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Ciliate telomerase RNA structural features

Monica McCormick-Graham and Daniel P. Romero*

Department of Pharmacology, School of Medicine, University of Minnesota, 435 Delaware Street SE, Minneapolis, MN 55455, USA

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ABSTRACT

Telomerase RNA is an integral part of telomerase, the ribonucleoprotein enzyme that catalyzes the synthesis of telomeric DNA. The RNA moiety contains a templating domain that directs the synthesis of a species-specific telomeric repeat and may also be important for enzyme structure and/or catalysis. Phylogenetic comparisons of telomerase RNA sequences from various *Tetrahymena* spp. and hypotrich ciliates have revealed two conserved secondary structure models that share many features. We have cloned and sequenced the telomerase RNA genes from an additional six *Tetrahymena* spp. (*T.vorax*, *T.borealis*, *T.australis*, *T.silvana*, *T.capricornis* and *T.paravorax*). Inclusion of these sequences, most notably that from *T.paravorax*, in a phylogenetic comparative analysis allowed us to more narrowly define structural elements that may be necessary for a minimal telomerase RNA. A primary sequence element, positioned 5′ of the template and conserved between all previously known ciliate telomerase RNAs, has been reduced from 5′-(C)UGUCA-3′ to the 4 nt sequence 5′-GUCA-3′. Conserved secondary structural features and the impact they have on the general organization of ciliate telomerase RNAs is discussed.

INTRODUCTION

Telomeres are the specialized DNA–protein structures found at the ends of eucaryotic chromosomes. They facilitate the complete replication of chromosomal termini, are necessary for chromosome stability and may direct chromosome attachment to the nuclear membrane (reviewed in 1–3). Telomeric DNA consists of a variable number of short, tandemly repeated G+C-rich sequences (reviewed in 2). The sequence invariably has a strand bias, with the G-rich strand oriented 5′→3′ toward the chromosome terminus.

Telomere terminal transferase, or telomerase, synthesizes the G-rich strand of telomeric DNA (reviewed in 4). Telomerase is a ribonucleoprotein enzyme with an essential RNA moiety (5) that was first identified in the holotrichous ciliate *Tetrahymena thermophila* (6). Subsequently, telomerase activity has been characterized from hypotrichous ciliates (7–9), as well as from mouse cells (10), *Xenopus* oocytes (11) and human tissue culture cells (12,13) and carcinomas (14).

Telomerase RNA genes from 14 ciliates and one yeast have been cloned and sequenced and all contain a region corresponding to the complement of their species-specific telomeric repeat (9,15–18). It has been shown both *in vitro* (9,17,19) and *in vivo* (18,20–22) that this complementary sequence serves as a functional template during telomeric DNA synthesis. Thus telomerase represents an unusual reverse transcriptase that dictates the synthesis of a species-specific telomeric DNA repeat by virtue of its own internal RNA template.

Comparative sequence analysis of telomerase RNA gene sequences from six *Tetrahymena* spp. and the closely related *Glaucoma chattoni* has led to the derivation of a conserved secondary structure for the *Tetrahymena* telomerase RNA (16,23). A similar phylogenetic study of seven hypotrichous ciliate telomerase RNA genes produced a secondary structure model for the hypotrich RNA (9). The primary sequences of the *Tetrahymena* and hypotrich telomerase RNAs, ranging in length from 154 to 190 nt, have diverged to such a degree that they cannot be aligned with any confidence beyond the template and six conserved nucleotides located 5′ of the template (5′-(C)UGUCA-3′). Despite the apparent absence of primary sequence conservation, a comparison of the two independently derived structure models indicates that most of the secondary structural features of the ciliate RNAs are conserved. The only major difference in the two structures is the presence of a stem–loop in the *Tetrahymena* model (helix II), which is not supported by the hypotrich telomerase RNA sequences (9,16).

Telomerase RNA from the yeast *Saccharomyces cerevisiae* is a remarkable 1301 nt in length (18). Primary and/or secondary structure features homologous to those conserved in the ciliate RNAs, other than the template, are not obvious at this time. Telomerase RNA genes from yeast species closely related to *Saccharomyces cerevisiae* must be cloned and sequenced before a secondary structure model for this RNA can be deduced.

In a continuing effort to more definitively identify conserved primary and secondary structural features that may be critical to telomerase function, we have cloned and sequenced telomerase RNA genes from six additional *Tetrahymena* spp., increasing the total number of known ciliate telomerase RNA sequences to 20. The resultant phylogenetic comparative analysis has helped to refine the ciliate telomerase RNA secondary structure model. The inclusion of these additional telomerase RNA gene sequences in the analysis has given us insight into what structural elements

* To whom correspondence should be addressed

may be minimally required for a functional ciliate telomerase RNA.

MATERIALS AND METHODS

General methods

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs and used following the procedures of Sambrook *et al.* (24). AMV reverse transcriptase was purchased from Life Sciences Inc. PCR reactions were carried out on a DNA Thermal Cycler with *Taq* DNA polymerase and PCR reagents from Perkin-Elmer-Cetus. Oligonucleotides were radiolabelled at the 5′-end with T4 polynucleotide kinase and [γ-^{32}P]ATP (sp. act. 7000 Ci/mmol; ICN) as described (24). Nick-translated probes were generated with a kit from Bethesda Research Laboratories and [α-^{32}P]dNTPs (sp. act. 3000 Ci/mmol; Amersham). Cloning into the polylinker of phagemids pUC118 and pUC119 and preparation of double-stranded and single-stranded DNA from the appropriate *Escherichia coli* host strains were as described (25). *Tetrahymena* spp. were cultured in a complex media as described (26). Total DNA was isolated from *Tetrahymena* spp. *T.australis* (strain AU 94-10), *T.borealis* (strain WZ 3), *T.capricornis* (strain MP 69A), *T.paravorax* (strain RP), *T.silvana* (strain MP 67) and *T.vorax* (strain V_2 S3-C) as described by Yu and Blackburn (26).

Radiolabeled probe for telomerase RNA gene cross-hybridizations

A 520 bp *Dra*I fragment from pCG1 containing the *T.thermophila* telomerase RNA gene (15) was cloned into the *Sma*I cloning site of pUC118. The *Hin*dIII–*Eco*RI fragment from this subclone was subsequently gel purified and used to generate a radiolabeled nick-translated probe. The fragment containing the telomerase RNA gene was recovered from 0.8% low melting agarose gel slices by multiple freeze–thaw cycles, phenol extraction and ethanol precipitation. Routinely, 0.3–0.6 μg of this fragment was nick-translated to a specific activity of 2×10^7 to 4×10^7 c.p.m./μg with 50 μCi [α-^{32}P]dATP as described (24).

Cloning and sequencing telomerase RNA genes

Total DNAs from *Tetrahymena* spp. were digested with a variety of restriction endonucleases and electrophoresed in 0.8% agarose gels for Southern blot analysis. Blots were probed at 30°C with the radiolabeled *T.thermophila* telomerase RNA gene (~1×10^7 c.p.m.) in a hybridization buffer containing 30% (v/v) formamide, 10% dextran sulfate (500 kDa), 5% SDS, 4 × SSC (0.6 M NaCl, 60 mM sodium citrate), 1 × Denhardt's solution, 25 mM sodium phosphate (pH 6.5), 10 mM EDTA, 0.25 mg/ml high molecular weight RNA. Blots were washed twice for 15 min at room temperature in 2 × SSC/0.1% SDS, followed by a final wash with 1 × SSC/0.1% SDS for 30 min at 30°C. Autoradiography of the Southern blots revealed cross-hybridization of the *T.thermophila* telomerase RNA gene to single restriction fragments for all species tested, independent of the restriction enzymes utilized (data not shown).

Preparative restriction digests of total DNA (30–40 μg) from various *Tetrahymena* spp. were electrophoresed in 0.8% low melting agarose gels and regions containing cross-hybridizing restriction fragments were excised from the gel. DNA fragments were recovered from gel slices as described above. Size-enriched DNA was then ligated into the polylinker of pUC118 to generate size-selected libraries. Ligation products were used to transform competent *E.coli* (strain MV1193) as described (24).

The transfer of bacterial colonies onto Nytran filters (Schleicher and Schuell) and screening of the size-selected libraries were as described by Sambrook *et al.* (24), with the identical radiolabeled probe and hybridization conditions used for Southern blot analyses. Cross-hybridizing clones were isolated and characterized by restriction digests. Cross-hybridizing restriction fragments were subcloned into the appropriate polycloning sites of pUC118 and pUC119. The complete sequences of both strands of the subclones were determined using the dideoxynucleotide termination method (27) with Sequenase™ (USB) and [α-^{35}S]dATP (1000 Ci/mmol; Amersham).

PCR amplification of the *T.capricornis* telomerase RNA gene

The PCR amplification, cloning and sequencing of the *T.capricornis* telomerase RNA gene was done as previously reported for two other *Tetrahymena* telomerase RNAs (16). In order to obtain the complete, unambiguous genomic sequence, it was necessary to clone and sequence an inverse PCR product. Approximately 0.1 μg *T.capricornis* total DNA was digested with *Dra*I, which results in a 0.5 kb fragment containing the entire telomerase RNA coding sequence, as determined by Southern blot analysis (data not shown). The DNA was diluted to ~1 μg/ml to favor intramolecular ligation and ligated with 0.1 U/μl T4 ligase (BRL) at 16°C for 20 h. One tenth of the ligated DNA was amplified by PCR in a 100 μl reaction containing 0.2 mM dNTPs, 0.2 μg each primer, 2.5 U *Taq* polymerase (Perkin-Elmer). The primer oligonucleotide sequences used corresponded to nucleotide positions +68 to +48 (21mer, – strand) and +91 to +120 (22mer, + strand) for the *T.capricornis* RNA (Fig. 2). Forty cycles of 94°C for 1 min, 46°C for 1 min and 72°C for 2 min were performed, finishing with 72°C for 10 min. The expected 0.5 kb PCR product was subsequently cloned and sequenced as described above.

Telomerase RNA sequences, sequence alignments, secondary structures and similarity values

The six telomerase RNA gene sequences generated in this study were aligned with the seven previously published *Tetrahymena* sequences (16). The addition of new sequences to the alignment resulted in some minor modifications in the identification of homologous nucleotides (Fig. 1). Helical regions that had been previously identified by phylogenetic comparative analyses (9,16) were further supported by compensatory base changes revealed by the new alignment (Fig. 3). Conserved nucleotides flanking the putatively base paired regions and those occurring in helical loops helped to reinforce helical structure identification. The assignment of stem–loops was dependent on context (the relative positions of highly conserved, and therefore homologous, flanking sequences and hairpin loops), as well as on the presence of compensatory base changes (28).

Pairwise comparisons of telomerase RNA sequences presented in Figure 1 were used to calculate similarity values H, where $H = m/(m + u + g/2)$. The factor m is the number of sequence positions with matching nucleotides in two sequences, u is the number of sequence positions with non-matching nucleotides and

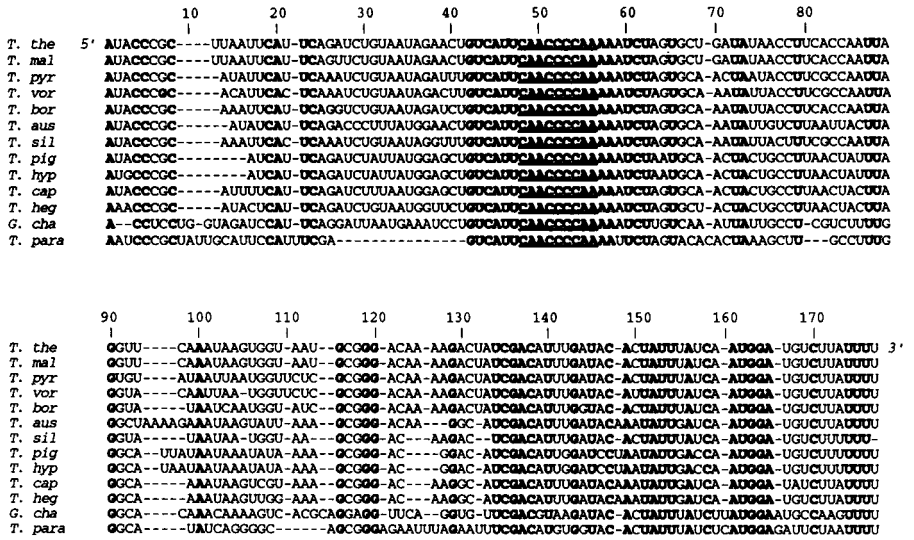

Figure 1. Alignment of *Tetrahymena* telomerase RNA sequences. The sequences of telomerase RNAs from 13 *Tetrahymena* species were aligned as described in the text. The following abbreviations are used: *T.thermophila* (*T.the*), *T.malaccensis* (*T.mal*), *T.pyriformis* (*T.pyr*), *T.vorax* (*T.vor*), *T.borealis* (*T.bor*), *T.australis* (*T.aus*), *T.silvana* (*T.sil*), *T.pigmentosa* (*T.pig*), *T.hyperangularis* (*T.hyp*), *T.capricornis* (*T.cap*), *T.hegewishii* (*T.heg*), *G.chattoni* (*G.cha*) and *T.paravorax* (*T.para*). Dashes (–) indicate alignment gaps. Bold nucleotides are conserved between all 13 species and the templating domain is underlined. Gapped positions are included in the assignment of numbers for nucleotide positions.

g is the number of sequence positions that have a gap in one sequence opposite a nucleotide in the other sequence (29).

The nucleotide sequence data described in this paper will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession numbers U22349–U22354 (incl.).

RESULTS AND DISCUSSION

We have cloned the telomerase RNA genes from six *Tetrahymena* species. Southern blots of total DNA from *T.australis*, *T.borealis*, *T.capricornis*, *T.silvana*, *T.paravorax* and *T.vorax* digested with various restriction enzymes were probed with the cloned *T.thermophila* telomerase RNA gene (15). There was cross-hybridization under low stringency to single restriction fragments for all six species, although the degree of hybridization of *T.paravorax* DNA with the probe was significantly weaker than that of the other species (data not shown). Size-selected libraries were constructed with a bacterial vector and screened as described previously (16). The telomerase RNA genes from five of the six species were successfully cloned and sequenced by this method. The telomerase RNA gene from the sixth, *T.capricornis*, was cloned and sequenced by a PCR and inverse PCR strategy (16).

Northern blot analysis of the six telomerase RNAs indicates that their lengths range between 148 and 160 nt (data not shown). The 5′-termini were subsequently mapped by primer extension of an oligonucleotide complementary to the highly conserved template region. Telomerase RNAs from five of the six species have 5′-ends that are fairly uniform in length and comparable to that from other *Tetrahymena* spp. The exception is the *T.paravorax* RNA 5′-terminus, which is shorter by ~10 nt (data not shown).

The predicted 3′-termini for each telomerase RNA gene coincides with between five and eight consecutive thymidine residues. This is a typical pol III transcription termination signal (30) and is a feature shared by all known ciliate telomerase RNA (9,15–17) and *T.thermophila* U6 snRNA genes (31). The region immediately upstream of the telomerase RNA 5′-terminus was determined for four of the six species. All four genes included conserved sequence elements believed to be required for pol III transcription, as previously noted for ciliate telomerase RNA and U6 snRNA genes (9,16,17,31,32). These conserved elements include a thymidine residue at position –1, a consensus upstream sequence element at –50 and a loose TATA-like element at position –25 (data not shown).

An alignment of 13 *Tetrahymena* telomerase RNAs is shown in Figure 1. The degree of primary sequence variation and relative constancy in overall length was such that an alignment of homologous nucleotides was fairly straightforward, differing only slightly from that previously published for seven telomerase RNA sequences (16). Thirteen nucleotides originally considered invariant are now identified as variable. This additional primary sequence variability contributes to compensatory base changes that support helices II–IV. A notable change in the structure model involves the conserved G and C residues at the base of helix II, which were originally represented as a G–C base pair. We now consider these nucleotides to be unpaired, in the light of the structure models for the hypotrich (9) and *T.paravorax* telomerase RNAs (see below).

The unstructured nucleotides that constitute the link between helices I and IV (5′-ACAA-3′) are conserved for the *Tetrahymena* spp. classified in the *T.pyriformis* complex (29,33,34). In contrast, the homologous region from species in the *T.pigmentosa* complex is the conserved dinucleotide sequence 5′-CA-3′ (Fig. 1, nucleotides 122–125). Telomerase RNA secondary structures of representative species from these two phylogenetic groupings are shown in Figure 2.

The sequence 5′-(C)UGUCA-3′, positioned 2 nt 5′ of the template, is conserved in all previously published ciliate telomerase RNAs (9,15–17). It has been speculated that this sequence element may be engaged in an RNA–RNA or RNA–protein structure directly involved in the telomerase active site (9). We have found that the homologous sequence from *T.paravorax*, 5′-GAGUCA-3′, includes nucleotide changes at the first two

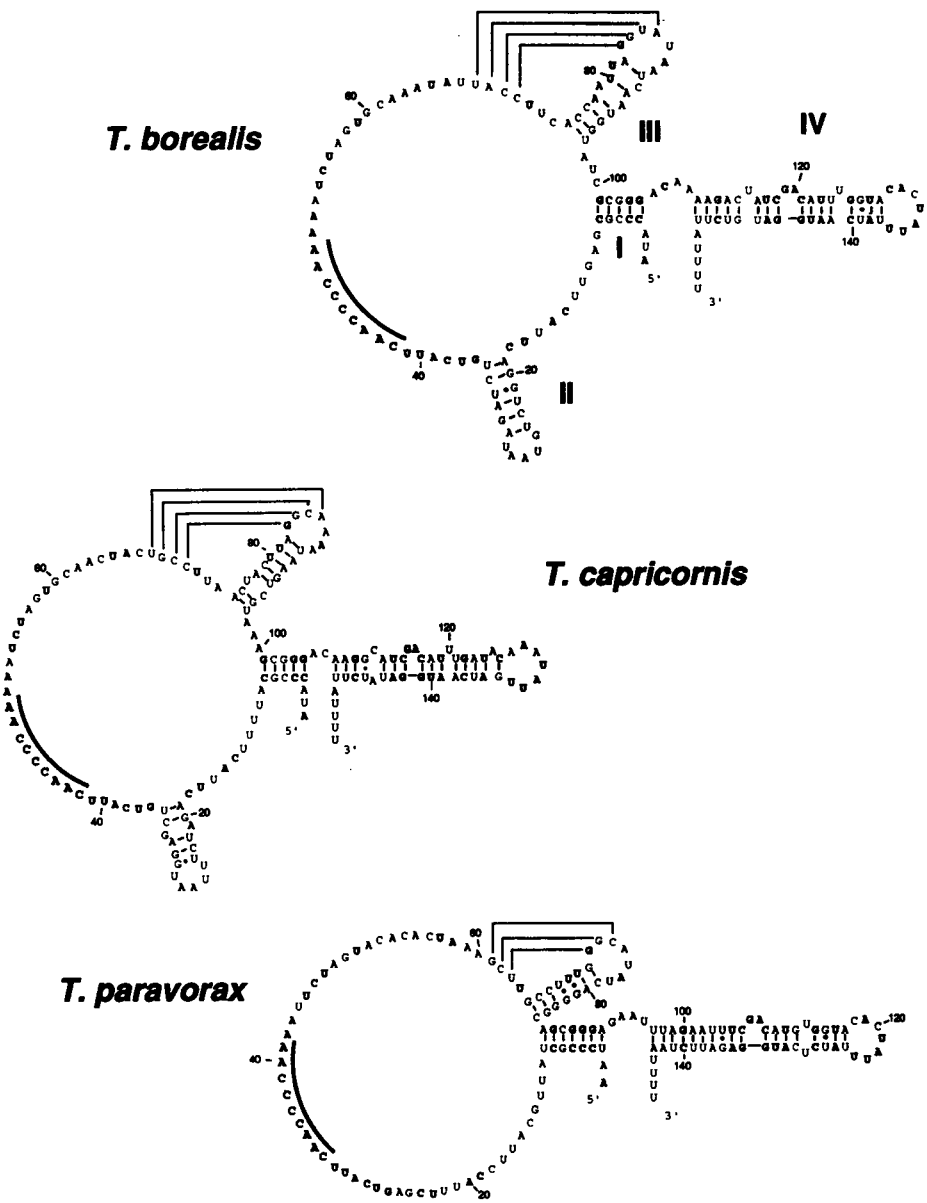

Figure 2. *Tetrahymena* telomerase RNA secondary structure models. The structures shown are representative of three distinct *Tetrahymena* ciliate groups (29,33,34): the *T.pyriformis* complex (*T.borealis*), the *T.pigmentosa* complex (*T.capricornis*) and *T.paravorax*. Helical structures are enumerated (I–IV) in a 5′→3′ direction. Conserved nucleotides are indicated by bold type and the templating domain is underlined. The numbering of nucleotides for the individual RNAs is independent of the assignment of nucleotide positions in Figure 1.

positions (Fig. 1). This reduces the absolutely conserved nucleotides positioned 5′ of the template to the sequence 5′-GUCA-3′.

The *T.paravorax* telomerase RNA secondary structure also differs somewhat from the other *Tetrahymena* RNAs (Fig. 2). Helix I is 7 bp in length rather than 5 bp, a characteristic shared by the *G.chattoni* RNA structure model (16). Somewhat suprisingly, the shorter 5′-terminus lacks the potential for a thermodynamically stable helix II, resembling the hypotrich telomerase RNA structure in this region (9). A comparison of telomerase RNAs from *T.paravorax*, all the other *Tetrahymena* spp. and the hypotrich *Oxytricha nova* (9) is shown schematically in Figure 3.

The absence of stem–loop II from the hypotrich and *T.paravorax* telomerase RNA structure models raises intriguing questions about the origin and functional importance of this structural feature. The absence of stem–loop II may represent a primitive trait of the ancestral ciliate telomerase RNA, a characteristic retained by the hypotrichs and *T.paravorax*. The relative evolutionary distance of *T.paravorax* from other *Tetrahymena* spp. supports this possibility. Pairwise comparisons of the aligned sequences in Figure 1 reveal an average similarity value (*H*) of 0.636 ($\sigma = 0.02$) for the *G.chattoni* RNA when compared with the other 12 sequences. Likewise, the average *H* for *T.paravorax* is 0.644 ($\sigma = 0.03$). In contrast, the average *H* values for the other 11 species range between 0.793 and 0.839 ($\sigma = 0.09$). These data are consistent with the divergence of *T.paravorax*, *G.chattoni* and an ancestral *Tetrahymena* species from a common progenitor at

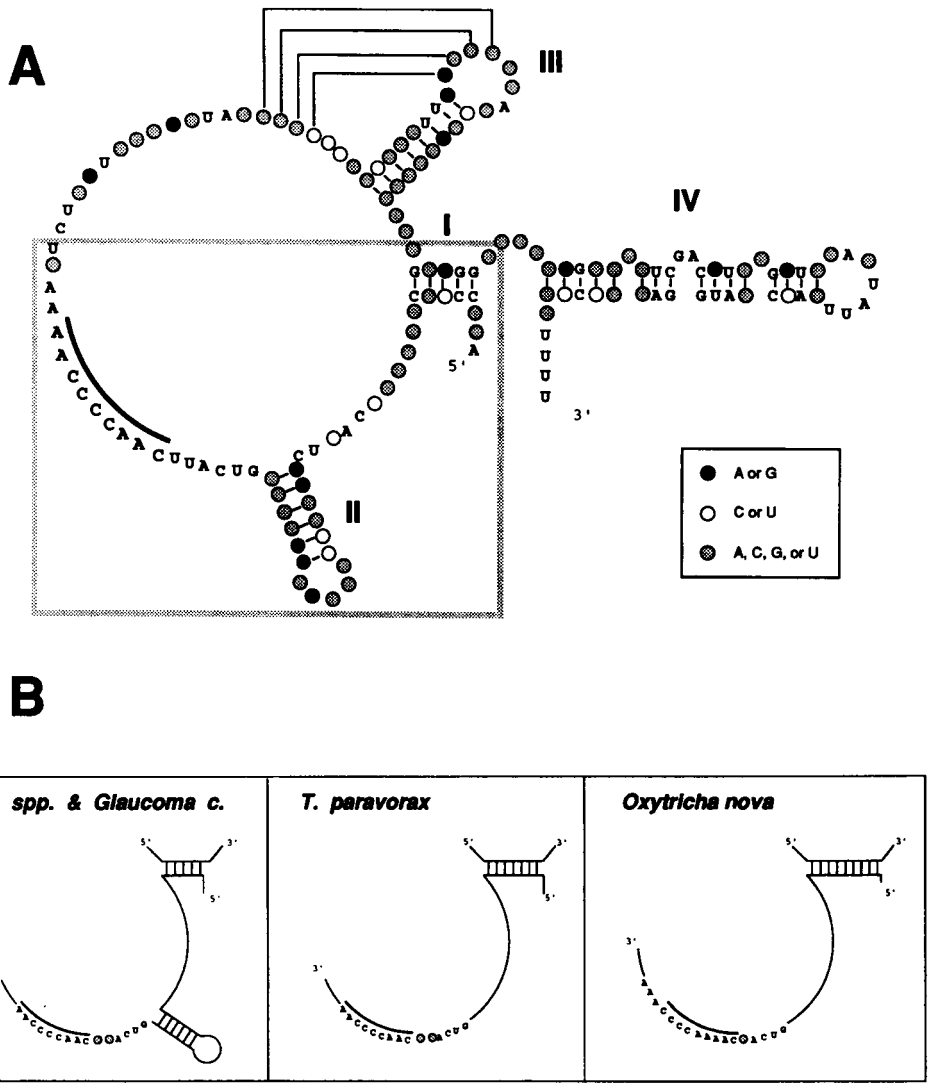

Figure 3. A consensus *Tetrahymena* telomerase RNA secondary structure. (**A**) Consensus structure model based on the 13 telomerase RNA sequences shown in Figure 1. The total length, relative position of helices and numbering of nucleotides in the consensus structure is based on the *T.thermophila* telomerase RNA (16). Conserved nucleotides are indicated by bold type and the templating domain is underlined. Helical structures are enumerated (I–IV) in a 5′→3′ direction. (**B**) The region highlighted by the gray box in the consensus structure model is shown schematically for three divergent ciliate groups. *T.* spp. includes all known *Tetrahymena* species with the exception of *T.paravorax*. The hypotrichous ciliate model is represented by the structure for *Oxytricha nova* (9).

approximately the same time. Phylogenies based on rRNA sequences and the histone H3II/H4II intergenic region indicate that *T.paravorax* is nearly as close to other ciliates as it is to the closest *Tetrahymena* spp. (33,34).

If the absence of stem–loop II is a primitive telomerase RNA structural feature, its acquisition by the ancestral *Tetrahymena* species must have occurred after the divergence of *T.paravorax*. Alternatively, if stem–loop II is a primitive feature of ciliate telomerase RNAs, its absence from *T.paravorax* and hypotrich telomerase RNAs may represent two independent deletion events and thus be an example of convergent evolution. The presence of helix II in telomerase RNAs from other, more distant holotrichous ciliates, such as *Paramecium*, would support the conclusion that helix II is a primitive, rather than an acquired, structural feature. Regardless of its origin, stem–loop II cannot generally be regarded as essential for telomerase RNA function. Deletion of stem–loop II from the *T.thermophila* telomerase RNA gene and the impact such a radical change might have on telomerase activity from that species has not yet been determined.

Stem–loop IV in the *T.paravorax* telomerase RNA consists of a long helical region interrupted by an absolutely conserved dinucleotide bulge (5′-GA-3′, see Figs 2 and 3A). Also conserved is the sequence 5′-UAUU-3′ present in the loop (Fig. 3A). The *T.paravorax* helix IV (18 bp, including two G–U pairs and a single U–U mismatch) is 2 bp longer than that from any other *Tetrahymena* telomerase RNA. In contrast, helix IV is slightly longer in hypotrich telomerase RNAs and is interrupted by two prominent bulges that range between 7 and 12 nt in length (9). The proximity of helices I and IV to each other may contribute to their stability by virtue of base stacking. It has been suggested that this helical region of the RNA may serve as a binding site for telomerase proteins (35). Recognition of helices I and IV, with the

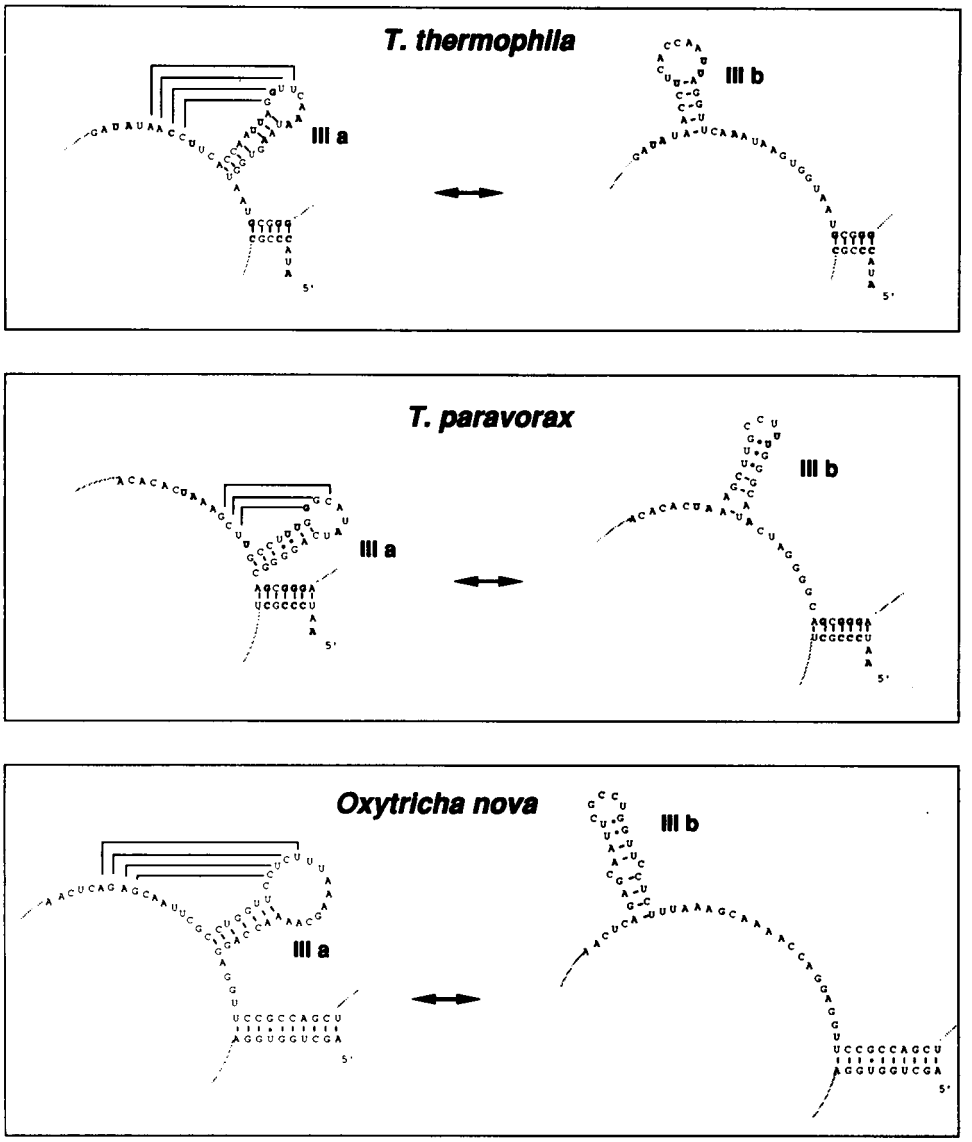

Figure 4. Potential conformational flexibility of the helix III region. Alternative base pairing of the helix III–pseudoknot region, designated helix IIIb, is shown for *T.thermophila*, *T.paravorax* and *O.nova*. Helix IIIb for *T.thermophila* is based on enzymatic and chemical probing of the *T.thermophila* telomerase RNA *in vitro* (from Bhattacharyya and Blackburn; 35).

characteristic bulged nucleotides in helix IV, may be important for telomerase protein recognition in a manner analogous to the specificity of the HIV Tat protein binding to the *TAR* RNA bulged helix (36,37). Other examples of RNA–protein interactions that involve the specific recognition of RNA helices include the binding of ribosomal proteins to rRNAs and the binding of tRNAs by their cognate tRNA synthetases (38,39).

Helix III, conserved in all ciliate telomerase RNAs, is a hairpin loop that contributes to a pseudoknot (9,23). The pseudoknot shown for *T.paravorax* (Fig. 2) differs somewhat from the homologous structure seen in all other telomerase RNAs. The size range of the first pseudoknot loop in other telomerase RNAs lies between 2 and 6 nt, whereas the homologous loop in the *T.paravorax* RNA is only 1 nt in length (U). The configuration is similar to that of a conserved pseudoknot seen in the 3′ non-coding region of tobamoviral RNAs (40) and in RNA2 of barley stripe mosaic virus (BSMV) (41). Mutational analysis of turnip yellow mosaic virus RNA has demonstrated a single nucleotide loop is of sufficient length to span the deep groove of the quasi-continuous double helix present in a pseudoknot of this kind (42). Pseudoknots with such a short first loop demand some level of distortion of the double helix in order to bring together the phosphates across the helical groove (43). This distortion may be contributed by the specific sequence of the base paired nucleotides or by partial destacking at the border between the two double helical segments. The length of the second loop of the *T.paravorax* pseudoknot, 4 nt in the hairpin loop of helix III (5′-AUAU-3′), is well beyond the lower limit for bridging the shallow groove of the helix (44).

There is evidence that pseudoknots such as those presented in Figure 2 may be in equilibrium between alternative stem–loops and pseudoknotted structures (42,45). It has been postulated that a metastable pseudoknot involving helix III may contribute to a translocation event during the synthesis of telomeric DNA by

telomerase (9). Chemical and enzymatic probing of the *T.thermophila* and *G.chattoni* telomerase RNAs *in vitro* indicate that helix III may in fact be in equilibrium with an alternative stem–loop structure, identified as helix IIIb (35). A review of the sequence data indicates the potential for a helix IIIb ranging between 5 and 9 bp in length for all known ciliate telomerase RNAs. A comparison of helix III with the alternative helix IIIb for three species is illustrated in Figure 4. Whether or not an alternative stem–loop such as helix IIIb is a critical telomerase RNA structural element remains to be tested.

The 18–20 nt situated between the template region and helix III are depicted as unpaired for all 20 ciliate telomerase RNA secondary structures. The overall length and lack of base pairing potential for this region is remarkably conserved. The accessibility of these nucleotides for heteroduplex formation with antisense oligonucleotides has been well documented for telomerase RNAs from *T.thermophila*, *Euplotes crassus*, *E.aediculatus*, and *O.nova* (9,15,17,46). Enzymatic and chemical probing of the *T.thermophila* and *G.chattoni* telomerase RNAs *in vitro* indicate that these nucleotides are in some sort of ordered structure, perhaps involving base stacking, that could not be deduced by phylogenetic comparative analysis (35). It has been suggested by Bhattacharyya and Blackburn (35) that the templating nucleotides and those residues situated immediately 3′, including all of helix III, constitute a domain whose structural flexibility contributes to the enzymatic function of telomerase. They also postulate that the remainder of the RNA (helices I, II and IV) is primarily involved as binding domains for telomerase proteins. It will be interesting to see if homologous RNAs from evolutionarily distant taxa, such as *Saccharomyces*, have retained similar traits in their general organization.

In summary, the addition of six *Tetrahymena* telomerase RNA genes to a phylogenetic analysis has helped to more narrowly define structural elements that may be necesssary for ciliate telomerase function. Of particular interest is the conserved motif situated 2 nt 5′ of the template, which has been reduced to the tetranucleotide 5′-GUCA-3′, and the absence of helix II in the *T.paravorax* telomerase RNA, which more closely resembles the hypotrich RNAs in this region. The potential for an alternative stem–loop positioned 3′ of the templating domain (helix IIIb) is present in all the RNAs. The general organization of the ciliate telomerase RNAs, including the apparent plasticity of the RNA structure immediately 3′ of the template, is amenable to functional analysis.

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