an in vitro reconstitution system stable base pairing of the P6.1 stem was required for the RNA–protein interaction between the CR4–CR5 domain and the telomerase reverse transcriptase (TERT) protein. Interestingly, two RNA mutations, one that extends the P6.1 stem and one that alters the conserved nucleotides of the L6.1 loop, allowed RNA–protein binding but significantly impaired telomerase activity. These data establish the presence of the P6.1 stem–loop and its importance for the assembly and enzymatic activity of the mammalian telomerase complex.

INTRODUCTION

Telomerase is a ribonucleoprotein enzyme that maintains telomere length by adding telomeric sequence repeats onto telomeres (1,2). Telomerase consists of two essential core components, the telomerase reverse transcriptase (TERT) (3) and the telomerase RNA. The RNA component of telomerase contains a short template element that determines the sequence of telomeric repeats added onto chromosome ends (4,5). Telomerase RNA genes have been isolated from a variety of organisms. The sizes and sequences of telomerase RNA vary dramatically among mammals (300–500 nt), yeast (≈1300 nt) and ciliates (150–200 nt) (6–10). Telomerase RNAs cloned from 20 different ciliates have a conserved secondary structure (11,12). A common secondary structure of vertebrate telomerase RNA was proposed based on a phylogenetic comparative analysis of 35 telomerase RNA sequences (10). Although there is no obvious sequence homology, the structure of vertebrate telomerase RNA displays a significant architectural homology to the ciliate RNA structure.

The conserved structural elements of telomerase RNA are likely to be involved in telomerase function. There are four highly conserved structural domains in the vertebrate telomerase RNA: the pseudoknot, the CR4–CR5 domain, the box H/ACA domain and the CR7 domain (see Fig. 1). The box H/ACA domain is required for RNA stability in vivo (13,14) and both the pseudoknot and the CR4–CR5 domains are essential for telomerase activity in vivo and in vitro (15–18). A possible base-paired region in the CR4–CR5 internal loop was suggested (13,15) but was not supported by phylogenetic covariation evidence because of the sequence conservatism in this region (10). Here, we report evidence for the presence of this stem–loop structure and its importance for telomerase assembly and enzymatic activity.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

A 1.1 kb BstYI-digested DNA fragment that contains the mouse telomerase RNA (mTR) gene was subcloned from a 5 kb EcoRI genomic DNA fragment (19) into the BamHI site of the pCAT3-promoter plasmid vector (Promega) and named pCAT-mTR. This 1.1 kb genomic DNA fragment included 0.4 kb of upstream promoter sequence, the mTR gene and 0.3 kb of downstream sequence. To generate mTR mutants, two KasI sites at positions 176 and 283 in the mTR gene were utilized for cloning PCR-mutagenized DNA fragments of the CR5 region (nt 254–266). This region of the mTR gene was PCR amplified using primers that contained mutant sequences and the fragments were cloned into the KasI-digested pCAT-mTR DNA. All mutant RNA genes were sequenced to confirm the presence of only the intended changes.

Cell culture and transfection

First generation mTR–/– mouse embryonic fibroblasts were routinely passaged in DMEM (Gibco) supplemented with 10% FBS and 1× penicillin/streptomycin/glutamine mixture.
(Gibco). Prior to transfection, cells were trypsinyzed and reseeded at 5 x 10^6 cells/100 mm dish or 1 x 10^6 cells/60 mm dish in antibiotic-free medium for 24 h. Plasmid DNA was prepared using the Endo-Neo Maxi kit (Qiagen) and cells were transiently transfected using FuGene 6 transfection reagent (Roche). Transfections were performed using the manufacturer’s instructions and a 3:2 ratio of FuGene transfection reagent to DNA. After 48 h, cells were trypsinyzed, divided into two equal fractions and centrifuged for 5 min at 750 r.p.m. Cell pellets used for RNA isolation were then immediately resuspended in TriZol (Life Technologies), frozen on dry ice and stored at −80°C until use. Cell pellets used to make telomerase extracts were washed once in PBS, centrifuged and resuspended in 1× CHAPS buffer (10 mM Tris–HCl, pH 8.0, 1 mM MgCl2, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA), 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10% glycerol, 5 mM β-mercaptoethanol and 1 mM DTT). One tablet of Mini Complete Protease Inhibitor Cocktail (Roche) was added to 10 ml of CHAPS buffer. After incubation on ice for 30 min, extracts were centrifuged for 15 min at 4°C at 13 000 r.p.m. Supernatants were then collected, frozen on dry ice and stored at −80°C until use. Transfection efficiency was monitored by co-transfection of a CMV-β-gal plasmid and consistently ranged between 60 and 70%.

**In vitro transcription of mouse telomerase RNA**

Telomerase RNA fragments were prepared by run-off *in vitro* transcription with T7 RNA polymerase using PCR fragments as the DNA template. DNA templates for various mTR fragments were constructed by PCR amplification from plasmid DNAs containing wild-type or mutant mTR genes. The pseudo-knot domain of mTR (nt 1–146) was amplified using primers mTR-T7F (5′-GGGGTACCTAATACGACTCACTATAGACCTAACCGTGATTTCATTAGCTGGGGTTC-3′) and mTR146R (5′-GGGCGTACGTTTTCGTTTGAGG-3′) and 1× CHAPS buffer (10 mM Tris–HCl, pH 8.0, 1 mM MgCl2, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA), 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10% glycerol, 5 mM β-mercaptoethanol and 1 mM DTT). One tablet of Mini Complete Protease Inhibitor Cocktail (Roche) was added to 10 ml of CHAPS buffer. After incubation on ice for 30 min, extracts were centrifuged for 15 min at 4°C at 13 000 r.p.m. Supernatants were then collected, frozen on dry ice and stored at −80°C until use. Transfection efficiency was monitored by co-transfection of a CMV-β-gal plasmid and consistently ranged between 60 and 70%.

**In vitro reconstitution and immunoprecipitation of telomerase**

The mouse TERT (mTERT) gene (a gift from Dr L. Harrington) was subcloned into a modified pcDNA-4a vector (Promega) that contains two influenza hemagglutinin (HA) epitope repeats to generate the pmTERT-HA construct. The mTERT-HA protein was expressed in *in vitro* using TrT rabbit reticulocyte lysates (Promega) in the presence of [35S]methionine at 30°C for 1 h. To assemble the telomerase complex, 1 µg in *in vitro* transcribed mTR was added to the *in vitro* transcription/translation reaction of mTERT-HA and incubated at 30°C for 1 h. The assembled telomerase complex was affinity purified on agarose beads conjugated with antibodies against the HA epitope. To ensure equal efficiency of immunoprecipitation, a portion of immunopurified material was electrophoresed in 7.5% SDS–PAGE gels. The gel was fixed in 25% isopropanol, 10% acetic acid and 2% glycerol for 30 min, soaked in Amplify (Amersham) for 30 min, dried under vacuum at 80°C and exposed to Hyperfilm MP (Amersham). To test the enzymatic activity of the purified telomerase complex, a portion of the sample was also assayed by telomeric repeat amplification protocol (TRAP; see below).

To detect the TERT-bound telomerase RNA by northern blotting, telomerase complex was eluted from agarose beads in gel loading buffer (8 M urea, 0.1% SDS, 10 mM Tris–HCl, pH 8.0, 20 mM EDTA, 0.1% xylene cyanol and 0.1% bromphenol blue) for electrophoresis and northern analysis.

**Northern blotting**

Total RNA was isolated from mouse embryonic fibroblasts using TriZol (Life Technologies). RNA concentrations were determined by OD260 using a GeneSpec I spectrophotometer (Hitachi Genetic Systems). Ten micrograms of total RNA was resolved on a 6% polyacrylamide–8 M urea denaturing gel and electrophotransferred to Hybond-XL membrane (Amersham) at 1 A for 1 h. The membrane was UV crosslinked and prehybridized at 68°C for 1 h in 50 ml of prehybridization buffer containing 5 ml of 1 M Tris–HCl, pH 8.0, 1 ml of 0.5 M EDTA, pH 8.0, 15 ml of 2 M NaCl, 25 ml of formamide, 5 ml of 10% SDS and 1 g blocking reagent (Boehringer Mannheim). Riboprobes were generated from PCR DNA polymerase using a MaxiScript (Hitachi Genetic Systems). riboprobes were synthesized and labeled with [α-32P]CTP using a MaxiScript Kit (Ambion). After incubation at 37°C for 30 min, 2 µl of RNase-free DNase I (2 U/µl) was added for an additional 15 min to remove the DNA template. Riboprobes were then purified using ProbeQuant G-50 microspin columns (Amersham). Membranes were hybridized at 68°C overnight in 20 ml of prehybridization buffer (see above) with ribobrobes added at 1 x 106 c.p.m./ml. The hybridized membrane was washed twice in 20 ml of 1× SSC (3.0 M NaCl and 0.3 M sodium citrate, pH 7.0/0.2% SDS) for 10 min and twice in 20 ml of 0.1× SSC/0.1% SDS for 30 min at 68°C. The blot was exposed to film for 3–6 h.
Telomerase activity assay

Telomerase activity from the transfected mouse cells was determined by the TRAP assay. The TRAP assay was carried out using a protocol modified from Kim et al. (20) or the TRAPEZE Telomerase Detection Kit from Intergen. Briefly, 2 μl of diluted cell extract was added to a 48 μl reaction mix. Protein concentration in the cell extracts was determined using a Bradford Protein Assay Kit (Bio-Rad). For the RNAase A controls, 2 μl of cell lysate was preincubated with 1 U RNase A at 37°C for 15 min. The final 50 μl reaction mixture contained 1× TRAP buffer (20 mM Tris–HCl, pH 8.3, 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween-20 and 1 mM EGTA), 50 μM dNTP, 100 ng 32P-end-labeled telomerase substrate, 1× kinase buffer (Gibco) and 10 U T4 polynucleotide kinase (Gibco). After incubation at 37°C for 30 min, the end-labeling reaction was terminated by heating at 65°C for 15 min. The TRAP reaction was carried out in a PCR thermocycler with one cycle of 60 min at 30°C and 27 cycles of 30 s at 94°C and 30 s at 59°C. After the TRAP reaction, 10 μl of PCR sample was added to 2 μl of 6× glycerol gel loading buffer (30% glycerol, 0.1% xylene cyanol and 0.1% bromophenol blue) and loaded onto a non-denaturing gel (10% polyacrylamide, 2% glycerol, 0.1% xylene blue) for electrophoresis. The gel was dried and exposed to film for 6–12 h.

RESULTS AND DISCUSSION

Two distinct regions of human telomerase RNA, the pseudoknot and the CR4–CR5 domain, are required for functional reconstitution of telomerase in vitro (15–17). The pseudoknot domain contains both a pseudoknot structural element and the template region for synthesis of telomere repeats. The CR4–CR5 domain contains a stem–loop structure formed by two sequence-conserved regions (CRs), CR4 and CR5 (10). Although the function of this essential CR4–CR5 domain is still largely unknown, our data, as well as previous evidence (14–18), indicate that a stable structure of the CR4–CR5 domain is essential for telomerase activity.

In the previously proposed secondary structure of vertebrate telomerase RNA, the CR4–CR5 domain comprises an internal loop, J5/S6 and J6/S5, with a number of highly conserved nucleotides (Fig. 1A). Although a potential paired region within this internal loop was suggested (Fig. 1B) (13,15), phylogenetic comparison did not support this base pairing because of the lack of sequence co-variation (10). To determine whether this putative helix exists, we generated mutations that either disrupt or restore the base pairings in this helix and analyzed the telomerase activity of the mutant RNAs in vivo. In one mutant (mTR-m1), 4 nt, 253AGAG256, on one side of the putative helix were changed to 253UCUC257 (Fig. 2A). In a second mutant (mTR-m2), 4 nt on the other side of the helix were changed to 263GAGA266. Both mutations disrupt the potential base-pairing of the putative P6.1 helix. A third mutation (mTR-m3) that combined the first two, 253UCUC257/263GAGA266, was made to restore the base-pairing ability, although the sequence of the stem was altered.

To test the ability of mTR mutants to reconstitute functional telomerase in vivo, plasmids that contained a genomic copy of the mTR gene with different mutations were transfected into mTR−/− mouse cells (21). Telomerase activity from the transfected cells was determined by the TRAP assay (see Materials and Methods). Both mTR-m1 and mTR-m2 abolished the ability to reconstitute telomerase activity in vivo (Fig. 2A, lanes 11, 12, 14 and 15). In contrast, the third mutant mTR-m3, which restored the base pairings in the P6.1 helix, restored telomerase activity to wild-type levels (Fig. 2A, lanes 17 and 18). Northern analysis showed that the mutant telomerase RNAs were expressed at similar levels in the transfected cells (Fig. 2B). This result establishes that the P6.1 helix exists and is essential for telomerase activity in vivo.

Previous work showed that deletion of the CR5 region (nt 254–266), which includes the P6.1 stem–loop, resulted in a lack of telomerase activity in vivo and the mutant RNA detected was larger than predicted (14). It was proposed that the larger size of that mutant RNA might be due to abnormal RNA processing. However, in our study the P6.1 stem RNA mutants mTR-m1 (254UCUC257) and mTR-m2 (263GAGA266) were the same size as the wild-type (Fig. 2B, lanes 4 and 5), indicating that they were processed normally in vivo. It is unclear why abnormal RNA processing occurred only in the P6.1 deletion mutant and not in the mutants that disrupt base pairing of this region. It is possible that deletion of the P6.1 helix may have caused a deleterious effect on the structure of other RNA domains, such as the box H/ACA domain, which has been shown to be important for RNA stability and possibly RNA processing (13).

Although the P6.1 base-paired sequences are absolutely conserved among all 35 vertebrate RNAs sequenced, it is the base pairing of helix P6.1, and not the sequence, that is important, as an altered sequence of the helix exhibits a normal level of telomerase activity. The sequence conservation seen in the 35 vertebrate species may have been a consequence of the way these sequences were cloned. Most of the genes were isolated through a PCR cloning strategy that amplified a region of the RNA gene using primers targeting two sequence-conserved regions, the CR2 and CR5 regions (10). Therefore, the apparent sequence conservation in the CR5 region was most likely due to the fact that only genes with a conserved sequence in this region were amplified. Genomic DNAs from a number of species failed to generate PCR amplification products of telomerase RNA genes using primers targeting the CR2 and CR5 regions (J.-L. Chen and C.W. Greider, unpublished observation) and were not pursued further. These genes may possess sequence variation in the CR2 and CR5 regions. A larger sequence collection from different species will be required to detect any sequence co-variation within these two regions.

To further understand the functional role of the P6.1 stem–loop structure, we reconstituted the mTR–protein complex in vitro. Two separate RNA fragments, one containing the pseudoknot domain and one containing the CR4–CR5 domain, are required and sufficient for reconstituting telomerase activity in vitro (15–17). We took advantage of this minimal reconstitution system to examine the effect of mutations in the CR4–CR5 domain on in vitro enzyme activity. The mTERT protein was tagged with the HA epitope at the C-terminus and expressed by in vitro transcription and translation in rabbit reticulocyte lysate. The expressed protein was assembled with
two RNA fragments of mTR, the pseudoknot fragment (nt 1–146) containing the pseudoknot domain and template and the CR4–CR5 fragment (nt 200–278) containing the CR4–CR5 domain and L6 (Figs 1 and 3A). Consistent with previous results (15), both the pseudoknot and CR4–CR5 RNAs are capable of binding TERT independently and both are required to reconstitute telomerase activity in vitro (Fig. 3B and D, lanes 1–4).

To assess the importance of the P6.1 stem–loop in the CR4–CR5 domain, five CR4–CR5 RNA fragments with different mutations in the P6.1 stem–loop were assayed for their ability to bind TERT and reconstitute telomerase activity in vitro (Fig. 3A). These mutant RNAs are designated CR4–CR5-m1 to CR4–CR5-m5, rather than mTR-m1 to mTR-m5, as the CR4–CR5 RNA fragment from position 200 to 278 was used in the in vitro reconstitution (Fig. 3A). In addition to the three
mutants (CR4–CR5-m1 to CR4–CR5-m3) with mutations in the P6.1 stem region, two more RNA mutants were generated. One mutant (CR4–CR5-m4) contains a 4 bp extension of the P6.1 stem. The other mutant (CR4–CR5-m5) has two point mutations in the L6.1 loop, U259A and G261C, at residues that are completely conserved in 35 vertebrate species (Fig. 3A).

In vitro reconstituted telomerase complex was immunoprecipitated with agarose beads conjugated with anti-HA antibodies. The immunopurified telomerase complexes were assayed for telomerase activity by TRAP and assayed for RNA–protein interaction by northern analysis. Consistent with the in vivo results described above, both of the RNA fragments with mutations that disrupted the P6.1 stem (CR4–CR5-m1 and CR4–CR5-m2) showed no telomerase activity when assembled together with the pseudoknot domain RNA (Fig. 3D, lanes 5 and 6). Interestingly, both the CR4–CR5-m1 and CR4–CR5-m2 RNA fragments failed to bind to TERT (Fig. 3B, lanes 5 and 6), while the wild-type CR4–CR5 and the CR4–CR5-m3 RNAs were able to bind the TERT protein (Fig. 3B, lanes 4 and 7) and reconstituted normal telomerase activity (Fig. 3D, lanes 4 and 7). This result indicates that a stable P6.1 stem is required for binding to TERT and reconstituting telomerase activity.

In contrast to the mutants that disrupted the P6.1 stem, the CR4–CR5-m4 and CR4–CR5-m5 RNAs exhibited normal binding to TERT (Fig. 3B, lanes 8 and 9). However, the CR4–CR5-m4 RNA failed to reconstitute detectable telomerase activity (Fig. 3D, lane 8). The extension of the P6.1 stem in the CR4–CR5-m4 RNA may have distorted the spatial...
position and orientation of the conserved L6.1 loop. A similar result was found for the CR4–CR5-m5 mutant; the point mutations at the two conserved nucleotides (259U and 261G) in the L6.1 loop significantly reduced telomerase activity while binding to TERT was unaffected (Fig. 3B, lane 9). The sequence alteration of the conserved loop 6.1 (PyUNG8) may disrupt an important RNA–RNA or RNA–protein contact and disturb the functional conformation at or near the catalytic site of telomerase. The inability of both CR4–CR5-m4 and CR4–CR5-m5 to fully reconstitute telomerase activity while maintaining TERT binding suggests that this loop may be involved in enzymatic catalysis.

The results described here support a helical structure, P6.1, in the CR4–CR5 domain of mammalian telomerase RNA, which is important for telomerase activity in vivo. While the P6.1 stem is required for assembly of the CR4–CR5 domain of telomerase RNA with TERT, the L6.1 loop is important for catalytic activity of the telomerase enzyme complex. Further structural and functional studies will be necessary to understand the detailed roles of this crucial stem–loop structure in the assembly of and catalysis by the telomerase complex.

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