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A Single Telomerase RNA Is Sufficient for the Synthesis of Variable Telomeric DNA Repeats in Ciliates of the Genus *Paramecium*

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***Paramecium* telomeric DNA consists largely of a random distribution of TTGGGG and TTTGGG repeats. Given the precise nature of other ciliate telomerases, it has been postulated that there are two distinct types of the *Paramecium* enzyme, each synthesizing perfect telomeric repeats: one with a template RNA that specifies the addition of TTTGGG and the second dictating the synthesis of TTGGGG repeats. We have cloned and sequenced telomerase RNA genes from *Paramecium tetraurelia*, *P. primaurelia*, *P. multimicronucleatum*, and *P. caudatum*. Surprisingly, a single gene encodes telomerase RNA in all four species, although an apparently nontranscribed pseudogene is also present in the genome of *P. primaurelia*. The overall lengths of the telomerase RNAs range between 202 and 209 nucleotides, and they can be folded into a conserved secondary structure similar to that derived for other ciliate RNAs. All *Paramecium* telomerase RNAs examined include a template specific for the synthesis of TTGGGG telomeric repeats, which has not been posttranscriptionally edited to account for the conventional synthesis of TTTGGG repeats. On the basis of these results, possible mechanisms for the synthesis of variable telomeric repeats by *Paramecium* telomerase are discussed.**

Telomeres, the DNA-protein structures found at the termini of eukaryotic chromosomes, are required for the complete replication and stable maintenance of chromosomes (reviewed in references 8 and 65). Telomeres have also been implicated in the correct positioning of chromosomes within the nucleus during cell division (1, 12, 29, 30). Telomeric DNA consists of a variable number of tandemly arrayed simple repeats, with a characteristic compositional strand bias: a G-rich strand is always oriented 5′ to 3′ toward the chromosome terminus. This generalized telomeric repeat sequence has been highly conserved throughout evolution and possesses particular structural properties which, in conjunction with associated proteins, may be critical to the telomere's roles in cell division and the maintenance of chromosome stability (16, 21, 27, 28).

The G-rich strand of telomeric DNA is synthesized by the ribonucleoprotein enzyme telomerase (reviewed in reference 10). The first telomerase to be characterized was that of the ciliate *Tetrahymena thermophila* (22, 23). The enzyme has subsequently been characterized in vitro from other ciliates, as well as from *Saccharomyces cerevisiae*, human, mouse, and *Xenopus laevis* cells (13, 33, 34, 36, 42, 47, 53, 64). This unique DNA polymerase synthesizes species-specific telomeric repeats as dictated by an RNA that is an integral part of the enzyme (24, 63). The template domain of telomerase RNAs is complementary to 1 1/2 telomeric repeats and has been defined functionally both in vivo and in vitro (2, 41, 54, 63).

Telomerase RNA genes have been identified, cloned, and sequenced from more than 20 ciliate species (24, 34, 37, 41, 48, 54). Although the telomerase RNA primary sequences have diverged considerably among the ciliates, the overall length (147 to 191 nucleotides [nt]) and secondary structure of this RNA are highly conserved (34, 48). In vitro and in vivo structure probing of the *T. thermophila* telomerase RNA has largely confirmed the secondary structure model derived from phylogenetic comparative analyses (7, 66). Telomerase RNAs from mammalian cells (11, 17) and the yeasts *S. cerevisiae* and *Kluyveromyces lactis* (11, 17, 39, 55) are considerably longer (450 and 1,300 nt, respectively); secondary structures for these RNAs have not yet been deduced.

The lengths and complexity of telomeric repeats can vary dramatically among species. The repeats for many eukaryotes are short and invariant, typically 6 to 8 bp in length. For example, the telomeric repeat sequences for the ciliates *T. thermophila* and *Oxytricha nova* are TTGGGG and TTTTGG GG, respectively (9). In contrast, some yeast species, such as *Candida albicans* and *K. lactis*, have telomeric repeats that are up to 26 bp in length (38, 40). The telomerase RNA template from *S. cerevisiae* (55) is complementary to the 13-bp sequence found at the junction between the subtelomere and telomere of new chromosome ends (31). However, the bulk of *S. cerevisiae* telomeric DNA consists of variable repeats with the general sequence of TG_{1-3}. The variability of *S. cerevisiae* telomeres is due to an inherent property of telomerase from this species. The *S. cerevisiae* enzyme synthesizes irregular repeats because of the high frequency of dissociation of an extended telomere before an entire repeat is synthesized, after which an imprecise positioning of the telomere end along the template occurs (13).

The variability in telomeres from ciliates of the genus *Paramecium* differs fundamentally from that seen with *S. cerevisiae. Paramecium* telomeres consist largely of a random assortment of two repeats, TT<u>G</u>GGG and TT<u>T</u>GGG (5, 18). It has been postulated that the variability of *Paramecium* telomeric repeats at the third nucleotide position may be accounted for by the presence of two distinct types of *Paramecium* telomerase enzymes, each synthesizing perfect telomeric repeats: one with a template RNA that specifies the addition of TT<u>T</u>GGG and the second dictating the synthesis of TT<u>G</u>GGG repeats (62). The random distribution of the two telomeric repeats is suggestive of distributive synthesis by two different enzymes competing for the same substrate. An alternative interpretation of the data is that the synthesis of variable repeats by *Paramecium* telomerase is due to an imprecise enzyme containing one RNA

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that serves as a template and that incorporates a variable nucleotide occurring at a single position along the template.

As a first step in an investigation of *Paramecium* telomerase, we have cloned, sequenced, and characterized telomerase RNA genes from *Paramecium tetraurelia*, *P. primaurelia*, *P. caudatum*, and *P. multimicronucleatum*. Our results indicate that all four *Paramecium* species transcribe a single telomerase RNA whose template is consistent with the conventional synthesis of TTGGGG telomeric repeats. Possible mechanisms for the synthesis of TTTGGG telomeric repeats by telomerase in paramecia are discussed.

MATERIALS AND METHODS

Materials. *P. caudatum* (J. R. Preer stock C-101), *P. tetraurelia* (T. M. Sonneborn stock 51), and *P. primaurelia* (T. M. Sonneborn stock 60) were obtained from the American Type Culture Collection (Rockville, Md.). *P. multimicronucleatum* was obtained from Carolina Biological Supply Co. (Burlington, N.C.).

General methods. *Paramecium* cultures were maintained at room temperature in ATCC Culture Medium 802 (a monoxenic infusion containing *Klebsiella pneumoniae*). Genomic DNA was isolated from *Paramecium* spp. as previously described (61). Total RNA was extracted from whole cells with guanidinium HCl as previously described (58). Oligonucleotides were radiolabelled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP (7,000 Ci/mmol; ICN) as previously described (50). Dideoxynucleotide sequencing of the *P. tetraurelia* telomerase RNA 5′ terminus with avian myeloblastosis virus reverse transcriptase was as described previously (25). Changes in free energy as a measure of RNA helical stability were determined with the Genetics Computer Group RNA folding program mFold.

Oligonucleotides. A description of the designs and applications of the following oligonucleotides (Amitof Biotech, Boston, Mass.) is included in the text. Degenerate nucleotide positions are indicated in parentheses. The oligonucleotides, which are written 5′ to 3′, are as follows: TEM1d, TTTTTGGG(G/T)TTG(A/T)(A/T)TGACA; TEM2d, TTTTTGGG(G/T)TTG(A/T)(A/T)TGAC; TEM3d, TTTTTGGG(G/T)TTGTTTGAC; TEM4d, TTTTTGGG(G/T)TTGAATGAC; TEM5d, TTTTTGGG(G/T)TTGATTGAC; TEM6d, TTTTTGGG(G/T)TTGTATGAC; TEM7, AACAATCATTATGTTCATTC; TEM8, GTTGTATGACTTCCTTTCC; TEM9, GTTGTATGATTTCCTTTCC; TEM10, GGTTTCTGGGGTTGTATGAC; TEM11, GGTTTCTGGGGTTTTATGAC; TEM12, GGTTTCTGGGTTTGTATGAC; $(G_4T_2)_3$, GGGGTTGGGGTTGGGGTT; $(G_3T_3)_3$, GGGTTTGGGTTTGGGTTT; PT5′, CGCCCCGGGACAATCATTATGTTC; PT3′, GCGTCTAGAAATAACTATTTAGAGC; T7P1, AATTCTAATACGACTCACTATA; and T7P2, TATAGTGAGTCGTATTAG.

Radiolabelled DNA probes. Nick-translated probes from gel-purified DNA fragments were prepared as previously described (37). A radiolabelled *P. tetraurelia* telomerase RNA gene probe was generated by PCR as previously described (4), with the following PCR cycling program modifications: melting at 94°C (30 s), annealing at 50°C (30 s), and extension at 72°C (45 s) for a total of 35 cycles (Perkin-Elmer Cetus TC-1). The two oligonucleotide primers are PT5′ (24-mer) and PT3′ (25-mer), whose 3′ termini correspond to nucleotide positions +1 to +16 and +207 to +191, respectively, of the *P. tetraurelia* telomerase RNA (see Fig. 3). The amplified PCR product is 219 bp.

Cloning *Paramecium* telomerase RNA genes. Southern blots of total DNA from *Paramecium* species, digested with a variety of restriction enzymes, were analyzed for hybridization with radiolabelled DNA probes (see above) as previously described (37). Hybridizing or cross-hybridizing fragments were gel purified and used to construct size-selected genomic libraries, which were screened as previously described (37). Cross-hybridizing clones were isolated and characterized by restriction digests. Appropriate subclones were constructed and complete sequences of both strands were determined by the dideoxynucleotide termination method (51) with Sequenase (U.S. Biochemical) and α-^{35}S-dATP (1,000 Ci/mmol; Amersham).

In vitro transcription of telomerase RNA. A transcription unit for in vitro transcription of *Paramecium* telomerase RNA by T7 RNA polymerase was constructed. The cloned *P. tetraurelia* telomerase RNA gene was amplified by a standard PCR protocol with oligonucleotide primers PT5′ and PT3′. The 219-bp PCR product contains the coding sequence for the entire *P. tetraurelia* telomerase RNA with *Sma*I and *Xba*I restriction sites at the 5′ and 3′ ends, respectively. The *Sma*I and *Xba*I restriction sites were utilized to clone the telomerase RNA coding sequence into the polylinker of the cloning vector pUC118. This intermediate plasmid construction was then digested at the polylinker with restriction enzymes *Sma*I and *Eco*RI. A synthetic T7 RNA polymerase promoter sequence, designed to include one blunt end and one *Eco*RI cohesive site (consisting of the complementary oligonucleotides T7P1 and T7P2), was ligated to the *Sma*I-*Eco*RI-digested plasmid construct as previously described (50). The plasmid construct (pPTER-T7), confirmed by dideoxynucleotide sequencing, includes an intact T7 promoter immediately upstream of the *P. tetraurelia* telomerase RNA coding sequence. Two additional G residues at the 5′ terminus of a telomerase RNA in vitro transcript were a consequence of cloning the T7 promoter juxtaposed to half of the *Sma*I restriction site. Linearization of pPTER-T7 with *Xba*I, followed by in vitro transcription with T7 RNA polymerase and ribonucleoside triphosphates (Promega transcription in vitro system) results in runoff transcription of a complete *P. tetraurelia* telomerase RNA with slightly modified 5′ and 3′ termini.

Site-directed mutagenesis of the telomerase RNA gene. Mutagenesis of the telomerase RNA was essentially performed as described previously (49). Single-stranded, uracil-containing pPTER-T7 was generated (60) and used in conjunction with oligonucleotides TEM9, TEM10, and TEM11 to introduce point mutations by the method of Kunkel et al. (32). The mutations (G43A, C49A, and C52A, respectively) were confirmed by single-strand DNA sequencing (51). The mutant telomerase RNAs were transcribed in vitro as described above for the wild-type RNA. These transcripts were suitable for the establishment of allele-specific hybridization conditions in the Northern (RNA) analyses illustrated in Fig. 4 and 6.

Northern blots. Total RNA (2 to 20 μg) was electrophoresed through an 8% polyacrylamide–7.5 M urea gel and electroblotted to Nytran membranes (Schleicher & Schuell). Blots were probed with radiolabelled oligonucleotides in 5× SSPE (5× SSPE is 0.75 M NaCl, 50 mM sodium phosphate and 5 mM EDTA [pH 7.4]), 1× SPED (2 mM sodium PP_i, 2 mM EDTA, 5× Denhardt's solution), and 0.5% sodium dodecyl sulfate (SDS) at 45°C for 10 to 12 h and subsequently washed in 2× SSPE–0.5% SDS for 60 min. Washing temperatures varied (52 to 58°C), depending on the melting temperature of the particular oligonucleotide probe. Allele-specific hybridization conditions for a given oligonucleotide probe were established with the telomerase RNA in vitro transcripts described above.

Southern blots and genomic library screening. The capillary transfer of DNA from agarose gels, hybridization of radiolabelled probes, and blot washing conditions were as previously described (37, 49). The transfer of bacterial colonies onto filters, hybridization, and washing conditions were also as previously described (48).

Telomeric DNA Southern blots were probed sequentially with oligonucleotides $(G_3T_3)_3$ and $(G_4T_2)_3$, both radiolabelled to a specific activity of 7,000 Ci/mmol. Wash temperatures, buffer volumes, and specific activities of hybridization solutions (0.4 μCi/ml) were carefully monitored to ensure an accurate, quantitative comparison. Exposures to X-ray film and the PhosphorImager were adjusted to correct for decay of the ^{32}P radiolabel. Hybridization of the $(G_3T_3)_3$ probe to *T. thermophila* DNA was approximately 13% of the total hybridization of $(G_4T_2)_3$ and $(G_3T_3)_3$ combined. This value for nonspecific hybridization was used to correct the relative percentages of *Paramecium* TTTGGG telomeric repeats present in the total DNA (Fig. 1).

Nucleotide sequence accession numbers. The nucleotide sequence data described in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers U45433, U45434, U45435, U45436, and U45437.

RESULTS

***Paramecium* telomeric DNA composition.** Telomeres of *P. primaurelia* and *P. tetraurelia*, both members of the *P. aurelia* complex, consist largely of equal, random assortments of TTGGGG and TTTGGG repeats (5, 18). To gauge whether this distribution is a universal property of all *Paramecium* telomeres, DNA from the four species used in this study were analyzed for relative amounts of TTGGGG and TTTGGG telomeric repeats. A Southern blot of total DNA from the four species, probed sequentially with oligonucleotides $(G_3T_3)_3$ and $(G_4T_2)_3$, is shown in Fig. 1. It is quite evident that telomeres from *P. caudatum* consist primarily of TTGGGG repeats (>95%), whereas those from the other three species consistently include a much larger percentage of TTTGGG repeats (approximately 60%). The degree of hybridization shown in Fig. 1 was sensitive to *Bal* 31 exonuclease treatment, indicating that the signal was telomere specific (data not shown). Interestingly, the mean length of *P. multimicronucleatum* telomeres in the *Hin*dIII restriction digest shown in Fig. 1 is clearly greater than that of the other *Paramecium* species (>8 kb). This result proved to be independent of the restriction enzyme used in similar restriction analyses (data not shown).

The *P. tetraurelia* telomerase RNA gene. An attempt to identify the *P. tetraurelia* telomerase RNA gene(s) by specific cross-hybridization to homologous genes from other ciliates was unsuccessful. We believe this is due to the fact that only a very limited region of ciliate telomerase RNA primary sequence, beyond that of the template region, exhibits any degree of conservation. However, we took advantage of the absolute

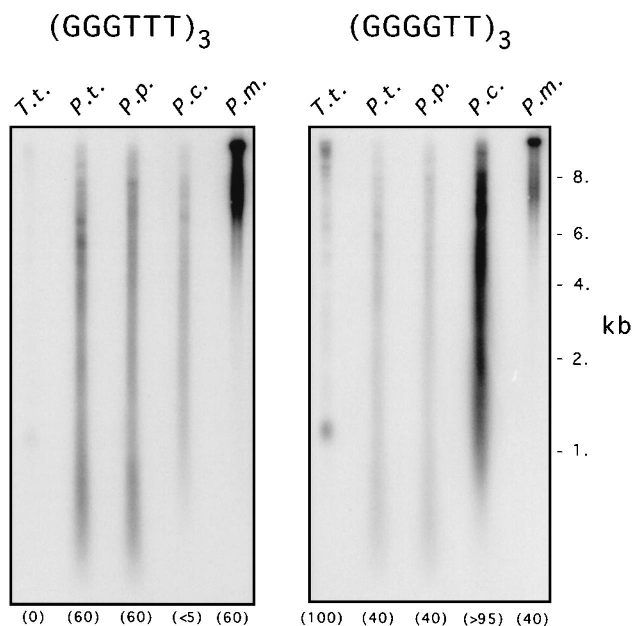

FIG. 1. *Paramecium* telomeric DNA composition. *Hin*dIII digests of *P. tetraurelia* (*P.t.*), *P. primaurelia* (*P.p.*), *P. caudatum* (*P.c.*), and *P. multimicronucleatum* (*P.m.*) total DNA (2 μg) were separated on an agarose gel and transferred to a Nytran filter. A *Bam*HI-*Pst*I digest of *T. thermophila* (*T.t.*) total DNA (1 μg), which contains only TTGGGG telomeric repeats, was included. The Southern blot was sequentially probed with radiolabelled oligonucleotides $(G_3T_3)_3$ and $(G_4T_2)_3$. The amounts of TTTGGG and TTGGGG telomeric repeats detected for each species were quantitated with a PhosphorImager and are represented at the bottom of each lane as percentages of the total DNA. The percentages were corrected for nonspecific hybridization of $(G_3T_3)_3$ to TTGGGG telomeric repeats (see Materials and Methods). Molecular size markers are given at the right.

conservation in the ciliate RNAs of 5 of 7 nt positioned immediately 5′ of the template (24, 34, 41, 48, 54). A consensus sequence for this region, 5′-UGUCA(A/U)(A/U)-3′, in conjunction with that of the predicted template(s), was included in the design of a degenerate antisense oligonucleotide (TEM1d; see Materials and Methods) used to probe a Northern blot of total RNA from *P. tetraurelia*. An RNA slightly longer than 200 nt hybridized strongly to the probe, as did the 159-nt *T. thermophila* telomerase RNA (data not shown). It should be noted that the presence of a second *P. tetraurelia* telomerase RNA that differs radically from all other ciliate homologs at the template region would not be detected by this method.

Primer extension analysis of oligonucleotide TEM2d with *P. tetraurelia* total RNA resulted in a major cDNA product 42 nt longer than the primer (data not shown). The design of TEM2d (nearly identical to that of TEM1d, with one less deoxynucleotide at its 3′ terminus) was based on sequence data from *Tetrahymena paravorax*, in which the absolutely conserved motif 5′ to the ciliate telomerase RNA template region had been reduced by 1 nt to the consensus sequence 5′-GUCA (A/U)(A/U)-3′ (37).

Four additional antisense oligonucleotides without degeneracies at two of three degenerate positions in TEM2d (see Materials and Methods) were tested in primer extension experiments. Only one of the four oligonucleotides (TEM6d) resulted in the elimination of all minor products and enhancement of the major extension product at 45°C. Dideoxynucleotide reverse transcriptase sequencing (25), with *P. tetraurelia* total RNA as the template and TEM6d as the primer, yielded an unambiguous sequence of the primer extension product. A sense oligonucleotide corresponding to this region (TEM7), in conjunction with the antisense TEM6d, were used as primers in a PCR to amplify the 5′ terminus of the putative telomerase RNA gene(s) from *P. tetraurelia* total DNA. The size of the PCR product (5′TER) was as predicted and the product was subsequently cloned and sequenced.

In order to identify the entire telomerase RNA gene(s), a Southern blot of *P. tetraurelia* total DNA was probed with 5′TER. Hybridization was confined to a single restriction fragment for all of the restriction enzymes tested (data not shown). A size-selected genomic library was constructed in a bacterial vector and screened with radiolabelled 5′TER as a probe. All positive clones isolated were identical and included a putative template region complementary to a TTGGGG telomeric repeat (5′-CAACCC-3′; see below).

Telomerase RNA genes from other *Paramecium* species. Total DNA from three additional *Paramecium* species (*P. primaurelia*, *P. caudatum*, and *P. multimicronucleatum*) were screened for the putative telomerase RNA coding sequences by Southern blot analysis with the radiolabelled *P. tetraurelia* gene as a probe. A single cross-hybridizing restriction fragment was detected for two of the three species (*P. caudatum* and *P. multimicronucleatum*) with a variety of restriction enzymes. In contrast, two cross-hybridizing fragments of equal intensities were consistently detected for *P. primaurelia* DNA (Fig. 2). To rule out the presence of a second, unknown *Paramecium* species in the *P. primaurelia* mass culture, clonal lines derived from single-cell isolates were established. Analysis of DNA from *P. primaurelia* clonal lines displayed cross-hybridization identical to that of the two restriction fragments (designated genes *A* and *B*) detected earlier from the mass culture (data not shown).

Size-selected genomic libraries from *P. multimicronucleatum*, *P. caudatum*, and *P. primaurelia* were constructed and screened for telomerase RNA genes. Positive clones were isolated and sequenced; an alignment of the *Paramecium* telomerase RNAs, as well as a portion of the 5′ nontranscribed

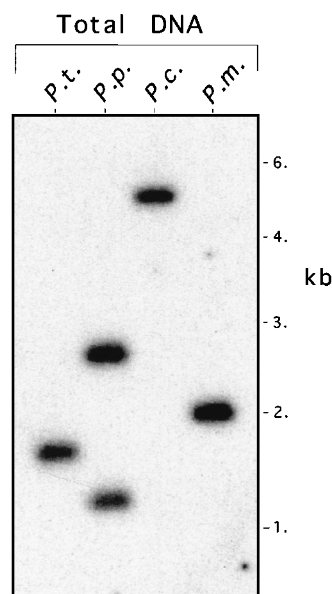

FIG. 2. Southern blot analysis of DNA from four *Paramecium* species. Approximately 2 μg of total DNA from each of four *Paramecium* species was digested with the restriction enzyme *Acc*I, separated by agarose gel electrophoresis, and transferred to a Nytran membrane. The blot was probed with the *P. tetraurelia* telomerase RNA gene. Molecular size markers are given at the right. Abbreviations are as noted in the legend to Fig. 1.

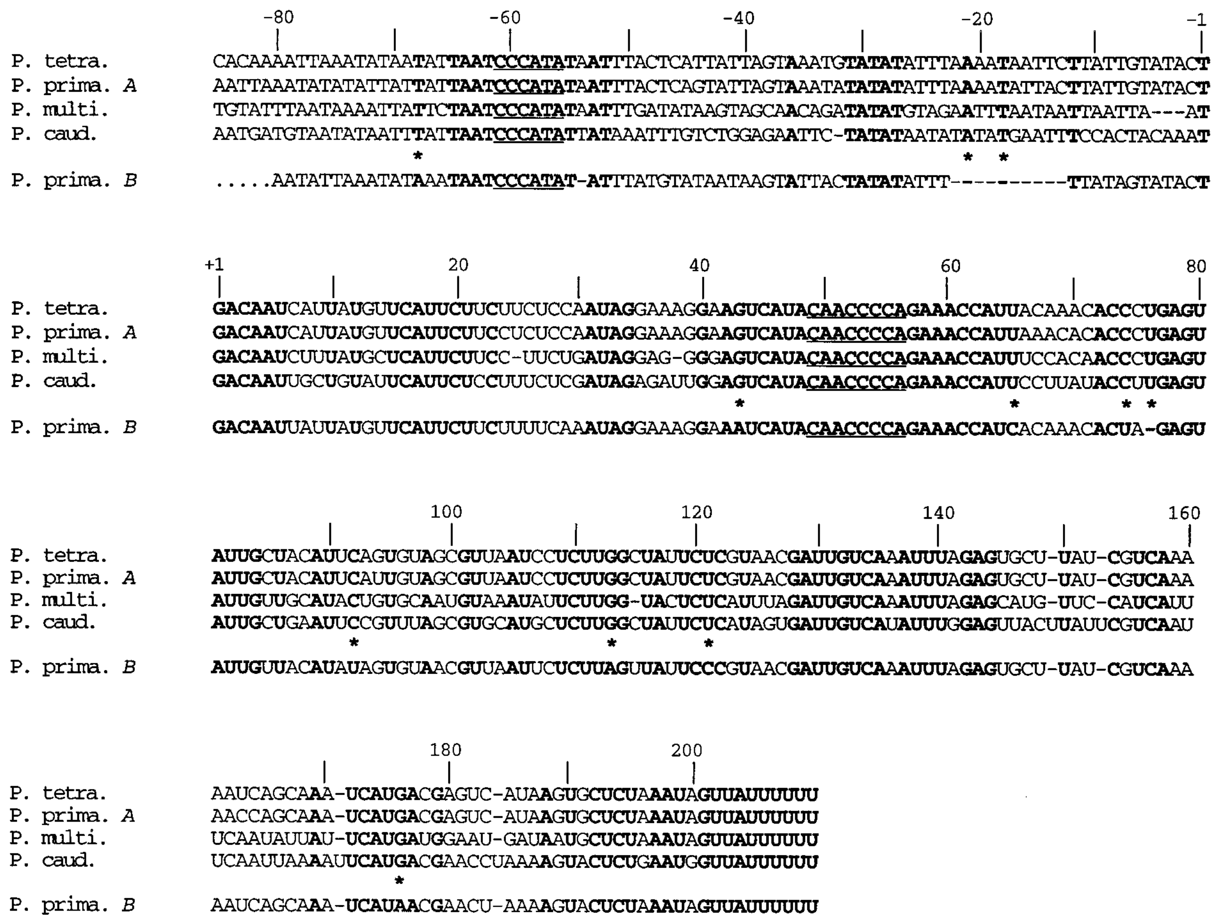

FIG. 3. Alignment of *Paramecium* species telomerase RNA genes. The sequences were aligned as previously described (46). Abbreviations: P. tetra., *P. tetraurelia*; P. prima, *P. primaurelia*; P. caud., *P. caudatum*; P. multi., *P. multimicronucleatum*. Dashes indicate alignment gaps and dots indicate an incomplete sequence. Nucleotides in boldface type are conserved within all five sequences, with the exception of those positions identified with an asterisk (*), which are not conserved in the second *P. primaurelia* gene sequence (P. prima. *B*). Gaps are included in the assignment of numbered nucleotide positions. The first nucleotide of each transcript was determined by primer extension (data not shown) and is indicated as nucleotide position +1 in the alignment. The telomerase RNA template nucleotides (positions +49 to +56, inclusive) are underlined, as is the conserved sequence element (positions −61 to −56, inclusive) situated upstream of all ciliate telomerase RNA coding sequences (34, 37, 48, 54).

region, is shown in Fig. 3. The alignment of homologous nucleotides was fairly straightforward, given the relative similarity in lengths and the lack of extensive gaps in the sequences. The 5′ termini were mapped by primer extension of oligonucleotide TER6d with total RNA from the four species and were shown to have a G residue at the first nucleotide position, as opposed to the adenine residue seen in all other ciliate homologs (data not shown). The 3′ termini are coincident with six to eight consecutive T residues, a feature shared by all ciliate telomerase RNAs (37) and shown to be a transcriptional termination signal for RNA polymerase III transcripts (19).

***Paramecium* telomerase RNA structural features.** Several additional features of the *Paramecium* telomerase RNA gene primary sequences are absolutely conserved. All five sequences presented in Fig. 3 contain template regions whose nucleotide sequences are consistent with the synthesis of TTGGGG telomeric repeats (5′-CAACCC-3′). All five genes include a conserved TATA box (5′-TATAT-3′) and proximal sequence element (5′-ATCCCATAT-3′) situated in the 5′ nontranscribed region, both of which are believed to be important RNA polymerase III transcriptional elements (45). The positions of these two upstream elements (at approximately −25 and −55, respectively) are similar to those seen for all other known ciliate telomerase RNA genes (24, 34, 37, 41, 48, 54), although the *Paramecium* proximal sequence element differs slightly from the ciliate consensus described by Lingner et al. (34). Inclusion of the new *Paramecium* data in formulating a consensus with other known ciliate genes reduces the conserved proximal sequence element from 5′-**ACCCATAA**-3′ to 5′-(A/T)**CCCATA**(A/T)-3′.

There are distinct differences between *P. primaurelia* telomerase RNA gene *B* and the other four sequences shown in Fig. 3, the most notable being a G-to-A transition at position 43. This G residue is included in the conserved motif 5′-**G**UCA-3′, which is positioned two nucleotides 5′ to all ciliate telomerase RNA templates and shown to functionally define the 5′ boundary of the template region (3). Seven additional point changes between *P. primaurelia* gene *B* and the transcribed regions of the other four telomerase RNA genes all occur at otherwise conserved positions (Fig. 3). A 10-bp deletion that coincides with nucleotide positions −22 to −13, inclusive, is also evident for gene *B* and raises the question of whether it is transcribed.

The relative transcription levels of *P. primaurelia* telomerase RNAs *A* and *B* were determined by allele-specific Northern blot hybridization. Two antisense oligonucleotide probes (TEM8 and TEM9) that differ at the single nucleotide position com-

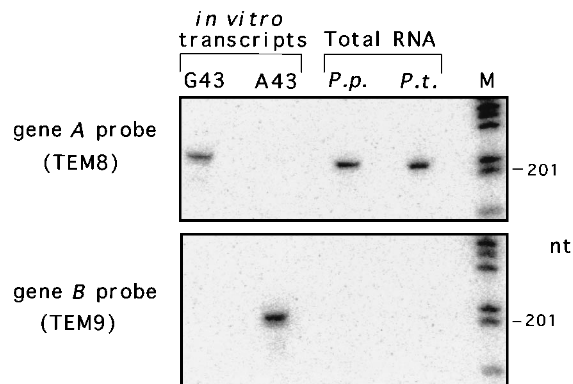

FIG. 4. Transcription of *P. primaurelia* telomerase RNA genes *A* and *B*. Total RNA (2 μg) from *P. primaurelia* (*P.p.*) and *P. tetraurelia* (*P.t.*) was analyzed by Northern blot hybridization. Putative transcripts from genes *A* and *B* differ at nucleotide position 43 (G43 and A43, respectively [Fig. 3]). Antisense oligonucleotide probes TEM8 and TEM9 are complementary to genes *A* and *B*, respectively, at nucleotide positions 34 to 52, inclusive. Specific hybridization conditions for the two oligonucleotide probes were established empirically with *P. tetraurelia* telomerase RNA in vitro transcripts (approximately 0.5 ng) that included either G-43 or A-43 (see Materials and Methods). Molecular size markers are given at the right.

plementary to G-43 and A-43 of putative RNAs *A* and *B*, respectively, were designed. Conditions for specific hybridization of TEM8 and TEM9 to the two alleles were established by probing Northern blots of *Paramecium* telomerase RNA in vitro transcripts containing either G-43 or A-43 (Materials and Methods). As Fig. 4 clearly shows, *P. primaurelia* telomerase RNA gene *A* is transcribed in vivo, whereas transcription of gene *B* was not detected within the limits of resolution of the 8% polyacrylamide gel (50 to 1,000 nt). Given this result, the presumably nontranscribed *P. primaurelia* telomerase RNA gene *B* was excluded from any further structural analysis.

The similarity percentages of telomerase RNAs from the four *Paramecium* species range between 70 and 98% (Fig. 3 and Table 1). Helical regions for the RNAs were readily deduced by the occurrence of compensatory base changes between the four RNAs and reinforced by an obvious resemblance to the conserved secondary structures of other ciliate telomerase RNAs (34, 48). Figure 5 shows the proposed foldings of *Paramecium* telomerase RNAs. More than 70% of the base pairs in this secondary structure are supported by at least one compensatory base change. Of particular note is the presence of helix II, which is entirely lacking in the hypotrich and *T. paravorax* models (34, 37). This indicates that stem-loop II is most likely a primitive ciliate telomerase RNA structural characteristic and that its absence from the hypotrich and *T. paravorax* RNAs is an example of convergent evolution. The potential for helix V, situated between helices I and III, is a structural feature unique to the *Paramecium* telomerase RNAs. The functional importance of structural elements other than the template, alignment, and template boundary domains (3) has not yet been established for any telomerase RNA.

Is the *Paramecium* telomerase RNA template edited? RNA editing, a process by which the sequence of an RNA molecule is altered posttranscriptionally, occurs in a number of eukaryotes (reviewed in reference 6). Whereas insertions and deletions make up the majority of RNA editing events, single-nucleotide substitutions have also been observed. Since the *Paramecium* telomerase RNA is a single-copy gene, the replacement of a single cytosine with an adenine residue at the telomerase RNA template could be responsible for the synthesis of TTTGGG telomeric repeats in vivo. RNA editing of a pyrimidine to a purine is rare but not unprecedented (35, 43, 44). A single-nucleotide replacement of C-49 with A-49 (C49A), or C-52 with A-52 (C52A), would result in a template region consistent with the conventional synthesis of TTTGGG repeats by a ciliate telomerase.

Allele-specific Northern blot hybridization similar to that shown in Fig. 4 was used to determine if *Paramecium* telomerase RNA templates are edited in vivo. Antisense oligonucleotide probes (TEM10, TEM11, and TEM12) were hybridized to in vitro-transcribed telomerase RNAs with C49A, C52A, and wild-type templates, a well as with *P. tetraurelia* total RNA. The results of these experiments (Fig. 6) indicate that the *P. tetraurelia* telomerase RNAs are not edited in the template region. The implication is that *Paramecium* telomerase synthesizes TTTGGG repeats by a mechanism that is fundamentally different from that of other ciliate telomerase enzymes.

DISCUSSION

***Paramecium* telomerase RNA structure.** Telomeres from *Paramecium* species in the *aurelia* complex (57) consist largely of a random assortment of two telomeric repeats that vary at a single nucleotide position, TT**G**GGG and TT**T**GGG (5, 18). Given the fact that telomerase activities from other ciliates synthesize invariant telomeric repeats both in vivo and in vitro (24, 53, 64), it was postulated that two types of telomerase with different templating RNAs account for *Paramecium* telomeric repeat variability (62). We have cloned the telomerase RNA genes from four *Paramecium* species and found, somewhat surprisingly, that a single telomerase RNA is transcribed in all four species.

Paramecium telomerase RNAs range between 202 and 209 nt in length (Fig. 3). Comparative sequence analysis reveals a conserved secondary structure that is common to that of other ciliates (34, 37, 48). In addition to being slightly longer than other ciliate telomerase RNAs, the *Paramecium* secondary structure model includes a novel helix V positioned between helices I and III (Fig. 5). The unstructured nature of the 17 to 20 nt situated between the template domain and the helix III pseudoknot is remarkably conserved in all the ciliates (37), including *Paramecium* spp. Stem-loop IV is extremely stable

TABLE 1. Similarities and nucleotide distances among *Paramecium* telomerase RNAs

Species	*H* value or no. of base changes and gaps[a] compared with sequence of:			
	P. tetraurelia	*P. primaurelia*	*P. multimicronucleatum*	*P. caudatum*
P. tetraurelia		0.976	0.734	0.761
P. primaurelia	5		0.734	0.746
P. multimicronucleatum	57	57		0.696
P. caudatum	52	55	67	

[a] The upper right triangular section of the table gives similarity values (*H*) for all pairwise comparisons of the telomerase RNA sequences in Fig. 3. *H* is defined according to Sogin et al. (56) as $m/(m + u + g/2)$, where m is the number of sequence positions with matching nucleotides in the two sequences, u is the number of sequence positions with nonmatching nucleotides in the two sequences, and g is the number of sequence positions that have a gap in one sequence opposite a nucleotide in the other sequence. The absolute number of base changes and gaps in the compared sequences is shown in the lower left triangular section of the table.

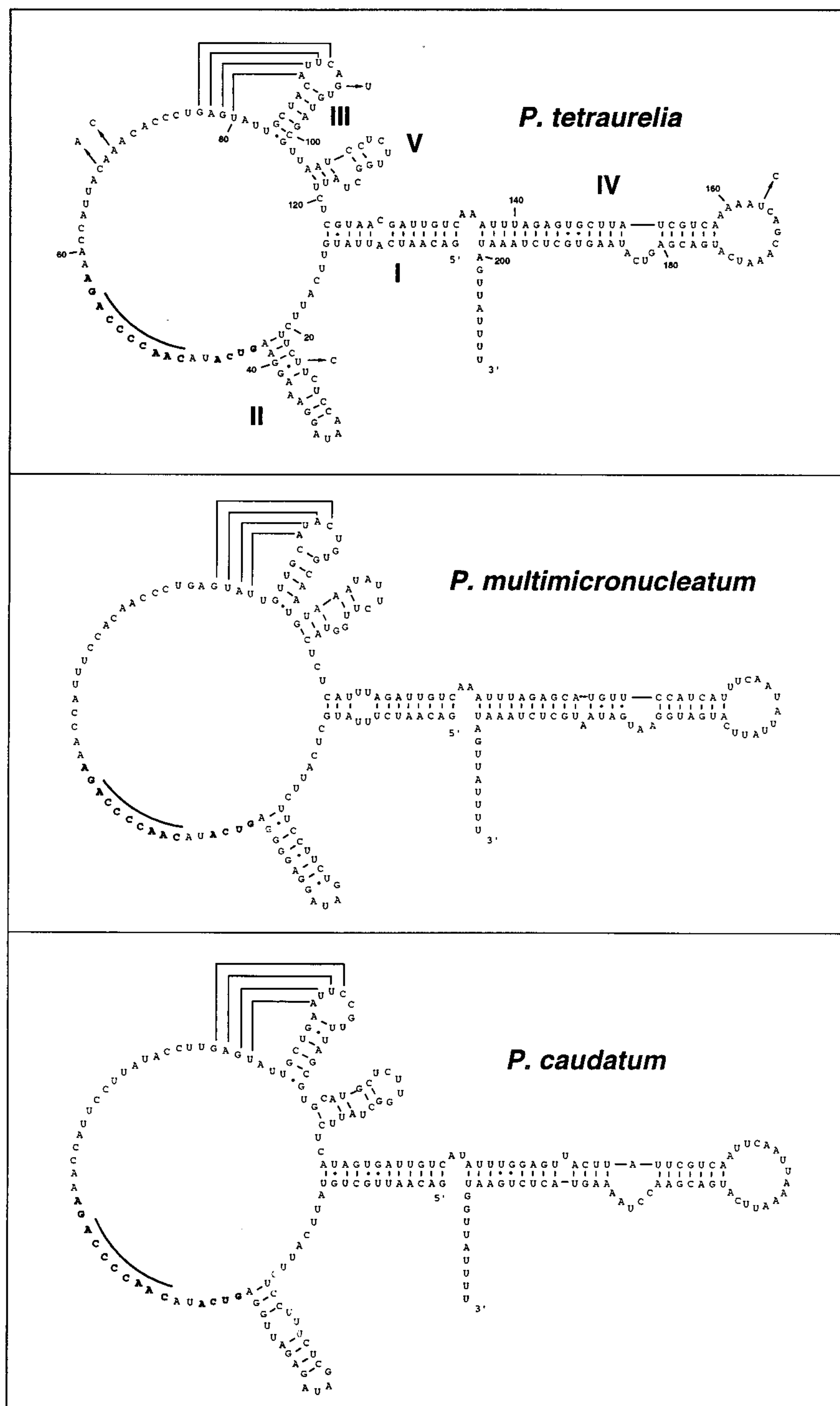

FIG. 5. *Paramecium* telomerase RNA secondary structures. The structures for *P. tetraurelia*, *P. caudatum*, and *P. multimicronucleatum* are shown. The five nucleotide substitutions between *P. tetraurelia* and *P. primaurelia* (gene *A*) are indicated in the *P. tetraurelia* structure by arrows. The numbering of helical regions (I to IV) is according to Romero and Blackburn (48). The template nucleotides are underlined. Nucleotide positions assigned to the *P. tetraurelia* structure coincide with those indicated in Fig. 3.

($\Delta G = -19.3$ kcal/mol [-80.8 kJ/mol], 37°C), is strongly supported by compensatory base changes, and includes a bulged nucleotide motif that is shared by all other ciliate telomerase RNAs. These bulged nucleotides introduce a kink in helix IV (7) that may be critical to telomerase assembly. A recently purified 80-kDa telomerase protein subunit from *T. thermophila* specifically binds telomerase RNA (15); recognition of the conserved stem-loop IV may represent an important telomerase protein binding domain. Also of note is the presence of the conserved pseudoknot of stem-loop III (59), postulated

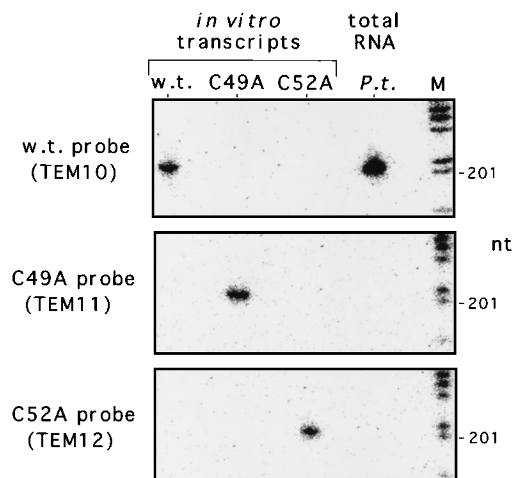

FIG. 6. Analysis of *Paramecium* telomerase RNA template sequences. Total RNA (2 μg) from *P. tetraurelia* (*P.t.*) was analyzed by Northern blot hybridization to determine if a telomerase RNA template substitution had occurred posttranscriptionally. The wash conditions for allele-specific hybridization of antisense oligonucleotide probes TEM10 (w.t.), TEM11 (C49A), and TEM12 (C52A) were established empirically by hybridization to wild-type, C49A, and C52A in vitro transcripts (see Materials and Methods). Lane M, molecular size markers.

to serve as a conformational switch during the synthesis of telomeric DNA (34).

The *P. primaurelia* genome contains two copies of the telomerase RNA genes (*A* and *B*) which have diverged from each other, though not in the region of template nucleotides. Northern blot data (Fig. 4) indicate that gene *B* is transcribed at extremely low levels, if at all. We postulate that gene *B* has been transcriptionally inactivated, possibly as a consequence of a deleterious substitution(s) within the structural portion of the RNA gene. Whether the 10-bp deletion at nt positions −13 to −22 (Fig. 3) is sufficient to prevent transcription of gene *B* has not been determined.

Variable telomeric repeats in *Paramecium* spp. When telomeric repeats other than TTGGGG are added to the ends of chromosomes in *T. thermophila*, there is a loss of telomere length regulation and the appearance of severe nuclear and cell division defects that lead to senescence (49, 63). The *Paramecium* telomerase RNA template domain, 5′-CAACCC-3′, is identical to that from *Tetrahymena* spp. and is consistent with the conventional synthesis of TTGGGG repeats by telomerase (Fig. 3). Yet the telomeres from only one of the four species, *P. caudatum*, consist primarily of TTGGGG repeats; telomeres from the other three species include a high percentage of TTTGGG repeats (Fig. 1). The implication is that the selective pressure to maintain TTGGGG repeats noted for *T. thermophila* is somewhat relaxed in the three *Paramecium* species with variable repeats. Conversely, the apparent high fidelity exhibited by *P. caudatum* telomerase in vivo indicates that this species may be under the same stringent requirement with regard to telomere sequence seen with *T. thermophila*. The apparent requirement for homogeneous repeats in *T. thermophila* may be mediated by specific recognition of telomeric DNA by telomere binding proteins (52).

How does *Paramecium* telomerase synthesize variable repeats? Synthesis by a conventional telomerase (as defined by other ciliate telomerase activities) cannot account for TTTG GG repeats, since the template domain 5′-CAACCC-3′ apparently is not altered to either 5′-**A**AACCC-3′ or 5′-CAA**A**CC-3′ by RNA editing (Fig. 6). It was not determined whether the template nucleotides were modified, and so a possible role for such base modifications in variable repeat synthesis cannot be ruled out.

The mechanism for variable repeat synthesis by *Paramecium* telomerase may be analogous to that described for *Saccharomyces castellii*. Telomerase from this yeast "stutters" by translocating the 3′ end of a de novo telomere back 2 nt before completing synthesis to the 5′ end of the template (13). This can occur multiple times during the extension of a telomeric primer; the net result is the interspersion of TG dinucleotide(s) within a template-encoded repeat sequence [TCTG GG(TG) versus TCTGGG(TG)$_{2-4}$ repeats observed in vivo]. By analogy, a single-nucleotide stutter at A-50 or A-51 of the *Paramecium* telomerase RNA template (5′-C**AA**CCC-3′) could result in the addition of a third adenine residue. Upon completion of a 6-nt repeat and dissociation or translocation (repositioning of the 3′ end of the telomere back to the 3′ end of the template), the net result would be a mixture of TTG GGG and TTTGGG telomeric repeats. Slippage of the telomere 3′ end relative to that of the template, followed by the templated addition of thymidine and/or guanidine juxtaposed to A-50 or C-49 of the template, could account for the rare TTTGGGG and TTTTGGG 7-nt repeats (<8%) documented for *P. tetraurelia* telomeres (18). A premature translocation or dissociation before the complete synthesis of a telomeric repeat could account for the occasional 5-nt TTGGG repeat (<3%) encountered in *Paramecium* telomeres from the *aurelia* complex (5, 18).

The relatively faithful synthesis of telomeric DNA seen for *P. caudatum* (Fig. 1) suggests that telomerase from this species rarely stutters. Comparisons of *P. caudatum* telomerase RNA primary and secondary structures with those from other *Paramecium* species do not reveal any obvious differences that might account for the apparent difference in the enzyme's fidelity. A comparative study of *P. tetraurelia* and *P. caudatum* telomerase activities in vitro may help to identify which telomerase component(s) is responsible for the synthesis of perfect telomeric repeats. These experiments must wait until *Paramecium* telomerase activities have been detected in vitro.

One consequence of the imprecise *Paramecium* telomerase described above is variability at the 3′ ends of de novo telomeres. The sequence of the 5 terminal nt could theoretically consist of GGTTG, GGGTT, GGTTT, or GTTTT just prior to dissociation from telomerase. Consequently, reassociation and proper alignment of the telomeric 3′ end with the RNA template for subsequent rounds of polymerization must be somewhat independent of canonical base pairing to the alignment domain. The *Tetrahymena* alignment domain has been functionally defined as the 3 nt (3′-AAC-5′) positioned 3′ of the template, complementary to the 3′ end of the telomere (5′-T TG-3′) extended by telomerase (2, 3). In contrast, the homologous sequence in *Paramecium* telomerase RNAs is 3′-GA C-5′ (Fig. 3 and 5). Even for the precise synthesis of TTGGGG repeats seen in *P. caudatum* telomeres, there is the potential for only 2 canonical bp at the alignment domain (5′-TTG-3′ paired to 3′-GAC-5′). It is important to note that a telomeric end is not critical for telomere addition in *Paramecium* spp.; telomeres are added to linear DNA introduced by microinjection, regardless of the sequence at the 3′ termini (20). Perhaps the anchor site, which is likely to involve integral telomerase proteins (15), plays a larger role than the alignment domain in telomeric primer positioning at the *Paramecium* telomerase active site.

Another possible mechanism for TTTGGG synthesis is the misincorporation of dTTP instead of dGTP at either C-49 or C-52 (Fig. 3 and 5). It is conceivable that similar misincorpo-

ration occurs in other ciliate enzymes but that the telomerase-associated exonuclease, previously described for *Tetrahymena* and yeast telomerases (13, 14), excises the misincorporated deoxynucleotide (T) and then the proper deoxynucleotide is added (G). This constitutes a proofreading mechanism that would presumably be more efficient in other ciliates and *P. caudatum* than in the other three *Paramecium* species used in this study. This model does not account for the synthesis of rare TTTGGGG and TTTTGGG 7-nt repeats or why dTTP would be misincorporated to the exclusion of dATP and/or dCTP. These questions make this alternative somewhat less compelling than the stuttering mechanism described above.

In vivo expression of a telomerase RNA gene with an altered template sequence may provide clues as to how *Paramecium* variable telomeric repeats are synthesized. Template substitutions can provide useful markers at the telomeric DNA sequence level, as has been demonstrated in *Tetrahymena*, yeast, and mammalian telomerase RNA studies (11, 17, 39, 55, 61). Evidence of a stuttering mechanism similar to that seen with *S. castelli* telomerase should be readily detected in the sequence of de novo telomeres synthesized in *Paramecium* transformants. The recent development of a selectable plasmid vector suitable for the efficient transformation of *Paramecium* spp. makes this experimental approach tenable (26).

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