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The mouse telomerase RNA 5′-end lies just upstream of the telomerase template sequence

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ABSTRACT

Telomerase is a ribonucleoprotein enzyme with an essential RNA component. Embedded within the telomerase RNA is a template sequence for telomere synthesis. We have characterized the structure of the 5′ regions of the human and mouse telomerase-RNA genes, and have found a striking difference in the location of the template sequence: Whereas the 5′-end of the human telomerase RNA lies 45 nt from the telomerase-RNA template sequence, the 5′-end of the mouse telomerase RNA lies just 2 nt from the telomerase-RNA template sequence. Analysis of genomic sequences flanking the 5′-end of the human and mouse telomerase RNA-coding sequences reveals similar promoter-element arrangements typical of mRNA-type promoters: a TATA box-like element and an upstream region containing a consensus CCAAT box. This putative promoter structure contrasts with that of the ciliate telomerase-RNA genes whose structure resembles RNA polymerase III U6 small nuclear RNA (snRNA) promoters. These and other comparisons suggest that, during evolution, both the RNA-polymerase specificity of telomerase RNA-gene promoters and, more recently, the position of the template sequence in the telomerase RNA changed.

INTRODUCTION

Due to the mechanism by which DNA is replicated, the ends of chromosomes would become progressively shorter during successive rounds of replication if they were not actively maintained. One way chromosomal ends are maintained is by *de novo* addition of short G-rich repeats to their 3′-ends by the specialized DNA polymerase telomerase. Telomerase is a ribonucleoprotein complex, in which a single RNA molecule and protein components are essential for activity. In organisms as diverse as ciliates, yeast and vertebrates, the telomerase RNA sequence contains a ‘template’ region complementary to the sequence of the telomeric repeats. In all organisms analyzed to date this template region is embedded in the telomerase RNA sequence and is responsible for both substrate alignment and for templating addition of the telomeric repeats (for review, see 1).

In ciliates, the telomerase-RNA gene is transcribed by RNA polymerase III and contains a promoter reminiscent of U6 small nuclear RNA (snRNA) genes (2,3). To characterize vertebrate telomerase RNA-gene transcription, we have mapped the mouse telomerase-RNA 5′ terminus and the human (4) and mouse (5) telomerase-RNA gene sequences flanking the telomerase-RNA transcriptional start site. The human and mouse telomerase-RNA gene sequences are related but, in contrast to the ciliate genes, reveal sequence elements typical of RNA polymerase II mRNA-type promoters. Further to our surprise, although these are two closely related species, the position of the transcriptional start site relative to the template-encoding sequence is very different. Whereas the human transcriptional start site lies 45 bp upstream of template-encoding sequence (4), the mouse transcriptional start site lies only 2 bp upstream of the template-encoding sequence.

MATERIALS AND METHODS

Plasmids and oligonucleotides

The molecular cloning of the mouse telomerase-RNA gene has been described (5). Molecular cloning of the human telomerase-RNA gene will be described in detail elsewhere (J.Feng, W.D.Funk, W.H.Andrews and B.Villeponteau, unpublished results). DNA sequence analysis was performed by the dideoxy method (6). Human (HoTel series) and mouse (MoTel series) telomerase-RNA gene-specific primers were synthesized. The HoTel-3 and MoTel-3 primers contain a T7 RNA polymerase promoter at the 5′-end (ATTAATACGACTCACTATAGGGAGA) followed by the gene-specific sequences listed below. The 5′- and 3′-ends for each primer are relative to the transcriptional start site for each gene; the 3′-ends of the resulting human and mouse probes are equidistant from the template sequence. HoTel series, HoTel-3: +197 CGAACGGGCCAGCAGCTGA +179; HoTel-5: –224

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GTTTCACCTTTAAAGAA –208; HoTel-11: –8 AGCGCACCG-GGTTGCGG +9; HoTel-12: +15 GGGCCTGGGAGGGGTGG +31; HoTel-13: +37 ATTTTTTGTCTAACCCTAACTG +58; HoTel-14: +59 AGAAGGGCGTAGGCGCC +75; HoTel-15: +81 TTTGCTCCCCGCGCGCT +97; MoTel series, MoTel-3: +161 GAACCTGGAGCTCCTGCGC +143; MoTel-5: –267 CCTACT-TTCAACTCTAG –251; MoTel-11: –51 AATCAGCGCGCGCC-ATG –35; MoTel-12: –29 TTTAAGGTCGAGGGCGG –13; MoTel-13: –7 CCTCGGCACCTAACCCT +10; MoTel-14: +16 TCATTAGCTGTGGGTTC +32; MoTel-15: +38 TTTTGTTCTC-CGCCCGC +54; MoTel-24: +141 GACGTTTGTTTTTGAGGC-TCGGGAACGCCG +112

RNase-protection analysis

Telomerase RNA was isolated from human HeLa and mouse NIH3T3 cells and probed with RNase A and T_1 as described (7). RNase-protection analysis was performed with radiolabeled antisense RNA probes and the protected fragments identified by electrophoresis through 6% denaturing polyacrylamide gels. α-^{32}P-Labeled RNA probes were synthesized by *in vitro* transcription with T7 RNA polymerase. Templates for *in vitro* transcription were generated by PCR with the 3′ gene-specific T7 RNA polymerase promoter-containing HoTel-3 or MoTel-3 primer and one of the 5′ HoTel or MoTel primers (primers 5 and 11–15).

Primer-extension analysis

Primer-extension reactions were modified from (8). Total NIH3T3 RNA (30 µg) was hybridized overnight at 60°C in 30 µl 1× hybridization buffer (40 mM PIPES, pH 6.7, 400 mM NaCl, 1 mM EDTA, 80% formamide) containing 1×10^6 c.p.m. of 5′-end-labeled mouse-specific MoTel-24 primer. After hybridization, the sample was precipitated with ethanol and resuspended for reverse transcription in 30 µl 50 mM Tris, pH 7.6, 60 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 5 mM dNTPs, 40 U RNAsin and 100 U Mu-MLV reverse transcriptase. The reaction was incubated 2 hr at 37°C after which the cDNA was precipitated with ethanol, resuspended in 4 µl 9 M urea/1% SDS and 16 µl 100% formamide/10 mM EDTA, and incubated 10 min at 85°C prior to loading onto a 6% denaturing polyacrylamide gel. To map the cDNA extension product, a dideoxy-sequencing ladder was generated using the same 5′-end-labeled MoTel-24 primer and was loaded beside the primer extension products.

RESULTS

In preliminary experiments using an RNase-protection assay to measure mouse telomerase RNA expression in NIH3T3 cells, we observed a protected product which migrated faster during polyacrylamide gel electrophoresis than predicted from the previously mapped human (4) and mouse (5) telomerase-RNA 5′-ends. The faster migration of the mouse telomerase RNA-protected probe could have resulted from (i) an unusually stable secondary structure causing anomalous migration during electrophoresis, or (ii) an alternate transcriptional start site lying downstream of the previously characterized site (5). To discriminate between these two hypotheses, we used an RNase-protection strategy that can distinguish between anomalous migration and an alternate start site.

RNase-protection analysis of the 5′-end of human and mouse telomerase RNA

Figure 1A illustrates the RNase-protection strategy, showing the predicted results for two telomerase-RNA transcripts starting at different positions, either distal or proximal to the telomerase template-encoding sequence. In this procedure, human or mouse telomerase RNA is hybridized to a nested set of six species-specific antisense probes (Probes 1–6) all sharing the same 5′-end but progressively shorter at the 3′-end. After RNase digestion, all of the probes extending past the 5′-end of the telomerase RNA will yield a protected fragment of equal size corresponding to the 5′-end of the telomerase RNA (arrows), whereas probes that do not extend to the 5′-end of the telomerase RNA will yield protected fragments corresponding to the size of the individual probes. Thus, for the distal start site in Figure 1A, Probes 1 and 2 will be digested to the same size, whereas Probes 3–6 will be progressively shorter. In contrast, for the proximal start site, Probes 1–4 will be digested to the same size, and only Probes 5 and 6 will be progressively shorter. Because these results depend on the position of the 3′-end of the antisense probes and not the intrinsic mobility of the protected fragments during electrophoresis, this strategy is not sensitive to an unusual secondary structure.

Figure 1B shows the results of mapping the 5′-end of the human (lanes 1–6) and mouse (lanes 7–12) telomerase RNAs with matched sets of human- and mouse-specific probes. RNase protection by human telomerase RNA of the two longest human-specific probes both generated digestion products of ~200 nt (Fig. 1B, lanes 1 and 2, arrow), indicating that these two probes extend past the 5′-end of the human telomerase RNA. In contrast, RNase protection of each of the remaining four progressively shorter probes by the human RNA, resulted in progressively shorter protected fragments (lanes 3–6), indicating that these probes do not extend past the 5′-end of the human telomerase RNA. The size of the protected fragments is consistent with the 5′-end originally defined by a primer-extension procedure: 45 nt upstream of the human template-encoding sequence (4).

Mouse telomerase RNA and the set of mouse-specific antisense probes generated a different RNase protection pattern (Fig. 1B, lanes 7–12). In this case, RNase protection by mouse RNA generated the same size digestion product for the four longest mouse-specific probes (160–170 nt; lanes 7–10; arrow), indicating that these four probes extend past the 5′-end of the mouse telomerase RNA. Only the two shortest probes, neither of which extends into the template-encoding sequence (Fig. 1A), did not extend past the 5′-end of the mouse telomerase RNA (lanes 11 and 12). Because the 3′-end of the shortest probe to be digested lies only 9 nt upstream of the template-encoding sequence (lane 10), these results indicate that the 5′-end of the mouse telomerase RNA lies very close to the telomerase template sequence.

Primer-extension analysis of the mouse telomerase RNA 5′-end

To map the 5′-end of the mouse telomerase RNA more precisely and to ensure that the RNase protection products were the result of the 5′-end of the telomerase RNA, and not, for example, the result of a splicing event, we performed a primer-extension analysis as shown in Figure 2. We used RNA isolated from NIH3T3 cells and a 5′-end labeled mouse-specific oligonucleotide primer (MoTel 24). The primer extension reaction resulted in a major extension product in the region corresponding to the

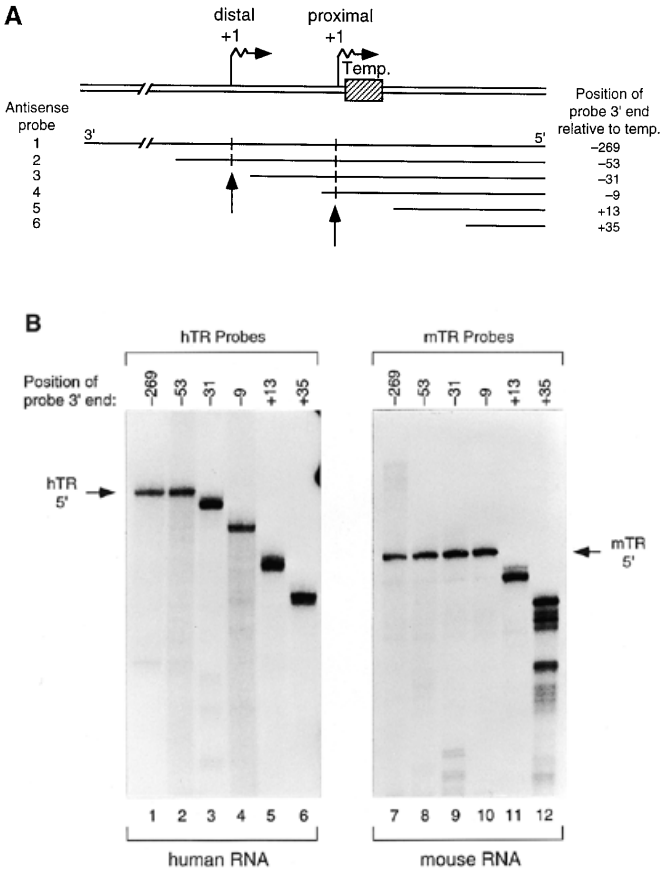

Figure 1. The 5′-end of the mouse telomerase RNA is closer to the template sequence than the 5′-end of the human telomerase RNA. (**A**) Schematic diagram of an RNase protection strategy to map the 5′-end of telomerase RNA. The double-lines represent a telomerase RNA gene with two possible 5′-ends (wavy arrows; proximal +1 and distal +1) relative to the template sequence (hatched box; Temp). Nested sets of six antisense species-specific probes (numbered relative to the 5′-end of the template sequence) all sharing the same 5′-end but progressively shorter at the 3′-end were generated. After digestion with RNase, probes that extend past the 5′-end of the human or mouse telomerase RNA will yield the same sized protected fragment corresponding to the 5′-end of the telomerase RNA (arrows) whereas probes that do not extend to the 5′-end of the telomerase RNA will yield different sized protected fragments corresponding to the size of the probe itself. (**B**) RNase protection analysis of human and mouse telomerase RNA. Six human (lanes 1–6; hTR probes) or mouse (7–12; mTR probes) species-specific telomerase RNA probes were used for RNA protection analysis with telomerase RNA isolated from human HeLa (human RNA) or mouse NIH3T3 (mouse RNA) cells. The position of the protected fragment corresponding to the 5′-end of the human (hTR 5′) or mouse (mTR 5′) telomerase RNA is indicated (arrow).

5′-end indicated by the RNase-protection analysis (Fig. 2, lane 1). Comparison with a parallel DNA-sequencing ladder (Fig. 2, lanes 2–5) reveals that the 3′-end of this extension product corresponds to just 2 nt upstream of the telomerase-RNA template sequence. This extension product was probably missed before (5) because the labeled primer used for extension analysis was directed to the template sequence, and thus was too close to the telomerase RNA 5′-end to allow detection of the extension product over the background of unextended primer. Together, the RNase-protection (Fig. 1) and primer-extension (Fig. 2; 4) indicate that the transcriptional start site for the human telomerase-RNA gene lies 45 bp

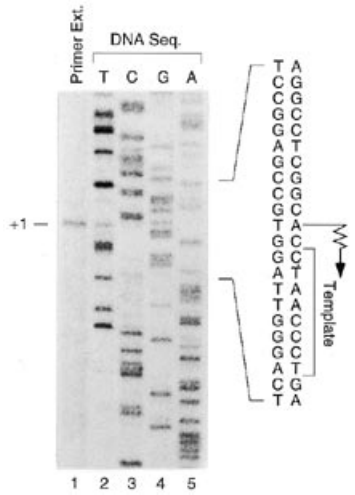

Figure 2. The transcriptional start site of telomerase-RNA gene is 2 nt upstream of the telomerase template sequence. A 5′-end-labeled mouse telomerase RNA-specific primer (MoTel 24) was used in a primer extension assay with telomerase RNA from mouse NIH3T3 cells (lane 1). The extended product mapping the 5′-end of the mouse telomerase RNA is indicated (+1). The same mouse-specific primer was used in a dideoxy DNA sequencing reaction to generate sequence from the antisense strand of the mouse telomerase-RNA gene (lanes 2–5). The sequence of the region encompassing the position of the primer-extension product (wavy arrow) and the telomerase template sequence (Template) is shown to the right of the sequencing reaction.

upstream of the template-encoding sequence and for the mouse telomerase-RNA gene lies 2 bp upstream of the template-encoding sequence.

DNA-sequence analysis of human and mouse telomerase RNA promoter regions

Identification of the likely transcriptional start sites for the human and mouse telomerase-RNA genes suggests that the regions upstream of these sites represent promoter sequences. To compare the human and mouse telomerase RNA-gene promoters, we determined the DNA sequence of these regions as shown in Figure 3. In Figure 3A, the human and mouse telomerase RNA-gene sequences have been aligned according to the transcriptional start sites (wavy arrow). Even though the position of the transcriptional start sites relative to the template-encoding sequence varies greatly between these species (from 45 to 2 bp upstream), the upstream promoter sequences exhibit considerable similarity (see bold and underlined residues). Significantly, this sequence similarity is clustered in two regions, which we refer to as Regions I and II (Fig. 3A).

Region I lies 19–30 bp and 19–31 bp upstream of the transcriptional start site of the human and mouse telomerase-RNA genes, respectively. The position and sequence of the 5′ region of this similarity, $T^{A}/_{T}T^{T}/_{A}TAAG$, suggests that it represents a TATA box, a key basal *cis*-regulatory element shared by many RNA polymerase II promoters and some RNA polymerase III promoters (e.g., the U6 snRNA promoter). Region II lies upstream of the putative TATA box, 47–64 bp upstream of the transcriptional start site in the human gene and 42–59 bp upstream in the mouse gene. This region of sequence similarity contains a consensus CCAAT box in both promoters. The CCAAT box is a promoter-specific

cis-regulatory element found in a variety of RNA polymerase II mRNA promoters and recognized by numerous different transcription factors (reviewed in 9). Significantly, although the telomerase RNA is a 'small nuclear RNA' there is no evident PSE promoter element as is found in small nuclear RNA (snRNA) promoters (10). Figure 3B summarizes our findings, highlighting the arrangement of conserved elements in the human and mouse telomerase-RNA gene sequences. Further studies will be required to determine the sequences important for transcription of the human and mouse telomerase-RNA genes.

DISCUSSION

By a combination of RNase-protection and primer-extension analyses, we have mapped the 5′-end of the mouse telomerase RNA and determined the DNA sequence of the 5′ flanking sequences of the human and mouse telomerase-RNA genes. For both the human (4; this study) and mouse (this study) telomerase RNAs, the RNase-protection and primer-extension analyses identified congruent 5′-ends suggesting that these 5′-ends correspond to the transcriptional start sites for these genes. These analyses reveal three important findings: (i) unlike all other known telomerase RNAs, the mouse telomerase RNA 5′-end lies just upstream of the telomerase template sequence; (ii) although the position of the human and mouse telomerase-RNA transcriptional start sites varies greatly with respect to the template-encoding sequence, the human and mouse telomerase-RNA genes contain related upstream flanking sequences and (iii) the human and mouse telomerase-RNA gene upstream flanking sequences contain TATA box and CCAAT box sequence elements typical of RNA polymerase II mRNA promoters (Fig. 3).

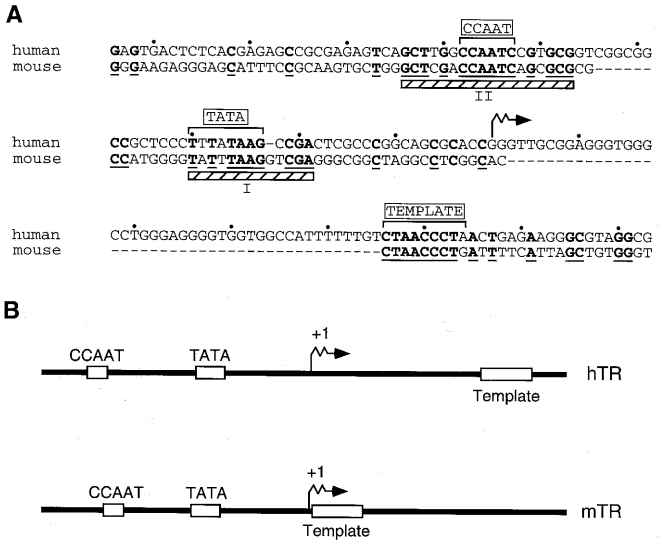

Figure 3. The human and mouse telomerase-RNA genes have similar promoter sequences. (**A**) Sequence alignment of the promoter and template-encoding regions of the human and mouse telomerase-RNA genes. The sequences are aligned relative to the transcriptional start site of each gene (wavy arrow). Gaps (dashes) and identical residues (bold and underlined) are indicated. The telomerase template-encoding sequence (TEMPLATE) as well as two regions of similarity (I and II) containing putative TATA and CCAAT boxes, respectively, are indicated for each gene. (**B**) Schematic diagram of the human and mouse telomerase-RNA genes emphasizing the different position of the template-encoding sequence (Template) to the transcriptional start site (wavy arrow; +1) in each gene. The position of putative CCAAT and TATA boxes is also indicated.

The telomerase RNA 5′-end can lie just upstream of the template sequence

The finding that the mouse telomerase RNA 5′-end lies very close to the template sequence shows that, in at least one organism, extended sequences upstream of the template sequence are probably not essential for telomerase function. This finding was unexpected because in all other organisms examined the telomerase RNA template sequence is located 35 or more nucleotides from the 5′-end (4,11–15). What effect might the position of the template have on telomerase activity?

Although with the ciliate *Tetrahymena* telomerase RNA deletion of as little as 19 nt from the 5′-end results in a loss of telomerase activity (C. Autexier and C.W. Greider, submitted), with human telomerase RNA removal of the first 43 nt 5′ of the template sequence leads to only some, not complete, loss of activity (16). These results, together with the proximity of the mouse telomerase RNA 5′-end to the template sequence, suggest that, in contrast to ciliates, the mammalian telomerase RNA sequences 5′ of the template sequence are not essential for telomerase function. They may, however, be important to modulate the function of telomerase. *In vitro*, mouse telomerase is less processive than human telomerase (17). Perhaps, the sequences 5′ of the template sequence in the human telomerase RNA help stabilize the correct folding of the telomerase RNA or aid in interactions either with other telomerase components or with the chromosome substrate; such interactions may afford greater processivity to the human enzyme.

The sequences 5′ of the human and mouse telomerase-RNA genes share conserved elements found in RNA polymerase II mRNA-type promoters

Our analysis of sequences 5′ of two mammalian telomerase-RNA genes, the human and mouse genes, reveals interesting similarities and differences with the 5′ flanking sequences of the distantly related ciliate telomerase-RNA genes. Analysis of the polymerase specificity of ciliate telomerase RNA-gene transcription indicates that these genes are transcribed by RNA polymerase III (2). Consistent with this specificity, comparison of the sequences 5′ of many ciliate telomerase-RNA genes (11–14) reveals basal promoter elements similar to those found in the ciliate RNA-polymerase III U6 snRNA-gene promoter: a TATA-like box and a USE, a sequence motif related to the snRNA-specific human PSE basal element (3). The RNA polymerase III specificity of U6 gene transcription is governed by the combination and arrangement of the PSE and TATA-box elements in these promoters (reviewed in 10). The sequence element arrangement and RNA polymerase III specificity of the ciliate telomerase-RNA promoters suggest that transcription of the ciliate telomerase-RNA genes is similar to that of the U6 snRNA gene.

The sequence of the mammalian telomerase-RNA gene 5′ regions shows that they share with the ciliate telomerase-RNA genes a TATA box-like element about 25 bp upstream of the putative transcriptional start site. In contrast to the ciliate genes, however, there is no evident snRNA gene-like PSE element further upstream. In its place is an extended region of sequence similarity specific to mouse and human: the 18 bp sequence from

–47 to –64 in humans and –42 to –59 in mice is 78% identical (14 out of 18 bp). This region contains a perfectly conserved CCAAT-box sequence, a promoter element not found in either RNA polymerase II or III snRNA-gene promoters but typical of RNA polymerase II mRNA-type promoters. These results suggest that, in contrast to the ciliate telomerase-RNA genes, the mammalian genes are transcribed by RNA polymerase II.

Consistent with the hypothesis that the mammalian telomerase-RNA genes are transcribed by RNA polymerase II, the mammalian telomerase-RNA genes contain multiple runs of four and more T residues (4,5). Such T-residue runs, which are not found in the ciliate telomerase-RNA genes (11–14), terminate transcription by RNA polymerase III and, therefore, indicate that the mammalian telomerase-RNA genes are unlikely to be transcribed by RNA polymerase III.

Together, the sequence analyses of the ciliate and mammalian telomerase-RNA genes suggest that, by an exchange of PSE- and CCAAT box-containing sequences upstream of a conserved TATA box, the polymerase specificity of ciliate and mammalian telomerase-RNA gene transcription was changed. A change in polymerase specificity would not be unprecedented: the U3 snRNA gene is transcribed by RNA polymerase III in plants, but by RNA polymerase II in vertebrates and lower eukaryotes (18).

Why might the ciliate and mammalian telomerase-RNA genes be transcribed by different RNA polymerases? We suggest two mutually compatible explanations: differences in the levels of promoter activity and differences in the requirements for cell-specific transcription in the different organisms.

First, during ciliate development thousands of subchromosomal fragments lacking telomeric repeats are generated by site-directed cleavage and amplification of micronuclear DNA (reviewed in 19). Telomerase is responsible for addition of repeats to these subchromosomal fragments thereby stabilizing them (20). Because of the large number of chromosomal ends generated during development, ciliates are expected to have more telomerase than other organisms. Consistent with this idea, both telomerase RNA and telomerase activity are much higher in Tetrahymena than in human cells (21,22; C.Greider, unpublished results). The promoters of snRNA genes are among the most active producing ~2–3 $\times$ 10^6 snRNA transcripts per cell per generation (23) and, therefore, ciliates may carry snRNA-like telomerase-RNA promoters because they are capable of producing large numbers of transcripts. In contrast, having many fewer telomeres, mammalian cells may not require a promoter with such high activity.

Instead, in mammals telomerase RNA-gene transcription may need to be regulated more stringently than in ciliates. Because snRNAs are required for mRNA splicing, most snRNA genes are ubiquitously expressed. In contrast, the expression of most mRNA-encoding genes is restricted temporally and spatially in the organism: an mRNA-type promoter could afford the mammalian telomerase-RNA gene greater flexibility to produce more complex and restricted expression patterns than are required in the ciliates.

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