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Secondary Structure of Vertebrate Telomerase RNA

Jiunn-Liang Chen, Maria A. Blasco,† and Carol W. Greider*
Department of Molecular Biology and Genetics
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Summary

Telomerase is a ribonucleoprotein enzyme that maintains telomere length by adding telomeric sequence repeats onto chromosome ends. The essential RNA component of telomerase provides the template for telomeric repeat synthesis. To determine the secondary structure of vertebrate telomerase RNA, 32 new telomerase RNA genes were cloned and sequenced from a variety of vertebrate species including 18 mammals, 2 birds, 1 reptile, 7 amphibians, and 4 fishes. Using phylogenetic comparative analysis, we propose a secondary structure that contains four structural domains conserved in all vertebrates. Ten helical regions of the RNA are universally conserved while other regions vary significantly in length and sequence between different classes of vertebrates. The proposed vertebrate telomerase RNA structure displays a strikingly similar topology to the previously determined ciliate telomerase RNA structure, implying an evolutionary conservation of the global architecture of telomerase RNA.

Introduction

Telomeres are necessary for eukaryotic chromosome stability and for the complete replication of chromosome ends (Blackburn, 1991; Zakian, 1996). Vertebrate telomeric DNA consists of short, tandemly repeated $(TTAGGG)_n$ sequences. Telomerase is a ribonucleoprotein enzyme that maintains telomere length by adding telomeric sequences onto chromosome ends (Greider and Blackburn, 1985, 1987). Telomerase contains an essential RNA component and a catalytic protein component, *te*lomerase *r*everse *t*ranscriptase (TERT). The RNA component of telomerase contains a short template sequence that specifies telomere repeats and is thus essential for the enzymatic activity of telomerase (Greider and Blackburn, 1989; Yu et al., 1990). The TERT component, first identified in *Euplotes aediculatus*, contains motifs found in reverse transcriptases that are essential for catalysis (Lingner et al., 1997). TERT homologs have been identified from a variety of organisms, including human, mouse, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae, Oxytricha trifallax, Tetrahymena thermophila*, and *Arabidopsis thaliana* (Counter et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997; Bryan et al., 1998; Collins and Gandhi, 1998; Greenberg et al., 1998; Fitzgerald et al., 1999; Oguchi et al., 1999).

Telomerase RNAs have been identified from 24 ciliate species (148 to 209 nucleotides [nt] in length), two yeasts (~1,300 nt), mouse (397 nt), cow (~443 nt), and human (451 nt) (Singer and Gottschling, 1994; Blasco et al., 1995; Feng et al., 1995; McEachern and Blackburn, 1995; Tsao et al., 1998; reviewed in Collins, 1999). Since these RNAs vary remarkably in their size and sequence, comparison of the telomerase RNA sequences from mammals, yeast, and ciliates reveals no obvious similarity among these three distantly related groups. A conserved secondary structure of telomerase RNAs from divergent groups of ciliates has been established using phylogenetic comparative analysis (Romero and Blackburn, 1991; ten Dam et al., 1991; Lingner et al., 1994; McCormick-Graham and Romero, 1995). Although RNA sequences from different groups of ciliates are very difficult to align, they all fold into a similar secondary structure, suggesting that a conserved structure is important for function.

Phylogenetic comparison has proven to be the most powerful approach for inferring higher-order RNA structure (Noller and Woese, 1981; James et al., 1988; Romero and Blackburn, 1991; Williams and Bartel, 1996). A general tenet of phylogenetic comparative analysis is that base pairs in homologous helical regions of an RNA will change in concert in different organisms while maintaining base-pairing ability. Thus, covariation of paired residues in a putative helical region offers support for that helix (reviewed in Pace et al., 1989).

To understand the structure of telomerase RNA in vertebrates, we cloned and sequenced full-length telomerase RNA genes from a variety of vertebrate species. Using phylogenetic comparative analysis, an evolutionarily conserved secondary structure model for vertebrate telomerase RNA was established. This proposed structure displays a significant architectural homology to the ciliate telomerase RNA. This remarkable conservation over a large evolutionary distance implies an important role for RNA structure in telomerase function.

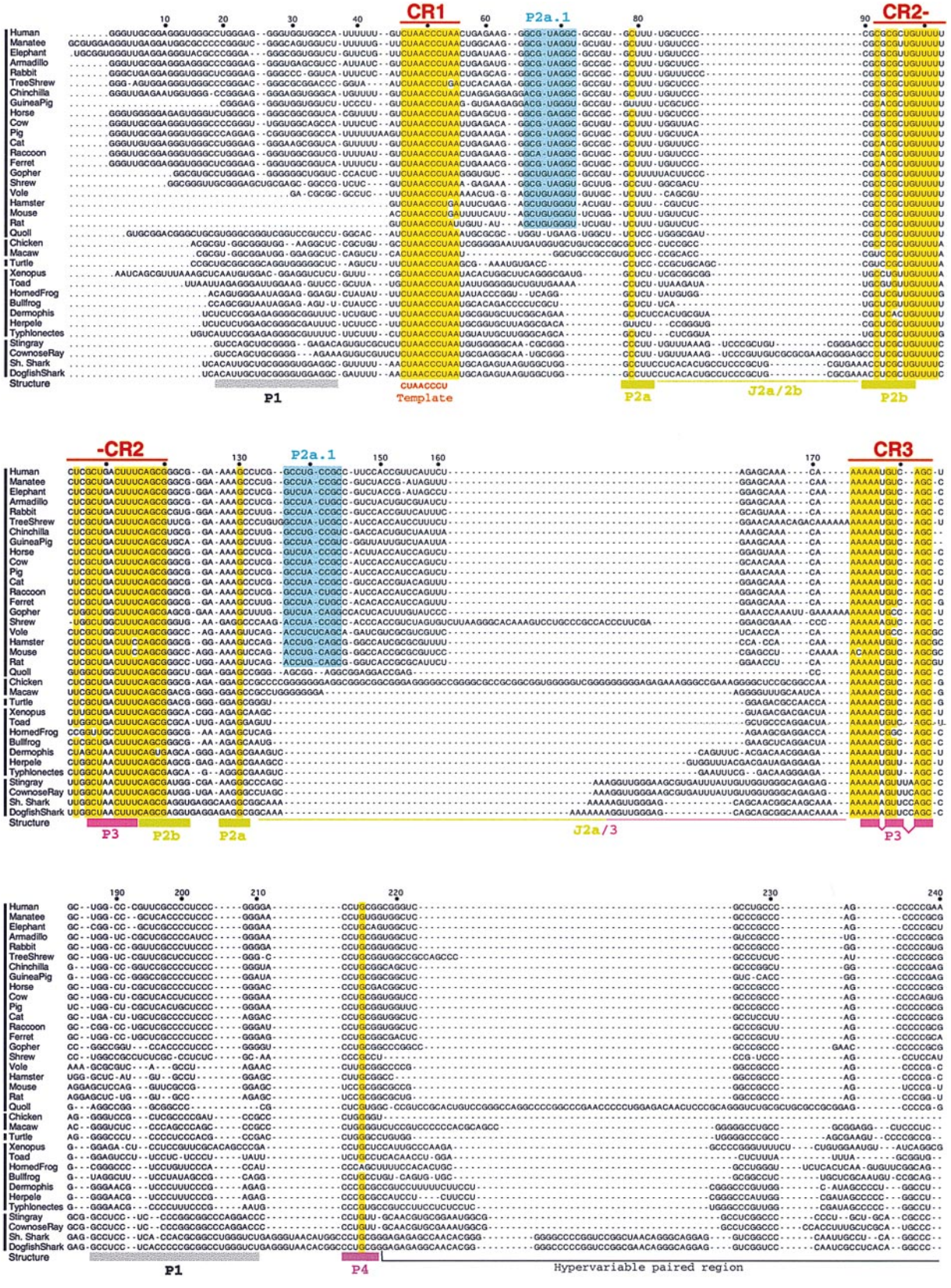
Results

Identification of New Telomerase RNA Sequences from Vertebrates

To establish the secondary structure of vertebrate telomerase RNA, we cloned 32 new telomerase RNA genes from five different classes in the phylum Vertebrata. The 32 species include 18 mammals, 2 birds (chicken and macaw), 1 reptile (turtle), 7 amphibians (two toads, two frogs, and three caecilians), and 4 fishes (two rays and two sharks). To assure a diversity of telomerase RNA sequences for comparative analysis, the 18 mammalian species were chosen from 11 different taxonomic orders (Figure 1).

Degenerate PCR was initially used to clone partial telomerase RNA genes, and then full-length genomic

*To whom correspondence should be addressed (e-mail: cgreider@jhmi.edu).
† Present address: Department of Immunology and Oncology, National Center of Biotechnology, Madrid, Spain.


CR1
P2a.1
CR2-
-CR2
P2a.1
CR3
Human
Manatee
Elephant
Armadillo
Rabbit
TreeShrew
Chinchilla
GuineaPig
Horse
Cow
Pig
Cat
Raccoon
Ferret
Gopher
Shrew
Vole
Hamster
Mouse
Rat
Quoll
Chicken
Macaw
Turtle
Xenopus
Toad
HornedFrog
Bullfrog
Dermophis
Herpele
Typhlonectes
Stingray
CownoseRay
Sh. Shark
DogfishShark
Structure
CUAACCCU
Template
P1
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P3
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Hypervariable paired region

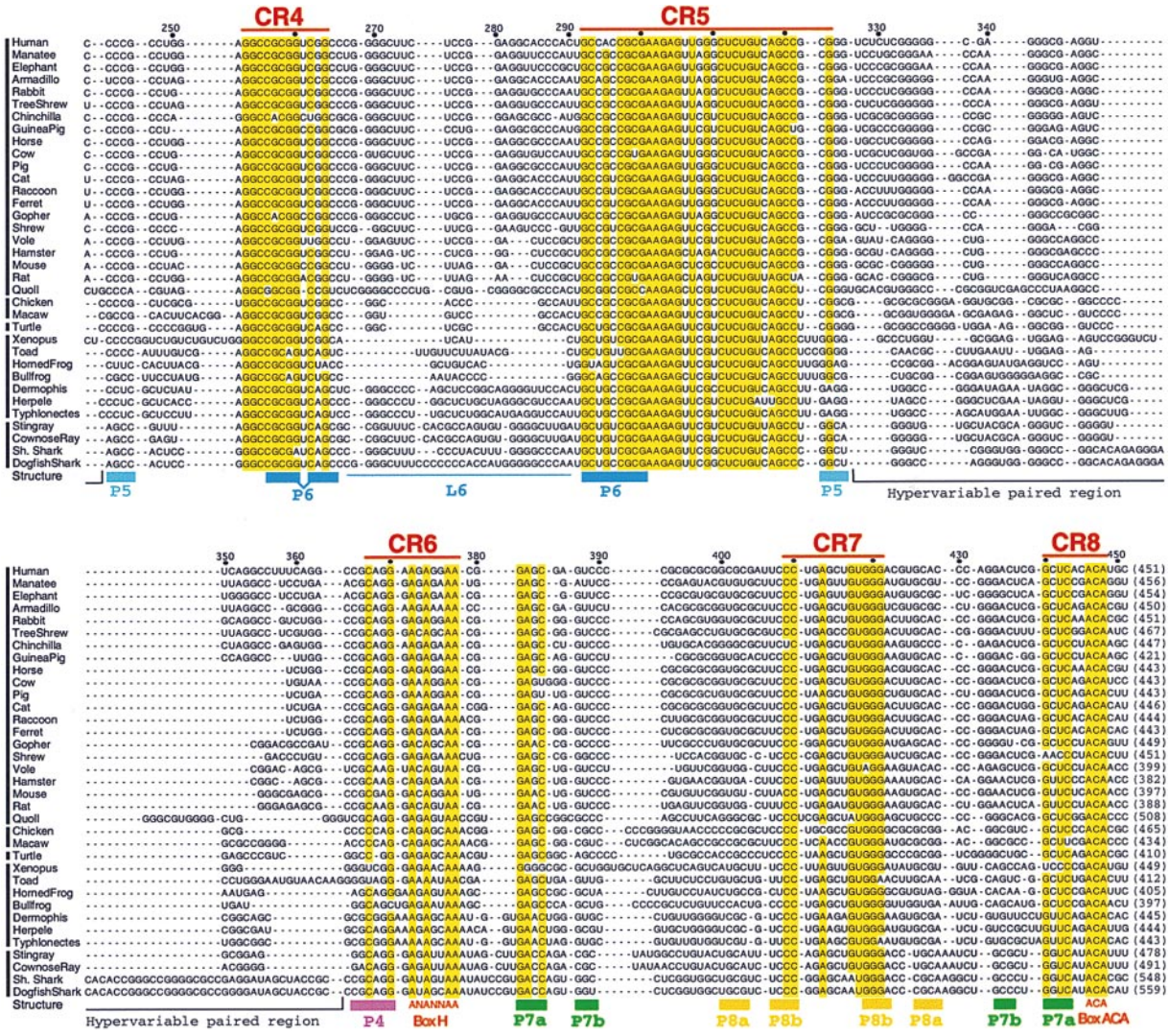

Figure 1. Alignment of Vertebrate Telomerase RNA Sequences

Eight conserved regions (CRs) are indicated with red lines above the aligned sequences; nucleotides with 90% or more of identity are shaded in yellow. Base-paired regions are indicated with thick lines in different colors under the specific sequence elements and the corresponding structural elements for each region are designated. The sequences that encompass the mammal-specific helix P2a.1 are shaded in light blue. Some single-stranded regions, J2a/2b, J2a/3 and L7, and the hypervariable paired regions are indicated by thin lines under the sequence alignment. The consensus sequences of the template region, Box H, and Box ACA motifs are also shown under the alignment. Dashes (-) denote alignment gaps. Every tenth nucleotide of the human sequence is marked with dots above the alignment. The total number of nucleotides in each RNA is indicated at the end of the respective sequence. Five vertical lines to the left of the species names denote five classes of vertebrates. Class Mammalia includes *Homo sapiens* (Human), *Trichechus manatus* (Manatee), *Elephas maximus* (Elephant), *Dasypus novemcinctus* (Armadillo), *Oryctolagus cuniculus* (Rabbit), *Tupaia glis belangeri* (TreeShrew), *Chinchilla brevicaudata* (Chinchilla), *Cavia porcellus* (GuineaPig), *Equus caballus* (Horse), *Bos taurus* (Cow), *Sus scrofa* (Pig), *Felis catus* (Cat), *Procyon lotor* (Raccoon), *Mustela putorius furo* (Ferret), *Suncus murinus* (Shrew), *Geomys breviceps* (Gopher), *Microtus ochrogaster* (Vole), *Cricetulus griseus* (Hamster), *Mus musculus* (Mouse), *Rattus norvegicus* (Rat), and *Dasyurus hallucatus* (Quoll). Class Aves includes *Gallus gallus* (Chicken) and *Anodorhynchus hyacinthinus* (Macaw). Class Reptilia includes *Chelydra serpentina* (Turtle). Class Amphibia includes *Xenopus laevis* (Xenopus), *Bombina japonica* (Toad), *Ceratophrys ornata* (HornedFrog), *Pyxicephalus adspersus* (Bullfrog), *Dermophis mexicanus* (Dermophis), *Herpele squalostoma* (Herpele), and *Typhlonectes natans* (Typhlonectes). Class Chondrichthyes includes *Dasyatis sabina* (Stingray), *Rhinoptera bonasus* (CownoseRay), *Rhizoprionodon porosus* (SharpnoseShark), and *Mustelus canis* (DogfishShark).

Table 1. Base Pairings in the Proposed Telomerase RNA Secondary Structure[a]

Helix	Paired Regions		Number of Base Pairs Supported by Covariation	Noncanonical Base Pairs (Other Than G/U Pair)	
	Base Pair (Human RNA Numbering)	Length (bp)		Type	Frequency[b]
P2a	78–82/137–141	5	4	C/C, C/U, U/U	5/175
P2b	90–98/116–124	9	4	—	—
P3	107–115/174–183 (177 bulged)	9	2	C/A	2/315
P4	211–216/365–370	6	5	C/A, C/C, C/U, G/G	8/210
P5	243–246/323–326	4	4	C/A, C/C, A/A	5/140
P6	256–265/292–300 (261 bulged)	9	5	C/A	5/315
P7a	381–384/440–443	4	3	G/A, C/A	4/140
P7b	387–389/434–436	3	3	G/A, C/A, C/C	6/105
P8a	400–403/426–429	4	4	G/A, C/A, G/G	6/140
P8b	407–410/419–422	4	2	G/A, C/A, C/U, U/U	5/140

[a] Only universal structural elements are included in this table.
[b] Frequencies are shown as the total number of noncanonical base pairs over the total base pairs of the paired region in the 35 sequences.

copies of each gene were cloned by either lambda phage genomic library screening or ligation-mediated PCR (see Experimental Procedures). After cloning the full-length gene, each sequence was further confirmed by PCR amplification and direct sequencing of the complete RNA gene from genomic DNA.

Although the human and mouse telomerase RNAs are encoded by single-copy genes (Blasco et al., 1995; Feng et al., 1995), pseudogenes were found in pig and cow (data not shown). The pig pseudogene had several deletions in highly conserved regions and a mutation in the templating region. The cow pseudogene had a very similar sequence to the previously published sequence (Tsao et al., 1998), but the upstream and downstream flanking sequences differed. Both of the telomerase RNA pseudogenes contained a 3′ terminal poly(dA) tail and lacked conserved promoter elements at the 5′ end, as is typical of pseudogenes (Vanin, 1985; Weiner et al., 1986).

Primary Sequence Comparison

The 35 vertebrate telomerase RNA sequences were aligned using conserved sequences as anchor points (see Experimental Procedures). The sequence alignment revealed eight highly conserved regions. A conserved region was defined as containing at least 3 nt that were identical in all species without a gap of larger than 3 nt. The conserved regions (CRs) were numbered from 5′ to 3′ and designated CR1 through CR8 (Figure 1). The first conserved region (CR1) has the consensus sequence 5′-CUAACCCU-3′ and represents the template region of telomerase RNA that specifies synthesis of the 5′-TTAGGG-3′ telomere repeats in vertebrates. The two longest conserved regions, CR2 and CR5, were used as the targets for degenerate primers in the PCR amplification (see Experimental Procedures). The remaining conserved regions (CR3, CR4, CR6, CR7, and CR8) were used as anchor points for the sequence alignment.

We used conserved sequence elements to predict the 5′ and 3′ end of the telomerase RNAs. The 5′ ends of the human and mouse telomerase RNA transcripts were previously mapped by both RNase-protection and primer-extension analyses (Hinkley et al., 1998). Although the 5′ end of human RNA is 45 nt upstream of the template (CR1) sequence, mouse 5′ end is only 2 nt upstream from the template (CR1) sequence. Biochemical evidence suggests that the human telomerase RNA gene is transcribed by RNA polymerase II (Avilion, 1995; Feng et al., 1995). The upstream region of each vertebrate telomerase RNA gene contains sequence elements typical of RNA polymerase II promoter (Supplemental Figure 1 [see Supplemental Data below]). To predict the transcriptional start sites of the RNA genes, we aligned the conserved CCAAT and TATA promoter elements found upstream of the template sequence. Based on this alignment, three rodent telomerase RNAs (mouse, rat, and hamster) appear to have the short 2 nt sequence upstream of the template region of the RNA (Supplemental Figure 1). Other RNAs have 14 to 52 nt upstream of the template region.

The 3′-end of the human telomerase RNA has been mapped to 3 nt downstream of the CR8 (ACA) motif (Zaug et al., 1996). The Box H/ACA snoRNAs also terminate 3 nt downstream of a consensus ACA motif (see Discussion and Balakin et al., 1996). We therefore used the ACA motif in the alignment to predict the 3′ ends of the telomerase RNAs (Figure 1). Given the predicted 5′ and 3′ ends, the sizes of vertebrate telomerase RNAs vary in length from 382 to 559 nt.

Conserved Secondary Structure of Vertebrate Telomerase RNA

To construct a secondary structure model for telomerase RNA, the aligned sequences were analyzed for nucleotide covariation (see Experimental Procedures). The covariation of a nucleotide pair that maintained Watson-Crick base-pairing was taken as positive support for a paired structural element. In some cases unusual base pairs replaced the canonical Watson-Crick base pairs, but maintained a stable helical structure. Such noncanonical base-pairings, such as G/U, G/A, and C/A, are frequently observed in the secondary structure of other RNAs, such as tRNAs (Sprinzl et al., 1998), ribosomal RNAs (Noller and Woese, 1981; Woese et al., 1983; Gautheret et al., 1994; Gutell et al., 1994), and

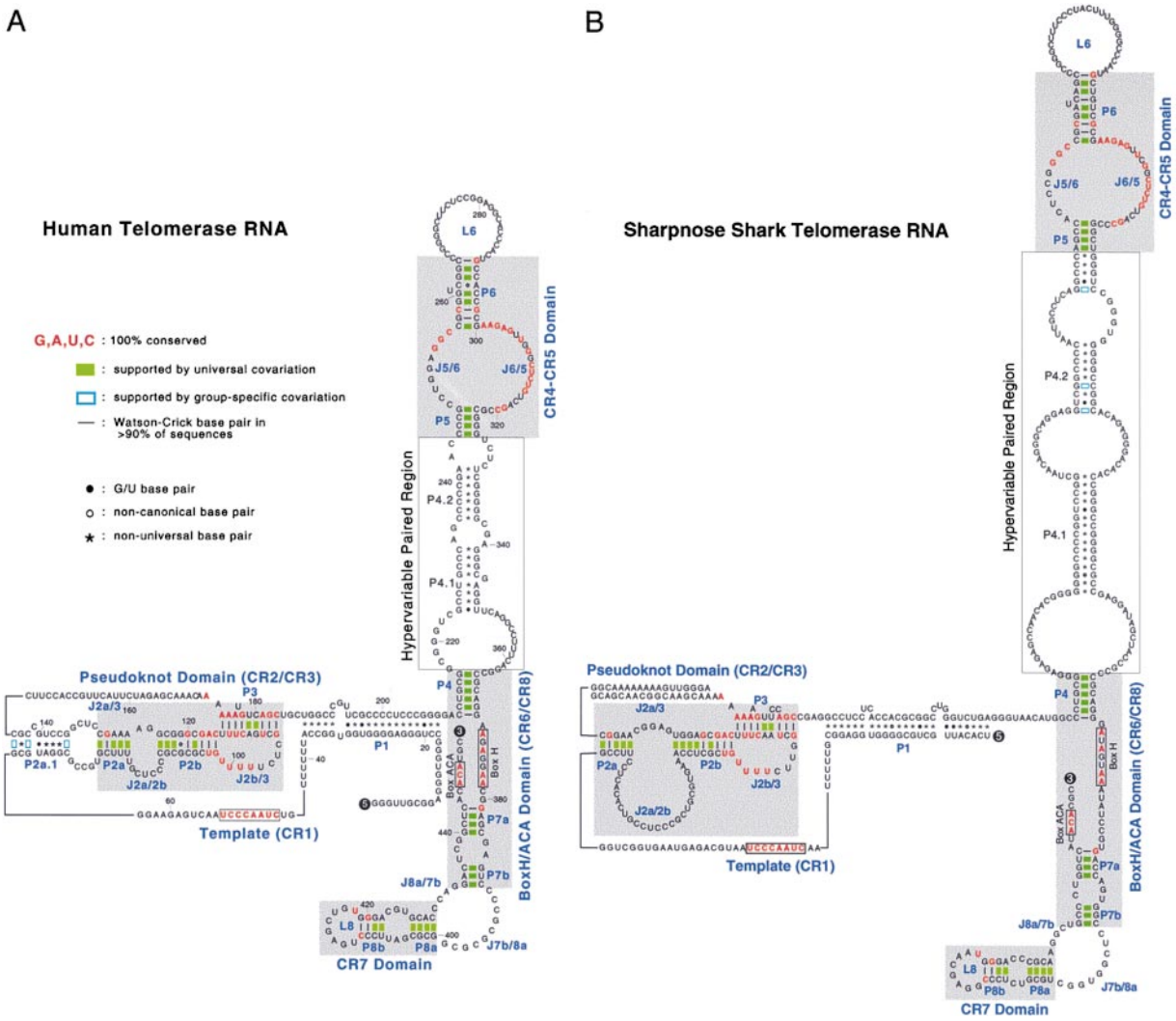

Figure 2. Proposed Secondary Structure of Vertebrate Telomerase RNAs

The proposed secondary structures of (A) human and (B) sharpnose shark telomerase RNAs were determined by phylogenetic comparative analysis as detailed in Experimental Procedures. Paired regions (P) are numbered from 5′ to 3′ as P1–P8. The junction regions (J) between two paired regions are named with reference to the flanking paired regions. Invariant nucleotides are shown with red letters in bold. Base pairs supported by covariation evidence are shown as green filled boxes. Base pairs supported by covariation only within a given group of species are shown as blue open boxes. Dashes represent potential Watson-Crick base pairs that exist in more than 90% of the sequences. Filled circles indicate G/U base pairs, while open circles represent noncanonical pairs. An asterisk between base pairs in a potential paired region represents the nonuniversal base-pairings. The nonuniversal paired regions exist only in an individual class of species and no homologous helical region could be confidently identified in other groups. The four universal structural domains described in the text are shaded in gray and labeled. The template region, Box H and Box ACA motifs are labeled and the conserved nucleotides are boxed. Every twentieth nucleotide of the human RNA is numbered.

RNase P RNAs (James et al., 1988; Brown et al., 1991; Brown et al., 1996). These noncanonical pairings allow possible hydrogen-bonding interactions and were treated as neutral evidence for a helical structure. Finally, rare noncanonical pairs, such as C/C, U/U, and G/G, were considered to be evidence against pairing (see Experimental Procedures and Table 1).

In general, a putative helix is considered supported when covariations occur at two or more positions (James et al., 1988; Pace et al., 1989). Here we propose ten conserved helical regions that are universally present in vertebrate telomerase RNA structure (Table 1). These helices constitute four distinct structural domains: the pseudoknot domain, the CR4-CR5 domain, the Box H/ACA domain, and the CR7 domain. Below we detail each domain of the secondary structure and refer to the human and shark RNA structures as representatives of two distantly related vertebrates (Figures 2A and 2B). These two RNA sequences share only 44% identity and yet fold into a similar structure. The nucleotide numbers given in the text refer to the human RNA structure shown in Figure 2A. Each paired region (P) is numbered from 5′ to 3′, and the junction region (J) between two paired regions is named with reference to the flanking paired regions. For example, the region between helices P2b and P3 is named J2b/3.

The sequence upstream of the template region pairs with the sequence located at nt 187–208 to form helix

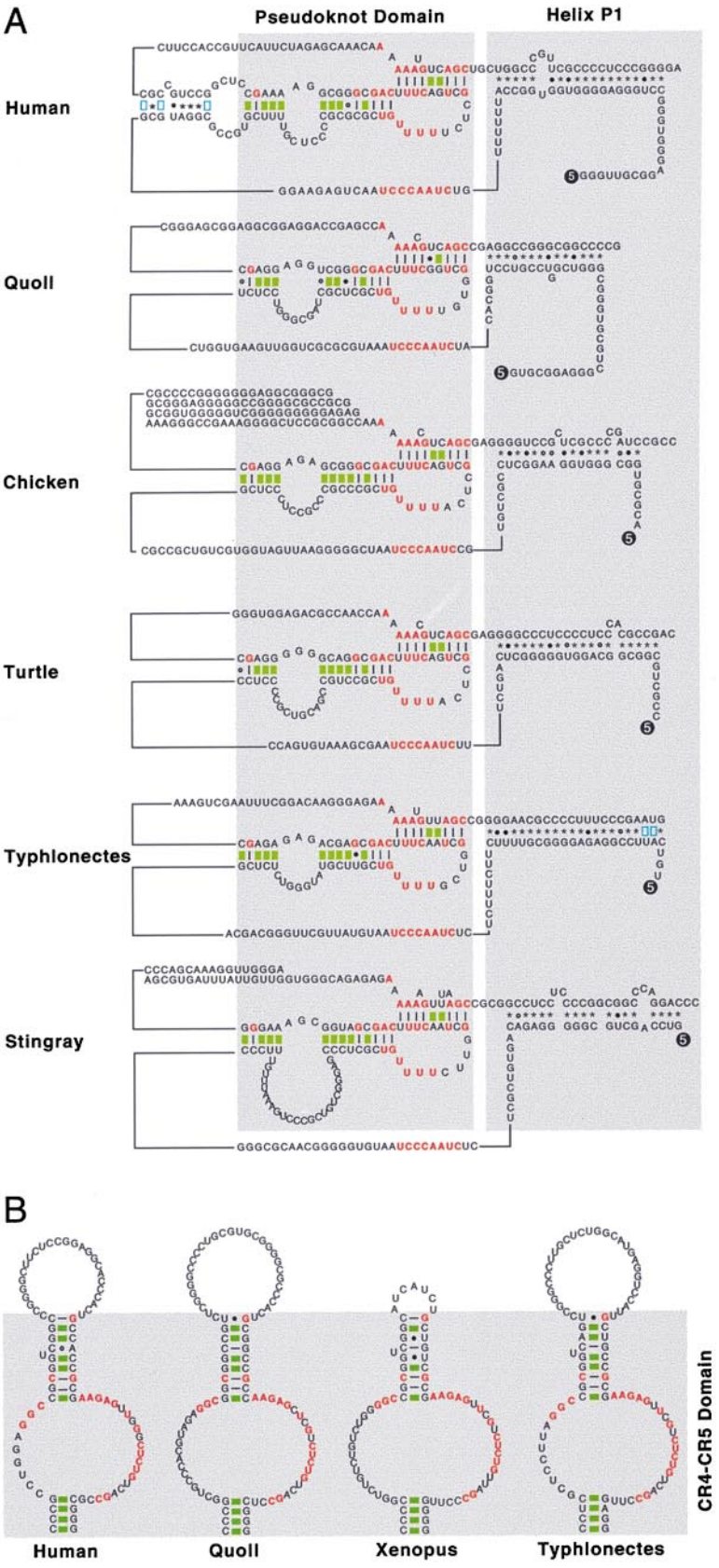

Figure 3. Species Variation of the Secondary Structural Elements

(A) Homologs of the pseudoknot domain and the P1 helix from human, quoll, chicken, turtle, *Typhlonectes* and stingray telomerase RNAs are shown. The pseudoknot domain and helix P1 are both shaded. Invariant nucleotides are shown in red. Base pairs supported by universal and group-specific covariations are shown as green filled boxes and blue opened boxes, respectively, as described for Figure 3.

(B) Homologs of the CR4-CR5 domain from human, quoll, *Xenopus*, and *Typhlonectes* telomerase RNAs are shown.

P1 in human and other vertebrate telomerase RNAs (Figures 2A and 2B). Although a sequence alignment of this region between distantly related groups of species was difficult to establish, group-specific covariations were found and are shown for the respective structures (Figure 3A). The 5′ end of mouse telomerase RNA, and other rodent RNAs, starts just 2 nt upstream of the template region; thus, these species lack the P1 paired region. However in all of the other species, a putative P1 helix can be formed (Figure 3A).

The pseudoknot domain contains the CR2 and CR3 sequences and is established by helices P2a, P2b, and P3. In addition to the base pairs that covary, we included putative base-pairings that extend the helix P3 and allow a coaxial stacking between P2b and P3 (Figures 2 and 3). Such coaxial stacking between two helical regions would significantly stabilize this type of pseudoknot structure (Pleij and Bosch, 1989; ten Dam et al., 1992). The J2b/3 region is conserved not only in sequence but also in length. This length conservation may also be important for maintaining a stable conformation of this pseudoknot element (Figure 3A). The helix P2a.1 was identified in only mammalian RNAs as an extension of the P2a helix (Figure 3A). Within the group of mammalian sequences, where a clear alignment of sequences in this region is possible, three positions in this P2a.1 helix are supported by covariations (Figures 1 and 3A).

The CR4-CR5 domain lies downstream of the pseudoknot domain and consists of a stem-loop structure established by helices P5 and P6. The intervening loop contains the conserved CR4 and CR5 sequence elements.

The Box H/ACA domain of the RNA includes P4, Box H, P7a, P7b, and Box ACA, which form a conserved structure similar to the structure found in the Box H/ACA family of small nucleolar RNAs (snoRNAs) (Ganot et al., 1997b; Mitchell et al., 1999). The conserved sequences of the Box H (CR6) and Box ACA (CR8) motifs are both single-stranded as seen in snoRNAs (Ganot et al., 1997b).

The CR7 domain defined by the highly conserved CR7 sequence contains 2 helices, P8a and P8b, and the L8 loop (Figures 1 and 2). In snoRNAs, this distal stem-loop is not conserved. The sequence and length conservation of the CR7 region suggests that this structure is specific to the vertebrate telomerase RNA.

Species-Specific Variation of the Secondary Structure

The single-stranded region J2a/3 that connects the P2a.1 and P3 helices is remarkably variable in length (Figures 1 and 3A). The size variation of J2a/3 suggests that this domain might play a less important structural role than the other highly conserved sequences.

The region between P4 and P5 is the most variable region in the structure of vertebrate telomerase RNA and is designated the hypervariable paired region (Figures 2A and 2B). The sequence and size variability makes a convincing alignment between distantly related species extremely difficult. However, two potential paired regions and three large internal loops could be identified for each individual class of species (Figure 2). In different vertebrate classes, the ability to base pair is maintained although the length of the paired region varies considerably. Three positions of nucleotide covariation were found in three distantly related groups of species (two rays, two sharks, and three caecilians species), where a reliable sequence alignment was possible (Figure 1 and data not shown). These group-specific covariations support proposed pairings in this hypervariable region (Figure 2B).

The distal loop region of helix P6, designated L6, varies in size and sequence between different classes of vertebrates. In amphibians, the L6 loop is as short as 7 nt, while in sharks and rays, it is as long as 33 nt (Figure 3B). Although no reliable covariations were identified, a potential helix might be able to form within the L6 region in some species.

Between the Box H/ACA domain and the CR7 domain, J8a/7b and J7b/8a together form an asymmetric internal loop, or a bulge (Figure 2). While the J8a/7b region remains 3 to 5 nt long in different species, the J7b/8a varies in length from 8 nt to 17 nt (Figure 1). The size and sequence variation of this internal loop suggests that it does not play a functional role in telomerase RNA.

Discussion

The Conserved Core Structure of Vertebrate Telomerase RNA

We propose a secondary structure model for vertebrate telomerase RNA based on phylogenetic comparative analysis. The most remarkable feature of this structure is the evolutionary conservation of four structural domains: the pseudoknot domain, the CR4-CR5 domain, the Box H/ACA domain and the CR7 domain. By mapping the set of vertebrate sequences onto the core structure, we inferred a minimum consensus telomerase RNA structure that is likely to represent the functional core of vertebrate telomerase RNAs (Figure 4). Each conserved structural domain in the consensus structure might play a distinctive role in either the function, stability, processing, or localization of telomerase RNA. These regions are also good candidates as binding sites for TERT or other telomerase accessory proteins.

This proposed structure of vertebrate telomerase RNA displays striking similarities in overall architecture to the structure of ciliate RNAs (Figure 5). The core structure of ciliate telomerase RNA comprises a template region followed by a pseudoknot structural element, named Helix III. The region 5′ of the template together with the region 3′ of the pseudoknot form Helix I. The P1 helix in the vertebrate RNA structure involves a similar long-range pairing and presumably is a homolog to the ciliate Helix I (Figure 5). The single-stranded template region and the pseudoknot structure are also both present in the ciliate and vertebrate RNAs. Although the vertebrate pseudoknot structure differs from the ciliate version in its complexity, they both belong to the same type of pseudoknot structure (Pleij and Bosch, 1989). Finally, the 3′-helical domain of ciliate RNAs, termed Helix IV, displays significant structural variation among different groups of ciliates (Romero and Blackburn, 1991; Lingner et al., 1994). This variable Helix IV of ciliate RNA may be analogous (or homologous) to the hypervariable paired region in the vertebrate RNA structure (Figure 5). In addition to the regions with structural similarity to ciliate

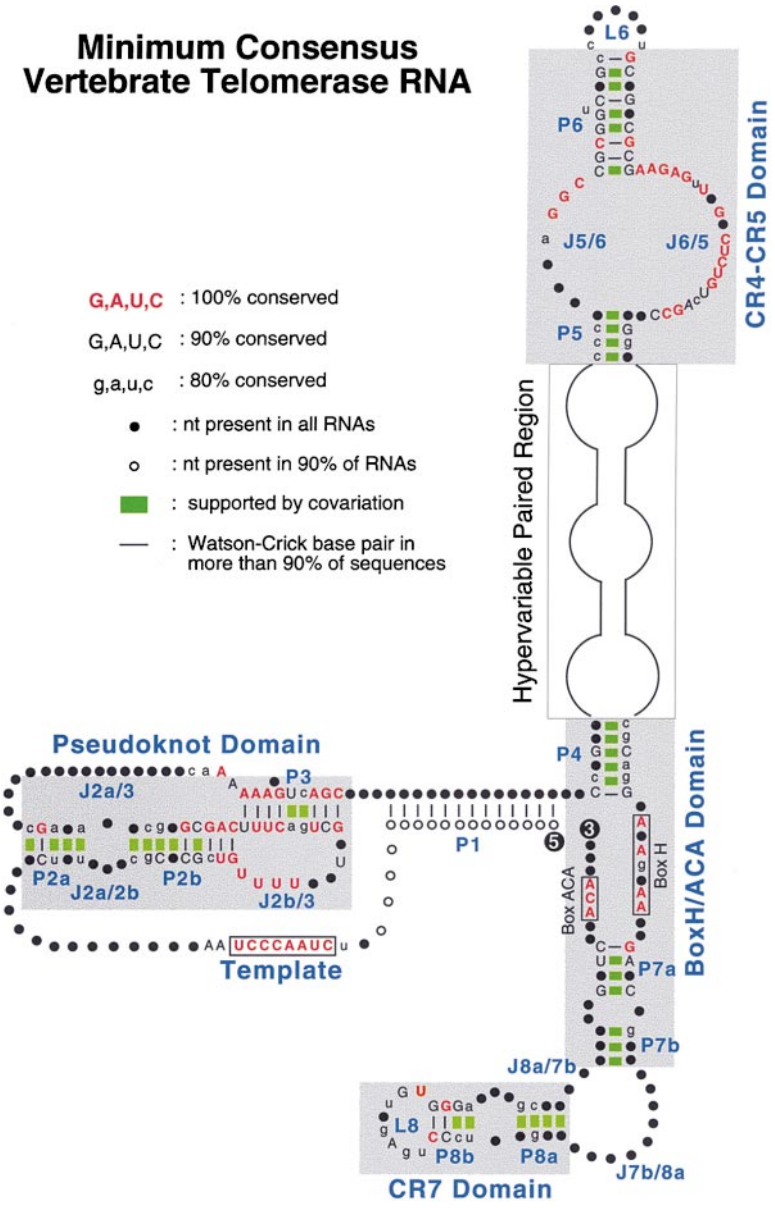

Figure 4. The Minimum Consensus Structure of Vertebrate Telomerase RNA

This minimal consensus structure includes only those nucleotides that are present in all available vertebrate telomerase RNA sequences. Invariant nucleotides are indicated with red capital letters in bold, 90% conserved residues are indicated by black capital letters, and 80% conserved residues are indicated by lowercase letters. Nucleotides that are universally present but vary in identity are indicated by filled circles. Nucleotides that are absent in the 5′ region of the mouse sequences are indicated by open circles. Helices are labeled according to the nomenclature shown in Figure 3. The base-pairings supported by covariation are marked by green filled boxes; the base pairs that show Watson-Crick pairing in more than 90% of sequences are indicated by dashes.

RNA, the vertebrate RNAs possess three additional structural elements not found in the ciliate RNAs: the CR4-CR5 domain, the Box H/ACA domain and the CR7 domain. These unique structures might play some vertebrate-specific roles for telomerase function.

Distinct Roles of the Conserved Structural Domains

The template region of telomerase RNA is essential to provide the sequence information for the addition of telomere repeats onto chromosome ends. The minimal conserved sequence of the template in vertebrate telomerase RNAs is 8 nt in length, CUAACCCU, indicating that this short region is sufficient for template function. In some species there are additional nucleotides that could potentially serve as a template for the synthesis of TTAGGG telomere repeats. As seen with ciliate telomerase RNAs, the region of the template domain that specifies nucleotide addition may vary in different species (Lingner et al., 1994). Determining which nucleotides serve a template function and which are used for primer alignment needs to be tested experimentally (Autexier and Greider, 1995; Gilley et al., 1995; Gilley and Blackburn, 1996).

The evolutionary conservation of the pseudoknot domain suggests that the pseudoknot plays an important role in telomerase function. In the ciliate RNAs, the pseudoknot domain is essential for telomerase assembly in vivo (Gilley and Blackburn, 1999). Experiments with human telomerase RNA have also shown that this pseudoknot structure is required for human telomerase function in vivo. A mutant human telomerase RNA with a 17-base insertion at position 176 failed to reconstitute telomerase activity (Feng et al., 1995; Autexier et al., 1996; Weinrich et al., 1997). This insertion maps in the pseudoknot next to the U-bulge in the helix P3 and likely disrupts the pseudoknot structure (Figure 3A).

A second line of evidence for the importance of the pseudoknot comes from deletion analysis of the human telomerase RNA. Three different assay systems have defined slightly different regions of the human RNA as

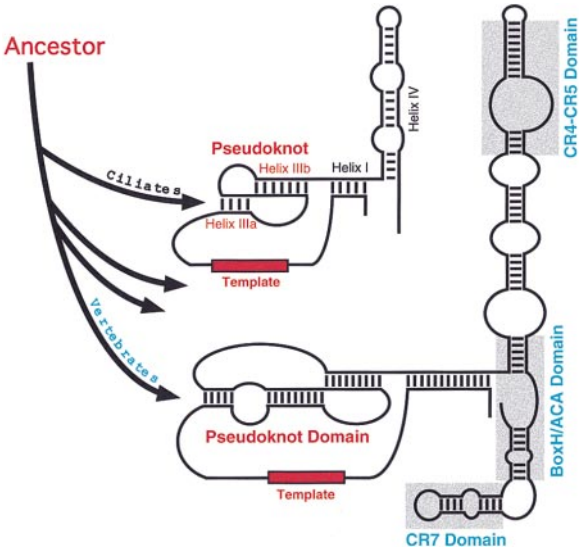

Figure 5. Comparison of Ciliate and Vertebrate Telomerase RNA Structures

The outline of the minimum-consensus structures of ciliate and vertebrate telomerase RNAs are shown. Template regions are indicated with black filled boxes. Vertebrate-specific structural elements are shaded. The diagram on the left illustrates a possible evolutionary course from the ancestral telomerase RNA to ciliate and vertebrate RNAs.

essential for telomerase activity. In each case, the pseudoknot domain was important for telomerase enzymatic function (Autexier et al., 1996; Beattie et al., 1998; Tesmer et al., 1999). Although in *Tetrahymena*, the pseudoknot domain is critical for telomerase assembly in vivo (Gilley and Blackburn, 1999), some in vitro results suggest that, in the assembled telomerase RNPs, the pseudoknot region is not essential for catalysis (Autexier and Greider, 1998; Licht and Collins, 1999).

The P1 helix is absent in mouse and some other rodent RNAs, suggesting that this long-range base-pairing is not essential for enzyme function (Figure 2 and Hinkley et al., 1998). Consistent with this suggestion, the deletion analysis of human telomerase RNA (Autexier et al., 1996; Beattie et al., 1998; Tesmer et al., 1999) showed that most or all of the 5′ sequence that precedes the template region is not required for reconstitution of high levels of telomerase activity. Disruption of the long-range base-pairing in Helix I of the ciliate RNAs also allows telomerase function in vitro (Autexier and Greider, 1998).

The conserved Box H/ACA domain in the vertebrate telomerase is likely to be required for proper 3′-end processing and stability of the RNA (Ganot et al., 1997b; Mitchell et al., 1999). Deletion or mutation of the Box H and Box ACA motifs in human telomerase RNA results in a loss of accumulation of the RNA in cells. In addition, while wild-type RNA localizes to the nucleolus when injected into *Xenopus* oocytes, mutation of the Box ACA motif results in a loss of nucleolar targeting (Narayanan et al., 1999). These results suggest that the conserved motifs of the Box H/ACA domain are required for RNA stability and nucleolar localization.

Although telomerase RNA contains a structural domain with two sequence motifs, Box H and Box ACA, similar to that found in snoRNAs, its function might be distinct from snoRNAs. SnoRNAs participate in site-specific modification of ribosomal RNAs (Tollervey and Kiss, 1997). Base-pairing between the internal loop in the Box H/ACA snoRNA and a conserved region of the ribosomal RNA allows targeted pseudouridylation of the rRNAs (Ganot et al., 1997a; Ni et al., 1997). In telomerase RNAs, two internal loops, J7b/8a-J8a/7b and J4/4.1-J4.1/4, adjacent to the Box H/ACA domain are potential analogs of the pseudouridylation pocket of the snoRNA. In contrast to the functional snoRNAs, there is no sequence complementarity between these two internal loops of telomerase RNA and ribosomal RNAs. In addition, the rRNA-binding pockets of snoRNAs presumably coevolve with their target sequences. Therefore, the sequences of these binding pockets are likely to be as conserved as ribosomal RNAs (Ofengand et al., 1995; Ganot et al., 1997a). However, in the telomerase RNA, neither the sequence nor the length of either internal loop is conserved. Thus, it is unlikely that the Box H/ACA domain of vertebrate telomerase RNA possesses a functional pseudouridylation pocket that targets rRNA, telomerase RNA, or any other RNAs to be modified at a conserved region.

Phylogenetic Comparison of Telomerase RNAs

The phylogenetic relationship of the 35 vertebrate telomerase RNA sequences is consistent with the vertebrate phylogeny based on sequences of other molecules, such as hemoglobin (Czelusniak et al., 1990). When compared to the 18S rRNA in the same species, telomerase RNA sequences display much greater divergence (see Supplemental Figure 2). The larger phylogenetic distance reflects a faster evolutionary rate for the telomerase RNA gene than for the rRNA. A similar rapid evolution of telomerase RNAs was also noted for the ciliates (Romero and Blackburn, 1991; Lingner et al., 1994). This rapid evolutionary rate is consistent with a relatively lower evolutionary pressure on the telomerase enzyme when compared to the ribosome.

Based on the striking similarity of the global RNA structure between ciliates and vertebrates, the essential core structure of this RNA seems to be preserved despite the extensive sequence divergence over a large evolutionary distance. However, given the relatively rapid evolutionary rate of the telomerase RNA, it is not possible to align the sequences of telomerase RNAs of ciliates, yeasts, and mammals. Further, until a phylogenetic secondary structure is defined for the large yeast telomerase RNAs, it is not possible to determine how the yeast RNA structure relates to the structures conserved in the ciliate and vertebrate RNAs.

An Sm protein-binding motif was recently identified in yeast telomerase RNA and shown to be important for Sm protein binding and telomerase activity in vivo (Seto et al., 1999). However, no evolutionarily conserved Sm sequence was identified for vertebrate species. Thus, the utilization of the Sm protein assembly pathway may be a unique feature of yeast telomerase, just as the acquisition of the Box H/ACA domain for 3′ end stability appears to be specific to the vertebrate telomerase RNAs.

The establishment of a secondary structure for telomerase RNA is a prerequisite for an explicit functional

analysis of the telomerase enzyme mechanism. The structure proposed here makes important predictions about which regions of the RNA component are likely to interact with proteins or which might be essential for the telomerase assembly and function. An understanding of the RNA–protein interactions of telomerase ribonucleoprotein will provide important insights into the global assembly and the function of this unusual RNP polymerase enzyme.

Experimental Procedures

Genomic DNA Isolation

50–100 mg of tissue was homogenized in 1 ml of DNAzol reagent (Molecular Research Center, Inc.). Genomic DNA was spooled onto a pipette tip after the addition of 0.5 ml of 100% ethanol. The DNA was washed once in 70% DNAzol and 30% ethanol, and then twice in 95% ethanol. After solubilization in 0.5 ml of 8 mM NaOH, the pH was adjusted to 8.0 and DNA was phenol/chloroform extracted. The DNA was dialyzed in a Slide-A-Lyzer mini dialysis unit (Pierce) overnight against 2 mM Tris-HCl, pH 7.5. For animal whole blood, DNAzol BD reagent was used in the initial extraction in place of DNAzol (Molecular Research Center, Inc.).

The sources of animal tissues used to isolate genomic DNAs are listed below: *Dermophis mexicanus* and *Herpele squalostoma* livers (Drs. James O'Reilly and Alan Savitzky); *Typhlonectes natans* liver (Dr. Sunny Boyd); stingray, cownose ray, and sharpnose shark livers (Dr. John Trant); *Ceratophrys ornata* and *Pyxicephalus adspersus* frog livers (Dr. Stephen Secor); *Mustelus canis* shark liver (Dr. Gavin Naylor); elephant blood (Laura Richman); pig liver (Thomas Morris); ferret liver (Jack Fallenstein); vole liver tissue (Dr. Brian Kirkpatrick); horse blood (Buckshire Corp.); manatee liver (Dr. Scott Wright); raccoon liver (Randy Ruby); shrew liver (Dr. Emilie Rissman); gopher liver (Drs. Rhett Stout and Mark Hafner); guinea pig liver (Dr. Leslie Tung); chinchilla liver (Dr. Lloyd Minor); tree shrew liver (Dr. John Siegwart); armadillo liver (Dr. Richard Truman); turtle liver (Dr. Allen Place). For other species, the genomic DNAs were obtained from the following sources: human, cow, cat, rat, Chinese hamster, chicken, and rabbit genomic DNAs (ClonTech Inc.); toad genomic DNA (Dr. Joseph Gall); *Xenopus laevis* genomic DNA (Dr. Norman Pace); quoll genomic DNA (Dr. Carey Krajewski).

Degenerate PCR

Partial telomerase RNA genes were amplified from genomic DNAs using degenerate PCR. Degenerate primers were initially designed based on the highly conserved sequences between human and mouse telomerase RNAs. New primers were subsequently designed as new sequences were obtained. The final primers used for degenerate PCR are listed below (I represents inosine): forward primers, TR89IF2 (5′-CCGCICGCTGTTTTTCTCGCTGACTTICAGCGGGC-3′), TR89IF5 (5′-CCGGIIGITGTTTTIITIGCTGACTTICAGCG-3′); and reverse primers, TR263R (5′-GCCGICCGCGGCCTIIAGGCGGG-3′), TR320R (5′-GCTIACAGAGICIAICTCTTCICGGCGGCA-3′), TR320R3 (5′-IGGCTIACAGCGICIAICTCTTCICGIIIGCA-3′).

PCR amplifications were carried out in a 50 μl reaction containing 1× Perkin-Elmer Taq buffer (with 1.5 mM $MgCl_2$), 1 μM of each forward and reverse primers, 100 μM dNTPs, 5% acetamide, 1 μg of genomic DNA, and 2.5 U of Taq-Gold DNA polymerase (Perkin-Elmer). Reactions were performed with 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 30 s, 50°C or 55°C for 30 s, and 72°C for 1 min, and one final cycle at 72°C for 7 min. DNA bands with the appropriate size were excised from an agarose gel. DNA was then extracted using QIAquick gel extraction Kit (Qiagen) and sequenced directly without further cloning procedures.

Cloning and Sequencing of the Full-length Telomerase RNA Gene

Ligation-Mediated PCR

To clone the full-length telomerase RNA genes, an adaptor-ligation-meditated PCR method was employed. The construction of adaptor-ligated genomic libraries and the PCR amplification of target sequences were carried out as previously described (Siebert et al., 1995) or using the Universal GenomeWalker Kit (ClonTech).

Phage Genomic Library Screening

The chicken DT40 genomic library in Lambda Fix was a generous gift from Dr. William R. A. Brown. The Chinese hamster ovary genomic library, bovine genomic library, and rat genomic library in EMBL3 SP6/T7 were purchased from ClonTech. The macaw genomic library in Lambda Fix-II was purchased from Stratagene. The phage genomic libraries were screened for the telomerase RNA gene following standard screening procedures (Sambrook et al., 1989). Either a partial sequence of the target gene or a closely related telomerase RNA homolog was used as a probe.

DNA was isolated from the purified single phage clone using Qiagen's Lambda DNA Midi preparation kit. Eluted phage DNA was dialyzed against 1 mM Tris-HCl, pH 7.5, in a Slide-A-Lyzer mini dialysis unit (Pierce) overnight. A genomic fragment containing the putative telomerase RNA gene was isolated and subcloned into a plasmid vector. A 3.7 kb and a 2.5 kb BstYI genomic fragment containing the chicken and macaw telomerase RNA genes, respectively, were subcloned into the BamHI-digested pJLC plasmid vector (Chen et al., 1998). For the Chinese hamster, rat and bovine telomerase RNA genes, DNA fragments were subcloned into the pBluescript plasmid vector. For the rat telomerase RNA gene, two SacI fragments were cloned into pBluescript KS^+, each containing a portion of the gene. For the Chinese hamster telomerase RNA gene and the cow pseudogene, a 5 kb BamHI fragment and a 4 kb EcoRI fragment were subcloned into pBluescript KS^+ plasmid vectors, respectively.

The chicken telomerase RNA gene was cloned by screening a phage genomic library, and six independent clones were identified and sequenced. A very G-rich insert was found between CR2 and CR3, and was absent in macaw and other vertebrate sequences. We confirmed that this sequence was present in all six independent phage isolates by Southern blotting (data not shown). In addition, confirmation PCR analysis of genomic DNA from five different chicken breeds indicated that this insert is present in all other chicken genomes and is not a cloning artifact.

Genomic PCR to Confirm Gene Sequence

To assure that our clones represented the correct sequence of each gene, full-length telomerase RNA genes from each species were reamplified from the genomic DNA using species-specific primers targeted to upstream and downstream regions flanking the RNA gene. The confirmation PCR was performed as described for the degenerate PCR except only 0.1 μg of genomic DNA was used and fewer cycles were carried out. Amplified PCR products were gel-purified and sequenced directly to avoid sequence errors that might occur in any given PCR product. PCR fragments were sequenced in both directions. For some sequences, apparent allelic polymorphisms were found. In these cases, other independent PCR products were sequenced for further confirmation. Because of their high GC contents, some telomerase RNA genes were resistant to routine PCR amplification. The PCR conditions employed to amplify these genes were specially modified using the Advantage-GC genomic Polymerase Mix (ClonTech) and GC-Melt reagent (ClonTech).

All of the telomerase RNA gene sequences used in this study were confirmed including those previously published. Thus, our cow telomerase RNA sequence has additional sequence at the 5′ end of the RNA gene that was not reported in the previously published sequence (Tsao et al., 1998). All isolated telomerase RNA sequences were deposited into GenBank with accession numbers from AF221906 to AF221942.

Sequence Alignment and Phylogenetic Comparative Analysis

Telomerase RNA sequences were manually aligned using SeqApp v1.9, a sequence-editing program (available at http://iubio.bio.indiana.edu). The sequences of closest relatives were aligned first on the basis of primary sequence similarity. Each group of aligned sequences was then aligned against each of the other groups. Sets of highly conserved nucleotides were identified and used as markers for aligning the more variable regions. For the extremely divergent regions, common secondary structural elements were predicted for each group of species using Zuker's thermodynamic algorithm for RNA structure prediction (Mathews et al., 1999). Finally, the variable sequences with no similarity were aligned based on their common secondary structure. The alignment was refined repeatedly as the

secondary structure model was developed. Comparative sequence analysis of aligned sequence was carried out as described (Pace et al., 1989; Larsen and Zwieb, 1991; Gutell et al., 1994). In the proposed structure, we distinguish between base pairs that are supported by covariation, and those that are not supported but are not contradicted. Nucleotide covariation was identified when the identities of any two nucleotides varied in concert in different sequences. If these two covarying nucleotides maintain Watson-Crick base-pairing, we assigned this as positive evidence for the existence of a base pair. However, in some cases mismatches of two nucleotides were observed and treated as either neutral or negative evidences. As a general rule, we included a base pair when there was more positive evidence for the pairing than negative evidence against it. If a base pair was supported in only one group of sequences, it was designated as a group-specific covariation.

Supplemental Data

Supplemental Figures 1 and 2 are available online at http://www.cell.com/cgi/content/full/100/5/503/DC1.

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GenBank Accession Numbers

All isolated telomerase RNA sequences were deposited into GenBank with accession numbers from AF221906 to AF221942.