

Expression of Mutated *Paramecium* Telomerase RNAs In Vivo Leads to Templating Errors That Resemble Those Made by Retroviral Reverse Transcriptase

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Telomeric DNA consists of short, tandemly repeated sequences at the ends of chromosomes. Telomeric DNA in the ciliate *Paramecium tetraurelia* is synthesized by an error-prone telomerase with an RNA template specific for GGGTT repeats. We have previously shown that misincorporation of TTP residues at the telomerase RNA templating nucleotide C52 accounts for the 30% GGGTTT repeats randomly distributed in wild-type telomeres. To more completely characterize variable repeat synthesis in *P. tetraurelia*, telomerase RNA genes mutated at C52 (A, U, and G) were expressed in vivo. De novo telomeric repeats from transformants indicate that the predominant TTP misincorporation error seen in the wild-type telomerase is dependent on the presence of a C residue at template position 52. Paradoxically, the effects of various other telomerase RNA template and alignment region mutations on de novo telomeres include significant changes in fidelity, as well as the synthesis of aberrant, 5-nucleotide telomeric repeats. The occurrence of deletion errors and the altered fidelity of mutated *P. tetraurelia* telomerase, in conjunction with misincorporation by the wild-type enzyme, suggest that the telomerase RNA template domain may be analogous to homopolymeric mutational hot spots that lead to similar errors by the human immunodeficiency virus proofreading-deficient reverse transcriptase.

Telomeres, the specialized DNA-protein structures found at the ends of eukaryotic chromosomes, are necessary for chromosome stability and to facilitate the complete replication of chromosomal termini. In the absence of a proper telomere “cap,” chromosomes may be recognized as damaged DNA, leading to cell cycle arrest (50, 22), as well as illegitimate fusions, degradation, and aneuploidy (reviewed in reference 7).

Telomeric DNA from most eukaryotes consists of a variable number of short, tandemly repeated sequences. The sequences invariably have a strand bias, with a typically G-rich strand oriented 5' to 3' toward the chromosome terminus. The telomeric G-rich strand is synthesized by the ribonucleoprotein enzyme telomerase. Telomerase RNA (TER) subunits, which have been isolated from a number of ciliates, yeast, and mammals, all share the characteristic feature of a templating domain that dictates the species-specific telomeric repeat added to the 3' end of the chromosome (reviewed in references 17 and 46).

Protein components associated with telomerase from ciliates, yeasts, and mammals have been isolated. A 123-kDa protein from the ciliate *Euplotes aediculatus* (35) and its homologs from *Tetrahymena thermophila*, *Oxytricha trifallax*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and humans (9, 13, 43, 44) all have the sequence and functional characteristics of a reverse transcriptase related to retrotransposon and retroviral reverse transcriptases (reviewed in reference 10). Mutational studies of the *S. cerevisiae* gene *EST2* (34, 36) and in vitro reconstitution experiments with the human and *Tetrahymena* recombinant proteins (3, 13, 53) have confirmed a catalytic function for these Ea p123 homologs. Two other proteins

that copurify with *Tetrahymena thermophila* telomerase, p80 and p95 (15), have also been described. It has been postulated that a p80-p95 complex functions to position the DNA substrate at the RNA template for extension by the telomerase holoenzyme (21). Mammalian homologs to *T. thermophila* p80 have also been identified (45, 27).

A nucleolytic activity associated with *T. thermophila* and *Euplotes crassus* telomerase cleaves the 3' termini of primers in vitro (14, 42). This activity either is an intrinsic property of telomerase or is catalyzed by a factor that remains tightly associated with telomerase through extensive purification. It has been postulated that the in vitro endonucleolytic removal of nontelomeric sequences from primers by telomerase may be part of a proofreading system in which mismatched nucleotides are removed prior to elongation (25). Such a proofreading mechanism may have evolved to ensure high fidelity for telomerase, since deviation from species-specific telomeric repeats can have severe consequences on cell viability and nuclear division (31, 38, 41, 48, 54). Alternatively, the associated nucleolytic activity may be involved in de novo telomere addition to restructured chromosomes during macronuclear development in the late stages of conjugation (reviewed in reference 16).

Naturally occurring variable telomeres have been documented for the yeast *Saccharomyces* spp., the malarial protozoan *Plasmodium falciparum* (8), and many species of the ciliate *Paramecium* (2, 12, 20, 40). Irregular repeat synthesis in *Saccharomyces* is a consequence of partial translocation and stuttering along the RNA template, which results in duplicate copying of one or more nucleotides during each round of polymerization (11). Despite a single telomerase RNA consistent with G₄T₂ repeat synthesis, wild-type telomeres in most *Paramecium* spp. consist of a random mixture of G₄T₂ and G₃T₃ repeats at 70 and 30%, respectively (40). This variability in *Paramecium* telomeres is due to a stereotypical misincorporation

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ration of TTP at templating nucleotide C52 (39). Paradoxically, *Paramecium caudatum* telomerase faithfully synthesizes invariant G₄T₂ repeats as dictated by its RNA template. The high fidelity exhibited by the *P. caudatum* enzyme is not solely a property of the telomerase RNA, since expression and utilization of the *P. caudatum* telomerase RNA in *P. tetraurelia* transformants do not impart high fidelity to the telomerase of that species in vivo (39).

To further characterize variable telomeric repeat synthesis by *P. tetraurelia* telomerase, we have extended our mutational analysis of the *P. tetraurelia* telomerase RNA template and alignment domains. Mutated genes were introduced into *P. tetraurelia* macronuclei by microinjection, and de novo telomeric repeats from transformant clonal lines were analyzed. We show that misincorporation of TTP by the enzyme at templating nucleotide 52 is dependent on a C residue at that position, part of a homopolymeric tract of four C residues. The effects of various other mutations on de novo telomere synthesis include significant increased and decreased fidelity, as well as the occurrence of aberrant, 5-nucleotide telomeric repeats. The patterns of altered fidelity and the appearance of novel, high-frequency deletion errors by mutated *P. tetraurelia* telomerase suggest that the telomerase RNA template domain may represent a homopolymeric mutational hot spot, similar to those documented for the human immunodeficiency virus type 1 proofreading-deficient reverse transcriptase.

MATERIALS AND METHODS

Materials. *P. tetraurelia* (*nd6/nd6* and *cam2/cam2* [29, 33]) was maintained at room temperature in a monoxenic wheat grass infusion, containing *Enterobacter aerogenes* (51). Restriction enzymes and other molecular biology reagents were purchased from New England Biolabs (Beverly, Mass.). The antibiotic G-418 was purchased from Sigma (St. Louis, Mo.).

General methods. Conventional PCR methods and molecular techniques (49) were used for plasmid constructions. Genomic DNA was isolated from *P. tetraurelia*, and radiolabeled telomerase RNA gene probe was generated by a PCR strategy that was described previously (40). Oligonucleotides were radiolabeled at the 5' end with T4 polynucleotide kinase and [γ -³²P]ATP (7,000 Ci/mmol; ICN). DNA sequencing was performed with either Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) or the CircumVent thermal cycle DNA-sequencing kit (New England Biolabs).

Transformation vector modification. To facilitate the efficient sequencing of de novo telomeric repeats from transformants, unique *XhoI* and *SalI* restriction sites flanking one of the "seed" telomeres were introduced into plasmid pPXVI (39). A 1.2-kb *BsaI*-*Bss*HIII fragment from pPXVI, which includes one of the seed telomeres and a portion of the β -lactamase gene, was subcloned into a modified version of pLITMUS 28 (New England Biolabs). The modified pLITMUS 28 had the M13 intergenic region cloned in a clockwise orientation, opposite that of the commercially available phagemid (47a). The *XhoI* site was introduced by oligonucleotide site-directed mutagenesis (32). This construct was linearized at a unique *SfiI* site, the termini were blunted with T4 DNA polymerase, and the following linker sequence introduced by ligation with T4 DNA ligase: 5'-GGG TCG ACG GGG TTG GGG TTG GGG CTG CAG GCC TAC GTG GCC CG-3'. This linker includes unique *SalI* and *PstI* sites, separated by 16 bp of telomeric sequence. The *SfiI* cloning site, destroyed as part of the cloning strategy, was reintroduced at the 3' end of this linker. The modified 1.2-kb *BsaI*-*Bss*HIII fragment was subcloned back into pPXVI, to yield the transformation vector pPXVII (see Fig. 2).

***P. tetraurelia* telomerase RNA mutagenesis.** Oligonucleotide site-directed mutagenesis of the TER gene in plasmid pPTEr (39) was performed by the method of Kunkel et al. (32). The TER genes were cloned into pPXVII by virtue of unique *SacI* and *XbaI* sites. The sequence of the modified genes in the resultant pPXVII-TER constructs (see Fig. 2) were confirmed by double-stranded DNA sequencing.

Transformation of *P. tetraurelia* by microinjection. Plasmids suitable for microinjection were prepared with the Wizard DNA purification system (Promega, Madison, Wis.). Plasmids pPXV-NEO (28), pPXVII, and various pPXVII-TER constructs were linearized by *SfiI* digestion, and microinjection of *P. tetraurelia* was performed precisely as previously described (39). Transformant clonal lines were maintained at a density of 200 cells/ml and expanded for 20 to 22 fissions following microinjection.

Southern blot hybridizations. Preparation of total DNA from transformant clonal lines, DNA transfer from agarose gels onto Nytran membranes, hybridization of radiolabeled DNA probes, and blot-washing conditions were all as previously described (40). A Molecular Dynamics PhosphorImager was used to

quantitate the relative ratio of the plasmid-encoded to endogenous TER genes present in total DNA from transformants.

Cloning de novo telomeres from *P. tetraurelia* transformants. Total DNA (5 μ g) extracted from transformants 20 to 22 fissions after microinjection was digested with *XbaI* in a 50- μ l reaction mixture, effectively removing one telomeric end from the microinjected pPXVII-TER plasmids. The *XbaI*-digested DNA was treated with 6 U of T4 DNA polymerase (New England Biolabs) in a standard reaction buffer (0.1 mM deoxynucleoside triphosphates [dNTPs]) at 12°C for 15 min to generate blunt ends suitable for ligation. Following phenol-chloroform (1:1) extraction and ethanol precipitation, 0.5 μ g of the DNA was incubated overnight at 16°C with T4 DNA ligase (100- μ l reaction volume). Competent *Escherichia coli* DH10B was transformed by electroporation with the ligation products. DNA prepared from ampicillin-resistant clones was screened for the TER gene by Southern blot hybridization.

Positive clones were screened for the presence of a unique *SalI* site situated between the remaining seed telomere and any de novo telomeric repeats added by telomerase in transformants. The seed telomere was removed from rescued plasmids by a restriction digestion with *XhoI* and *SalI*, followed by a ligation of the resultant compatible, cohesive ends with T4 DNA ligase. The secondary-rescue ligation products were used to transform competent *E. coli* (DH10B) by electroporation. The C strands of cloned, de novo telomeric repeats were sequenced by using a primer complementary to plasmid vector sequence (5'-TAA CTT TTA CTC AAT GTC AAA G-3') that is adjacent to the *XhoI*-*SalI* site. The identities of TER genes on rescued plasmids were also confirmed by sequencing through the plasmid-encoded genes with the minus strand primer (5'-GCG TCT AGA AAT AAC TAT TTA GAG C-3'), complementary to nucleotides +209 to +191, inclusive, of the TER gene.

RESULTS

Telomerase RNA mutations. We have previously reported that the naturally occurring variability in *P. tetraurelia* telomeres is due to misincorporation of TTP at telomerase RNA templating nucleotide C52. Expression of a C52A TER template mutation in *P. tetraurelia* transformants leads to a dramatic increase in the number of de novo G₃T₃ repeats (39). To address whether the stereotypical misincorporation observed for the wild-type telomerase is dependent on a novel rC · dT base pair during the polymerization cycle, we have included single C52U and C52G substitutions in a parallel analysis (Fig. 1A).

We had also observed inefficient utilization of an A50G template mutation by transformants. Only two G₄TC telomeric repeats (the predicted sequence for an A50G template) were detected in 10 de novo telomeres isolated from this class of transformants (39). One consequence of G₄TC repeat synthesis from the A50G template is a diminished base-pairing potential of de novo repeats with the telomerase RNA alignment region (Fig. 1B). Therefore, we have introduced an alignment nucleotide mutation, A56G, both singly and in combination with the A50G template substitution. Introduction of this alignment mutation tests whether A56G can compensate for the A50G template, the net effect being efficient G₄TC repeat synthesis in A50G A56G transformants. We have also constructed an A51G template mutation, predicted to give rise to G₄CT repeats. In direct contrast to the A50G mutation, de novo repeats from the A51G template should have the potential to form three Watson-Crick base pairs with the alignment region (Fig. 1B).

***P. tetraurelia* transformation by microinjection.** Mutated telomerase RNA genes were cloned into pPXVII, a plasmid suitable for *Paramecium* transformation (39). Prior to comicroinjection, both pPXVII-TER (Fig. 2) and pPXV-NEO (28) were digested with *SfiI*. The resultant linearized molecules have tracts of approximately 45 G₄T₂ telomeric repeats at their termini (Fig. 2), which increases the efficiency of transformation. Plasmid pPXV-NEO provides resistance to the antibiotic G-418, a selectable marker for transformants. Approximately 2 \times 10⁶ copies of each linearized plasmid were comicroinjected into the macronuclei of postautogamous *P. tetraurelia*.

Following microinjection, single-cell isolates were fed and

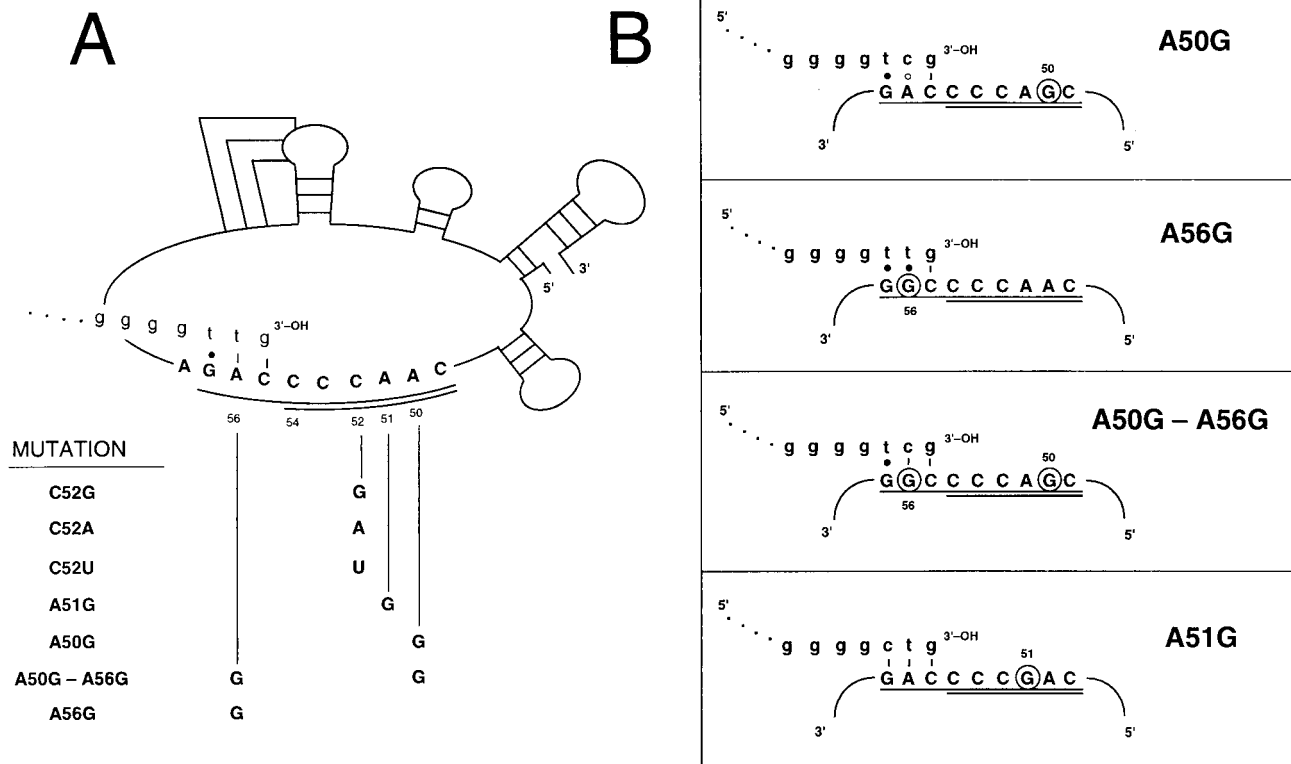


FIG. 1. (A) *P. tetraurelia* telomerase RNA nucleotide substitutions. A schematic of the *Paramecium* telomerase RNA secondary structure and nucleotide positions is taken from reference 40. Alignment nucleotides (1) are underlined and templating nucleotides are double-underlined. The 3' end of a de novo telomere is shown (lowercase letters) base paired with the alignment nucleotides prior to elongation by telomerase. The various mutations analyzed are identified by the wild-type telomerase RNA position followed by the altered nucleotide. (B) Base-pairing potential of telomeric repeats with mutated telomerase RNAs. Only the telomerase RNA alignment (underlined) and templating (doubly underlined) nucleotides are shown. Substituted nucleotides are circled, and the 3' end of the predicted de novo telomere is shown in lowercase letters, base paired with the alignment region prior to elongation. Dashes represent Watson-Crick base pairs, solid circles represent rG · dT pairs, and open circles represent mismatched nucleotides.

allowed to expand to approximately 100 cells over 3 to 4 days at room temperature, at which time they were tested for G-418 resistance. Resistant clonal lines were maintained at 200 cells/ml for an additional 7 to 10 days, and total DNA and RNA were isolated approximately 20 to 22 fissions after microinjection. There were no obvious growth or morphological phenotypes for any of the various pPXVII-TER transformants compared to uninjected controls or cells transformed with pPXV-NEO alone (data not shown).

Total DNA from transformants was digested with *SacI* and *XbaI* to determine the relative amounts of the plasmid-borne TER gene (0.7-kb fragment) and the endogenous gene (4.5-kb fragment) present in clonal cell lines. Quantitation of Southern blots probed with the TER gene (Fig. 3) by PhosphorImager analysis indicates that the plasmid-borne gene accounts for no less than 98% of the telomerase RNA gene present in transformants (data not shown). Northern blot analyses indicate that the amount of TER transcribed from the plasmid-borne gene relative to that from the endogenous gene is directly proportional to the gene dosage (reference 39 and data not shown).

De novo telomere synthesis in transformants. The fixed length of microinjected plasmid termini represent a convenient reference point in determining de novo repeat synthesis by transformant telomerase (39). In vivo extension of a *SacI* telomeric fragment that contains the plasmid-encoded TER gene (1.2 kb; Fig. 2) was measured for all classes of transformants 20 to 22 cell divisions after microinjection (Fig. 4). De novo telomeres in C52A and C52U transformants were on average 200

to 250 bp longer than those in cells transformed with the wild-type telomerase RNA gene. In contrast, the same telomeric fragment was consistently shorter than the 1.2-kb input length in cells expressing the C52G template mutation.

De novo telomere synthesis in A50G transformants was similar to that of the C52G mutants, with minimal telomere extension in these cells. The relative efficiency of telomere extension was greater in the A50G A56G double mutation, approaching that of the wild-type control (Fig. 4). Finally, the average lengths of de novo telomeres in A56G and A51G transformants exceeded those of wild-type transformants, although not to the same degree as those from cells expressing the C52A and C52U template mutations.

Telomere extension in five different transformants was also gauged by Southern blot hybridization of total DNA to various telomeric oligonucleotides (Fig. 5). There was a very low level of (G₄TC)₂ hybridization to A50G telomeres (lane 3), in contrast to the strong hybridization of this probe to A50G A56G transformant DNA (lane 4). Telomeres from the A51G transformant (lane 2) hybridized strongly to the (G₄CT)₂ probe and somewhat weakly to (G₃CT)₂ (see Discussion). The C52G transformant DNA hybridized to the wild-type telomeric repeat (G₄T)₂, but not at all to the sequence G₃CT₂, which was predicted for this template mutation (lane 5).

Telomere addition to the microinjected plasmid termini has made it possible to routinely clone and sequence the de novo telomeric repeats shown in Fig. 4 from *P. tetraurelia* cells expressing mutated telomerase RNA. Briefly, total DNA from

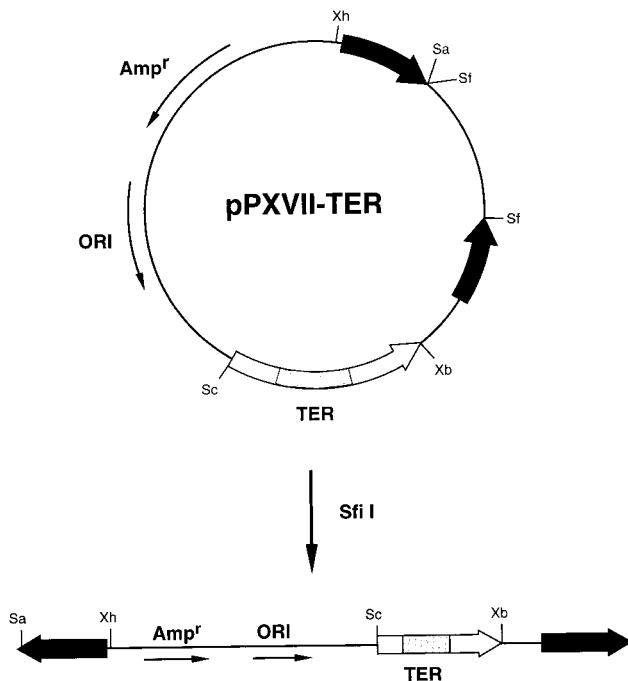


FIG. 2. Plasmid for *P. tetraurelia* transformation. Plasmid pPXVII-TER was constructed as described in Materials and Methods. Wild-type and mutated TER genes, including nucleotides -240 through $+325$, were cloned so that the direction of transcription is toward the terminus of linearized plasmids. Shaded regions are indicative of transcribed nucleotides. A bacterial origin of replication (ORI) and ampicillin resistance marker (Amp^r) are as indicated. Solid arrows represent tandem G_4T_2 telomeric repeats, located at the termini when plasmids are linearized by *SfiI* digestion. Restriction sites: Sa, *SalI*; Sc, *SacI*; Sf, *SfiI*; Xb, *XbaI* (Xb); Xh, *XhoI*. The approximate size of the pPXVII-TER construct is 4.3 kb (figure not drawn to scale).

transformants is digested to completion with *XbaI*, effectively removing one telomere from the linearized pPXVII-TER plasmids (Fig. 2). The *XbaI*-treated DNA is incubated with T4 DNA polymerase and dNTPs, resulting in blunt ends that facilitate the circularization of molecules with T4 DNA ligase. Transformation of competent *E. coli* with the ligation products

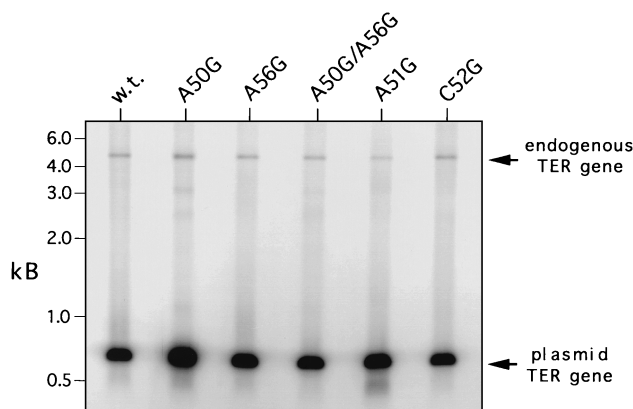


FIG. 3. Endogenous and plasmid-encoded telomerase RNA genes in transformants. Southern blots of total DNA ($1 \mu\text{g}$), isolated from transformants and digested with *SacI* plus *XbaI*, were probed with the radiolabeled *P. tetraurelia* TER gene. The endogenous and plasmid-encoded gene fragments migrate to 4.4 and 0.7 kb, respectively. All transformants analyzed included the TER gene as indicated. Analyses of C52A and C52U transformants are not shown. w.t., wild type.

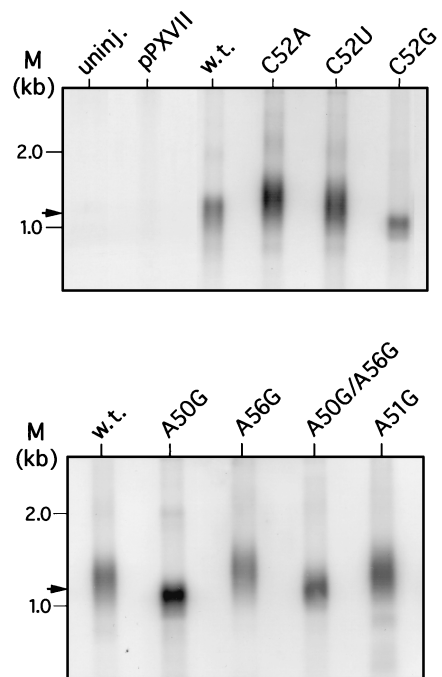


FIG. 4. Southern blot analyses of a defined telomeric fragment from *P. tetraurelia* transformants. Digestion of the pPXVII-TER series of linearized, microinjected plasmids with *SacI* generates a telomeric fragment of approximately 1.2 kb that includes the telomerase RNA gene (arrow; see Fig. 2). Southern blots of total DNA ($1 \mu\text{g}$) from transformants digested with *SacI* and probed with the radiolabeled *P. tetraurelia* telomerase RNA gene are shown. uninj., DNA from uninjected cells; PXVII, DNA from clones coinjected with pPXVII (without TER) and pPXV-NEO. All other samples were from clones coinjected with pPXV-NEO and pPXVII-TER, with the telomerase RNA substitutions as indicated. w.t., wild type.

and selection for ampicillin resistance leads to the rescue of circular plasmids from transformant DNA containing de novo telomeric repeats. Due to the exonucleolytic activity associated with T4 DNA polymerase, the most distal telomeric repeats may be lost during blunt end formation.

Cloned telomeric repeats were sequenced as described in Materials and Methods; representative examples from various transformants are shown in Fig. 6 and a compilation of de novo repeats is presented in Table 1. Wild-type transformants produced a mixture of G_4T_2 and G_3T_3 repeats, consistent with the composition of wild-type *Paramecium* telomeres (2, 20). As previously reported (39), the vast majority of de novo telomeric DNAs dictated by the C52A template mutation consist of G_3T_3 repeats. The C52U substitution leads to an equally high percentage of G_3AT_2 repeats, indicative of an enzyme that does not misincorporate TTP at template position 52. Finally, there was no extension of seed telomeres on the microinjected plasmids recovered from cells expressing the C52G template. This was somewhat anticipated, given the lack of de novo telomere extension in these cells (Fig. 4 and 5). There was also the apparent loss of distal repeats from the microinjected plasmid telomeres in C52G transformants, since none of the rescued plasmids retained a unique *SalI* restriction site 30 bp proximal to the telomere end (see Materials and Methods) (Fig. 2). Further evidence of telomere attrition in C52G transformants is that the number of repeats per seed telomere from rescued plasmids was smaller than that initially present during transformation (data not shown).

De novo telomeric repeats were not detected in plasmids rescued from cells expressing the A50G template mutation. In

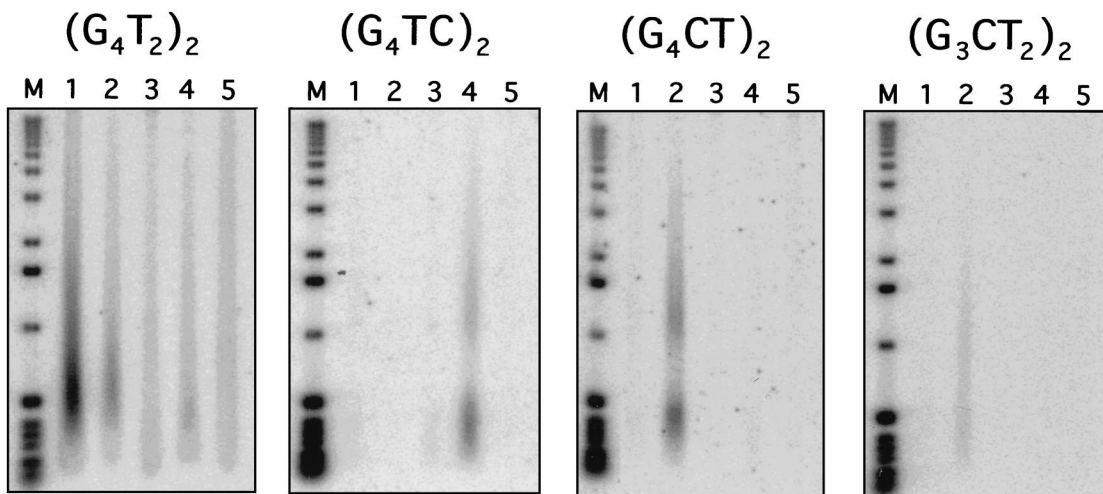


FIG. 5. Southern blots of telomeres from transformants. Total DNA (8 µg) was digested with *DraI* and separated on 0.8% agarose gels (in quadruplicate) prior to capillary transfer of the DNA to Nytran filters. The radiolabeled oligonucleotides used as probes are as indicated. Telomerase RNAs expressed in transformants are as follows: lane 1, wild type; lane 2, A51G; lane 3, A50G; lane 4, A50G A56G; lane 5, C52G.

contrast, cells expressing the A50G A56G double mutation efficiently synthesized G_4TC repeats, as well as novel G_3TC repeats (66 and 24%, respectively). Five-nucleotide repeats (G_3T_2) have been documented for wild type *Paramecium* telomeres, albeit at less than 3% (39) (Table 1). There was an apparent increase in telomerase fidelity in cells expressing telomerase RNA with the A56G mutation alone. The percentage of de novo G_4T_2 repeats increased from 72% in wild type transformants to 85% in cells with the A56G mutation (Table 1). Finally, the A51G template substitution leads to a surpris-

ingly high percentage of 5-nucleotide G_3CT repeats (62%). The 6-nucleotide repeat predicted for this template mutation, G_4CT , accounts for 31% of the de novo telomeric DNA sequenced.

DISCUSSION

Error discrimination by DNA polymerases can occur at a number of steps along the polymerization cycle (reviewed in reference 19). Binding of the correct dNTP to a template-

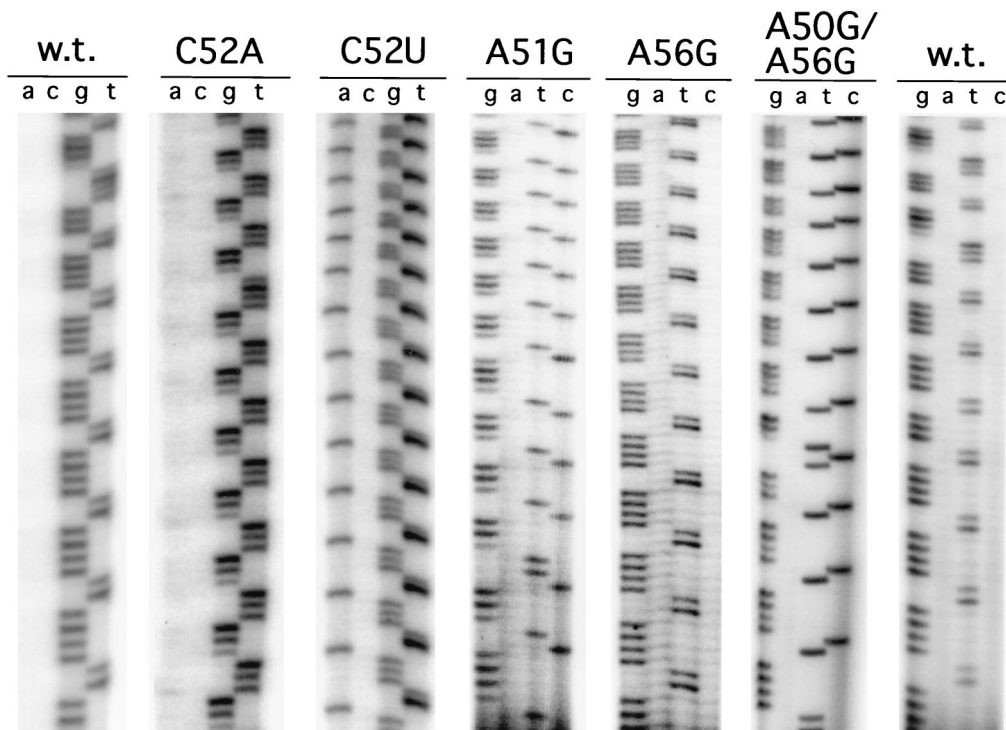


FIG. 6. Representative telomere sequences from *P. tetraurelia* transformants. The C-rich strand of de novo telomeres cloned from transformants were sequenced as described in the text. The mutated telomerase RNAs expressed in transformants are as indicated. w.t., wild type.

TABLE 1. Sequence of de novo telomeric repeats from transformants expressing mutated telomerase RNAs^a

Construct	% of repeats									No. of repeats
	G ₄ T ₂	G ₃ T ₃	G ₃ T ₂	G ₃ AT ₂	G ₄ CT	G ₃ CT	G ₄ TC	G ₃ TC	Other	
Wild type	70	26	2						2	110
C52A	7	93								210
C52U	5	1	1	93						224
C52G										0
A50G										0
A51G	3	1			31	62			3	113
A56G	85	10							5	197
A50G/A56G	2	1					66	24	7	80

^a Telomeric repeats were cloned and sequenced from transformant DNA approximately 20 to 22 generations after microinjection, as described in Methods. The percentages of the various telomeric repeats are as indicated, and the total numbers of de novo repeats sequenced for each transformant class are shown on the right. The percentages of repeat sequences predicted for a given telomerase RNA template are shown in boldface.

primer complex is favored over that of the incorrect dNTPs, due to hydrogen bonding and the geometry of the base-pair and base-stacking interactions at the catalytic site. The rate of phosphodiester bond formation is presumably higher for the cognate dNTP, and subsequent extension is more efficient with a correctly paired 3'-hydroxyl terminus. Crystallographic studies of *Bacillus stearothermophilus* and bacteriophage T7 DNA polymerases complexed with their substrates reveal that the basis for error discrimination is the steric complementarity between the enzyme and a correctly formed Watson-Crick base pair at the active site (18, 30). Mismatched nucleotides cannot bind with the same geometry, resulting in misalignment of the primer 3'-OH terminus, which effectively prevents misincorporation.

DNA polymerases, with the exception of retroviral reverse transcriptases, can also preferentially remove misincorporated nucleotides with an associated nucleolytic activity. Subsequently, error rates for proofreading DNA polymerases, which range from 10⁻⁷ to 10⁻¹¹ error per nucleotide, are orders of magnitude lower than those for reverse transcriptase (37). Misincorporation error frequencies as high as 10⁻² have been documented for spleen necrosis virus reverse transcriptase (47). Studies with avian myeloblastosis virus and human immunodeficiency virus type 1 reverse transcriptases have also shown that certain template sequences constitute mutational hot spots, where misincorporation, insertion, and deletion errors occur more frequently (reviewed in reference 6). These hot spots all have a common feature: they are homopolymeric nucleotide sequences (4). Furthermore, single-nucleotide substitutions within and adjacent to the hot spots can significantly decrease polymerization error frequencies (5).

Our in vivo data on both wild-type and mutated *P. tetraurelia* telomerase suggest that the telomerase RNA residues C52 to C55 comprise a homopolymeric mutational hot spot, similar to those described for retroviral reverse transcriptases. Substitutions within and adjacent to these template and alignment nucleotides have a direct effect on telomerase fidelity in vivo. A net effect of the *P. tetraurelia* telomerase RNA templating nucleotide C52 substitutions was to reduce the homopolymeric tract of C residues from four to three. Coincident with two of these mutations (C52A and C52U) was a decrease in misincorporation of TTP at this templating position. As anticipated, the C52A substitution simply changes the template to specify G₃T₃ repeats, with greater than 90% of the de novo repeats synthesized the cognate G₃T₃ (39) (Fig. 6 and Table 1). The importance of C52 in TTP misincorporation was clearly demonstrated by telomere sequences from C52U transformants. The composition of de novo telomeres from cells expressing the C52U template was 93% G₃AT₂ repeats. If TTP addition

were independent of an rC residue at this template position, a substantial percentage of de novo G₃T₃ repeats would have been detected in telomeres from C52U transformants.

In contrast to the C52A and C52U transformants, plasmids rescued from cells expressing the C52G mutation lacked de novo telomeric repeats. There was a cumulative net loss from the input seed telomere over the approximately 26 fissions following microinjection (Fig. 4), and the predicted G₃CT₂ repeats from this template mutation were not detected (Fig. 5 and data not shown). Although telomere attrition was also seen with the A50G mutation (Fig. 4), two cognate G₄TC repeats dictated by this template were detected in a previous study (39) and are present at low levels in transformants (Fig. 5). Whereas the A50G template mutation results in an inefficient telomerase, the effect of the C52G substitution appears to be somewhat more severe. There is efficient incorporation of dC residues in other telomeric repeats in vivo (see below), and so the inclusion of dC in the 3' strand of a *Paramecium* telomere per se cannot be the sole reason for a block of activity. Some *Tetrahymena* telomerase RNA template mutations are also apparently nonfunctional in vivo (54). It is formally possible that telomeres from C52G transformants include G₃CT₂ repeats that are not protected by telomeric proteins from degradation. The exact nature of the apparent block in telomere maintenance by C52G transformants cannot be determined at this level of analysis.

A single nucleotide substitution in the TER alignment region (G57A) increases the incidence of G₃T₃ errors from 30 to 50% in *P. tetraurelia* transformants (39). Paradoxically, an A56G substitution that decreases the base-pairing potential between de novo repeats and the alignment region (5'-GGG **TTG**-3' base paired to 3'-GGC-5', where boldface type indicates the base-paired nucleotides [Fig. 1]) increases telomerase fidelity, leading to a decrease in G₃T₃ misincorporation that is statistically significant ($\chi^2 = 12.4$; $P < 0.005$). This phenotype is reminiscent of that previously characterized for *P. tetraurelia* expressing a C49A template substitution. The predominant telomeric repeat synthesized by C49A transformants was G₄T₃. There was a complete absence of G₃T₄ repeats detected in these cells, although it was the predicted sequence if there had been continued TTP misincorporation at C52 (39).

Inefficient maintenance of telomeres by A50G transformants is not due to the failure of telomeric proteins to recognize and protect the mutated telomere. The A50G template mutation, coupled with a compensatory A56G substitution in the alignment region, results in efficient extension of telomeres with G₄TC repeats (Fig. 5 and 6; Table 1). The Watson-Crick base-pairing potential between the 3' end of the de novo telomeric repeat (5'-GGG**T**CG-3') and the mutated alignment

region (3'-GGC-5') is restored in these transformants and approximates that of the wild-type interaction (5'-GGGTTG-3' base pairing with 3'-GAC-5'). Similarly, there is perfect base-pairing potential of de novo repeats from A51G transformants with the wild-type alignment region (5'-GGGCTG-3' base-paired to 3'-GAC-5'); the cognate G₄CT repeats were easily detected in telomeres from transformants (Fig. 5 and Table 1). These data support the conclusion of an *in vitro* study with *Tetrahymena* telomerase that correct positioning and efficient elongation of primers is largely dependent on an analogous interaction with the alignment domain (23).

Telomeres from *P. tetraurelia* expressing wild-type telomerase RNA include a small percentage of 5-nucleotide repeats, G₃T₂ (39) (Table 1). Surprisingly, both A50G A56G and A51G transformants synthesized high percentages of G₃TC and G₃CT de novo repeats (24 and 62%, respectively). This high incidence of G₃CT repeats accounts for the observed hybridization of a (G₃CT)₂ probe to DNA from A51G transformant DNA under low-stringency wash conditions (Fig. 5): only 2 of 12 bp are mismatched between this probe and the 12-nucleotide telomeric sequence (G₃CT)(G₄CT)G.

Taken together, our results demonstrate that substitutions within or adjacent to the homopolymeric tract from C52 to C55 of the *Paramecium* telomerase RNA template can either increase or decrease TTP misincorporation *in vivo* or can promote stereotypical deletion errors at greater than 50%. Errors by retroviral reverse transcriptase at homopolymeric mutational hot spots are also affected by changes at neighboring nucleotides, with error rates at particular hot spots never being greater than 2% (5). Experiments with *Tetrahymena* telomerase also indicate that altered TERs can promote misincorporation and premature disassociation of primers, although error frequencies for mutated telomerase were not monitored in these *in vitro* studies (23, 24). Together, these data support the paradigm of a complex catalytic site for telomerase, with a role for the telomerase RNA beyond that of a passive template. In the case of wild-type and mutated *P. tetraurelia* telomerase, templating nucleotide C52 appears to be at the center of both misincorporation and deletion errors that can occur at a high frequency.

Based on the sequence of cloned telomeres and the analysis of reaction products from activity assays, the error frequency of *Tetrahymena* telomerase has been estimated at no greater than 10⁻² (24). Nucleolytic activities associated with both the *Tetrahymena* and the equally precise *Euplotes* enzyme (14, 42) have been postulated to be part of a proofreading system, in which misincorporated nucleotides are removed from de novo repeats prior to elongation (25). It is conceivable that telomerase from most *Paramecium* species lack an efficient mechanism to prevent or correct TTP misincorporation, resulting in a high percentage of G₃T₃ errors. A notable exception may be the *P. caudatum* telomerase, whose telomeres consist solely of G₄T₂ repeats (40). A comparison of a precise *P. caudatum* telomerase with an imprecise *P. tetraurelia* enzyme *in vitro* may determine whether a proofreading nucleolytic activity contributes to ciliate telomerase fidelity. Interestingly, telomerase from the malarial parasite *Plasmodium falciparum*, whose telomeres consist of G₃T₃A and G₃T₂CA repeats (52), lacks an associated nucleolytic activity (8).

The current model for telomere length regulation is that of a competitive relationship between telomere binding proteins and telomerase for access to the telomere end (reviewed in reference 26). There is an apparent loss of length regulation in *P. tetraurelia* transformants that do not synthesize high percentages of both G₄T₂ and G₃T₃ (39) (Fig. 4 and Table 1). When the vast majority of repeats are G₃T₃ (C52A) or when

G₄T₂ repeats predominate (A56G), there is a concomitant increase in telomere length. However, telomere extension did not differ from that in the wild type when the ratio of the two naturally occurring repeats was shifted from 70:30 to 50:50 in G57A transformants (39). It is possible that the juxtaposition of G₄T₂ and G₃T₃ repeats at regular intervals is necessary for efficient binding by *P. tetraurelia* telomere binding proteins. It follows that the affinity of the *P. tetraurelia* telomere binding protein for (G₄T₂G₃T₃)_n would be greater than that for either (G₄T₂)_n or (G₃T₃)_n. Conversely, a *P. caudatum* telomere binding protein would be predicted to have a higher affinity for (G₄T₂)_n. A more complete comparative analysis of the various factors that dictate telomere synthesis and regulation in *P. tetraurelia* and *P. caudatum* may shed light on the determinants that dictate telomerase precision.

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