Variable telomeric repeat synthesis in *Paramecium tetraurelia* is consistent with misincorporation by telomerase

Monica McCormick-Graham,
W. John Haynes¹ and Daniel P. Romero²

Department of Pharmacology, Medical School, University of Minnesota, Minneapolis, MN 55455 and ¹Laboratory of Molecular Biology, Department of Genetics, University of Wisconsin, Madison, WI 53706, USA

²Corresponding author

Telomeric DNA at the ends of chromosomes consist of short, tandem repeat sequences. The telomeres of *Paramecium tetraurelia* are made up of variable repeats, whereas *Paramecium caudatum* telomeric repeats are largely invariant. To investigate variable repeat synthesis in *P. tetraurelia*, mutated telomerase RNA genes were expressed *in vivo*. We demonstrate that the *P. caudatum* telomerase RNA can participate in telomere synthesis when expressed in the *P. tetraurelia* macronucleus, despite 24% primary sequence divergence of the RNAs between the two species. *De novo* telomeric repeats from transformants indicate that *P. tetraurelia* telomerase fidelity is dramatically affected by template substitutions and that misincorporation at a single templating position is likely to account for the majority of *P. tetraurelia* telomeric DNA variability. Furthermore, we show that fidelity is not solely a function of the RNA moiety, as the *P. caudatum* telomerase RNA does not impart high fidelity to the chimeric enzyme.

Keywords: mutagenesis/*Paramecium*/telomerase RNA/transformation

Introduction

Telomeres, the specialized structures found at the ends of chromosomes, consist of simple, tandem DNA repeats and the proteins which bind them (reviewed in Zakian, 1989; Biessmann and Mason, 1992). These structures function as a chromosome 'cap', protecting the termini from exonucleolytic cleavage and preventing illegitimate end-to-end chromosome fusions. Telomeres also ensure the complete replication of chromosomes and in this way prevent the loss of genetic information at the subtelomere. In addition, telomeres have been proposed to help direct chromosome attachment to the nuclear envelope (Agard and Sedat, 1983; de Lange, 1992).

Telomeric DNA generally consists of short G-rich tandem repeats on the strand oriented 5'→3' toward the end of the chromosome. The ribonucleoprotein enzyme telomerase synthesizes the G-rich strand of telomeric DNA. The RNA moiety of telomerase was first identified in *Tetrahymena thermophila* (Greider and Blackburn, 1989) and has subsequently been identified in over 20 ciliates (Shippen-Lentz and Blackburn, 1990; Romero and Blackburn, 1991; Lingner *et al.*, 1994; McCormick-

Graham and Romero, 1995, 1996), yeast (Singer and Gottschling, 1994; McEachern and Blackburn, 1995), mouse (Blasco *et al.*, 1995) and human (Feng *et al.*, 1995). All telomerase RNAs include a templating domain that dictates the telomeric repeat sequence added to the 3'-end of the chromosome. Two putative telomerase proteins from *T. thermophila* (Collins *et al.*, 1995) and *Euplotes aediculatus* (Lingner and Cech, 1996) have been identified and partially purified. The apparent stoichiometry of telomerase components is a 1:1:1 ratio of the RNA and protein subunits. It has been suggested that the 80 kDa protein subunit from the *T. thermophila* enzyme binds the telomerase RNA, whereas the 95 kDa protein binds the telomeric primer.

Telomerase RNA genes from four *Paramecium* species were cloned and sequenced in an earlier study (McCormick-Graham and Romero, 1996). The telomeres from three of the four *Paramecium* species consist of variable repeats, composed primarily of a random mixture of G₄T₂ and G₃T₃ (Baroin *et al.*, 1987; Forney and Blackburn, 1988; McCormick-Graham and Romero, 1996). In contrast, the telomeric DNA from the fourth species, *P. caudatum*, is composed primarily of G₄T₂ repeats (>95%). A single telomerase RNA gene is transcribed in all four species, with templating nucleotides consistent with the synthesis of G₄T₂ repeats. In addition, there is no RNA editing of the telomerase RNA templating nucleotides that might account for the conventional synthesis of G₃T₃ repeats by the *Paramecium* enzyme (McCormick-Graham and Romero, 1996). These data suggest that *Paramecium* telomerase is inherently imprecise, with the notable exception of *Paramecium caudatum* telomerase, which synthesizes G₄T₂ repeats with reasonably high fidelity.

The mechanism by which G₃T₃ repeats are synthesized by *Paramecium* telomerase is not known. It is possible that the observed variability is due to misincorporation by telomerase of dTTP instead of dGTP at one of two positions along the template. Another possibility is that telomerase 'stutters' in the middle of synthesizing a telomeric repeat, by translocating back one position on the template so that the telomere is extended by three sequential dT residues before the addition of dG. Such a stuttering mechanism would be similar to that documented *in vitro* for telomerase from the yeast *Saccharomyces castelli* (Cohn and Blackburn, 1995) and demonstrated both *in vivo* and *in vitro* for the *T. thermophila* telomerase utilizing a mutated telomerase RNA (Yu and Blackburn, 1991; Gilley *et al.*, 1995).

We have utilized an *in vivo* approach to investigate telomere synthesis in *Paramecium*. Linearized plasmids containing the telomerase RNA gene mutated at various positions along the template were microinjected into the macronucleus of *P. tetraurelia*. The effect of the mutations

on telomerase fidelity was determined by sequencing *de novo* telomeric repeats. Similarly, the *P. caudatum* telomerase RNA was expressed in the *P. tetraurelia* macronucleus to establish if the *P. caudatum* RNA could function in a heterologous system and whether it could impart high fidelity to the *P. tetraurelia* telomerase. The sequence of *de novo* telomeric repeats from the various transformants and the implication of these results for *Paramecium* telomere synthesis are discussed.

Results

Telomerase RNA mutations

Mutagenic studies have been useful in delineating the telomerase RNA templating domain in ciliates and yeast, both *in vivo* (Yu *et al.*, 1990; Singer and Gottschling, 1994; McEachern and Blackburn, 1995) and *in vitro* (Autexier and Greider, 1994; Gilley *et al.*, 1995). To investigate how variable telomeric repeats are synthesized in *P. tetraurelia*, the telomerase RNA gene sequence was altered at the four nucleotide positions shown in Figure 1A. Two of the four nucleotide substitutions (C49A and A50G) are identical to template mutations expressed in *T. thermophila* (Yu *et al.*, 1990; Gilley *et al.*, 1995). A prediction for a third template mutation, C52A, is the synthesis of G₃T₃ repeats. The fourth is the substitution of G57 with an A residue, a nucleotide position which is conserved among all other ciliate telomerase RNAs as an A residue.

Transformation of *P. tetraurelia* by co-microinjection

A plasmid suitable for the stable transformation of *P. tetraurelia* by microinjection has been described (Haynes *et al.*, 1995). This plasmid, pPXV-NEO, confers resistance to the antibiotic G-418 through the activity of aminoglycoside 3'-phosphotransferase II (APH-3'-II). The expression of APH-3'-II is driven by the constitutive *P. tetraurelia* calmodulin promoter (Kanabrocki *et al.*, 1991). A second plasmid, pPXVI, was derived from pPXV-NEO by replacement of the APH-3'-II cassette with polycloning sites. *Paramecium tetraurelia* telomerase RNA genes, with and without various template mutations, were cloned into the polycloning site of pPXVI as described in Materials and methods and illustrated in Figure 1.

Prior to co-microinjection, both plasmids were linearized by digestion with the restriction enzyme *Sfi*I. The ends of the plasmids include a linker sequence ~30 bp long, located distal to a tract of 45 G₄T₂ repeats (Haynes *et al.*, 1995). The telomeric sequence helps protect the plasmids from degradation and also increases the efficiency of transformation in *Paramecium* (Bourgain and Katinka, 1991). Following co-microinjection of ~2 × 10⁶ copies of each linearized plasmid into the macronucleus of post-autogamous *P. tetraurelia*, the division of individual cells was monitored until cultures had expanded to ~100 cells (3–4 days). Clonal cell lines resistant to G-418 were expanded and harvested prior to autogamy (between 20 and 22 generations following microinjection). The generation times of transformants were similar to uninjected controls and cells microinjected with pPXV-NEO alone (~13 h) and there were no obvious morphological differences between transformants and controls when examined in a

Fig. 1. Transformation of *P. tetraurelia* with mutagenized telomerase RNA genes. (A) Single nucleotide substitutions in the *P. tetraurelia* telomerase RNA templating domain. Nucleotide positions and a schematic of the *Paramecium* telomerase RNA secondary structure are from McCormick-Graham and Romero (1996). Mutagenized nucleotides are circled and templating positions underlined. (B) Plasmids for microinjection of *P. tetraurelia* macronuclei. The plasmid pPXV-NEO includes a selectable marker (G-418 resistance), utilizing *trans* regulatory sequences from the *P. tetraurelia* calmodulin gene for constitutive expression (Haynes *et al.*, 1995). The APH-3'-II cassette in pPXV-NEO was replaced at *Pvu*II restriction sites with a bacterial vector polylinker to facilitate convenient cloning of various telomerase RNA gene constructs (pPXVI). Wild-type and mutated telomerase RNA genes (TER), including nucleotides -240 to +325, were cloned so that the direction of transcription is toward the terminus of linearized plasmids (pPXVI-TER). Shaded regions are indicative of transcribed nucleotides. Black arrows represent tandem G₄T₂ telomeric repeats, situated at the termini when plasmids are linearized by *Sfi*I digestion. Restriction sites are designated as follows: *Sfi*I (Sf), *Pvu*II (P), *Sma*I (Sm), *Sac*I (Sa), *Xba*I (X). Approximate sizes of the three plasmids are indicated (figure not drawn to scale).

phase contrast microscope. Examination of transformants stained with 4',6-diamidino-2-phenylindole (DAPI) did not reveal any abnormal nuclei or other unusual phenotypic differences between transformants and controls (data not shown).

Plasmid-encoded versus endogenous telomerase RNA expression

The degree to which microinjected telomerase RNA genes were retained and replicated in transformants was determined by Southern blot analysis. A double digestion of total DNA from *P. tetraurelia* transformants with *Sac*I and *Xba*I results in 0.75 and 4.0 kb fragments that include the plasmid-borne and endogenous telomerase RNA genes respectively. Southern blots were probed with the radio-

Table I. Ratios of plasmid-borne to endogenous telomerase RNA genes from transformants and their relative levels of transcription

Construct	DNA	RNA	Percentage
Wild-type (1)	15	n.d.	n.d.
	25	n.d.	n.d.
Wild-type (2)	11	n.d.	n.d.
	9	n.d.	n.d.
A50G (1)	11	7	87.5
	17	9	90.0
A50G (2)	68	57	98.3
	46	53	98.1
G57A (1)	22	11	91.7
	25	24	96.0
G57A (2)	2	2	66.6
	1	1	50.0
C49A (1)	11	2	66.6
	7	3	75.0
C49A (2)	11	1	50.0
	14	5	83.3
C52A (1)	35	31	96.9
	40	31	96.9
C52A (2)	10	7	87.5
	7	7	87.5

Each plasmid construct was microinjected on two separate occasions. Samples from two individual clonal lines were analyzed for each microinjection. The relative abundances of plasmid-encoded to endogenous telomerase RNA genes were quantitated from the Southern blots of total DNA (see Figure 2A), as described in Materials and methods. Telomerase RNA ratios were similarly determined by allele-specific hybridizations of Northern blots, as represented in Figure 2B for two C52A transformants. Quantitation of wild-type telomerase RNA transcribed from microinjected plasmids could not be determined by this method (n.d.). The ratios of plasmid-encoded to endogenous telomerase RNAs were converted to the percentage (%) of the total telomerase RNA component in transformants that was transcribed from microinjected plasmids.

labeled telomerase RNA gene and the ratio of plasmid-encoded to endogenous telomerase RNA genes was quantitated by phosphorimaging (Figure 2A and Table I). The amount of the plasmid telomerase RNA gene relative to the endogenous gene was somewhat variable between microinjections, with a range between 1 and 68 (Table I).

Allele-specific hybridization was used to determine if plasmid-encoded telomerase RNA genes mutated at the template were transcribed (it was not possible to differentiate the plasmid-encoded wild-type telomerase RNA from the endogenous RNA by this method). Oligonucleotides complementary to each mutated RNA were used to sequentially probe a series of Northern blots of total RNA isolated from transformants. Quantitation by phosphorimaging indicated that the ratios of mutated telomerase RNA to endogenous telomerase RNA transcribed ranged from 1 to 57 (Table I and Figure 2B). The transcription of plasmid-encoded telomerase RNA relative to the endogenous RNA correlates roughly with the gene dosage of the introduced gene relative to that of the endogenous gene (Table I).

Extension of telomeres in transformants

Having established that microinjected telomerase RNA genes were both maintained and transcribed in *Paramecium*, we then determined what effect, if any, the transcripts had on telomere synthesis. The length of the microinjected plasmid termini was known (Figure 1B), which afforded a convenient reference point in measuring *de novo* telomere

Fig. 2. Endogenous and plasmid-encoded telomerase RNA gene maintenance and expression in transformants. **(A)** Telomerase RNA gene maintenance. A Southern blot of total DNA (1 µg), isolated from transformants and digested with *SacI* and *XbaI*, was probed with the radiolabeled *P.tetrahymena* telomerase RNA gene (TER). The endogenous and plasmid-encoded gene fragments migrate to 4.4 and 0.7 kb respectively. C, uninjected cells; PXVI, pPXVI transformant; A50G, pPXVI-A50G transformant; G57A, pPXVI-G57A transformant; C49A, pPXVI-C49A transformant; C52A, pPXVI-C52A transformant. **(B)** Telomerase RNA transcription in transformants. A representative allele-specific Northern blot hybridization experiment for a *P.tetrahymena* transformant is shown. Oligonucleotides complementary to both the wild-type and C52A mutant template regions were used to sequentially probe total RNA (2 µg) from two independent samples of uninjected controls and from cells transformed with pPXVI-C52A. Hybridization conditions for oligonucleotide probe specificity were established empirically with *P.tetrahymena* telomerase RNA *in vitro* transcripts (~0.5 ng). Quantitation of the relative amounts of plasmid-encoded to endogenous telomerase RNA genes (Southern blots) and RNAs (Northern blots) is described in Materials and methods.

addition in transformants. Southern blot analyses of total DNA from four cell lines transformed with each plasmid construct, harvested 20–22 generations after microinjection, were digested with *SacI* and probed with the telomerase RNA gene. Plasmid telomere extension varied between the different classes of transformants (Figure 3). Cells microinjected with the G57A construct extended their telomeres to the same extent as cells expressing the plasmid-encoded wild-type RNA (an average value of 100 bp or 17 telomeric repeats). Telomeres in the A50G construct were approximately the same length as the input and G₄TC repeats were undetected by Southern blot analysis (data not shown). Cells expressing the C49A and C52A template mutations had longer telomeres than any of the other transformants (average increased length of 300 and 250 bp respectively). The same relative increase

Fig. 3. Southern blot analysis of a defined telomeric fragment from transformants. Digestion of the pPXVI-TER series of linearized, microinjected plasmids with the restriction enzyme *SacI* generates a telomeric fragment ~1.2 kb in length that includes the telomerase RNA gene (black arrow; see Figure 1B). A Southern blot of total DNA (1 µg), isolated from transformants and digested with *SacI*, was probed with the radiolabeled *P.tetraulera* telomerase RNA gene. PXVI, pPXVI transformant; G57A, pPXVI-G57A transformant; A50G, pPXVI-A50G transformant; C49A, pPXVI-C49A transformant; C52A, pPXVI-C52A transformant; w.t., pPXVI-w.t. transformant; uninj., uninjected cells.

or decrease in telomere extension was also detected when telomeres from pPXV-NEO were similarly analyzed (data not shown). It should be noted that the endogenous telomerase RNA is still present and is assumed to also contribute to transformant *de novo* telomere addition.

De novo telomere sequence analysis

Extension of the microinjected plasmid telomeres is representative of *de novo* telomere addition in transformed cells (Figure 3). The presence of an ampicillin resistance marker in these linear molecules made it possible to routinely isolate *de novo* telomeric repeats for more detailed sequence analysis. Briefly, total DNA from transformants was digested with the restriction enzyme *XbaI* to effectively remove one telomeric end from the microinjected plasmids (Figure 1B). The *XbaI*-treated DNA was incubated with T4 DNA polymerase and dNTPs to generate blunt ends, in order to facilitate circularization of the linear molecules with DNA ligase. Transformation of *Escherichia coli* with the ligation products followed by selection for ampicillin resistance resulted in the isolation of 'rescued' circular plasmid clones containing *de novo* telomeres. Due to the exonucleolytic activity associated with T4 DNA polymerase, the most distal repeats may be lost during the generation of blunt ends.

A compilation of *de novo* telomeric repeats from the various transformants is shown in Table II. Cells microinjected with either pPXVI or the plasmid expressing wild-type telomerase RNA had ~70% G_4T_2 repeats, consistent with the composition of previously reported *Paramecium* telomeres (Baroin *et al.*, 1987).

The C52A template substitution leads to a dramatic increase in G_3T_3 repeat synthesis in transformants. When 97% of telomerase RNAs in transformants included the C52A mutation, *de novo* telomeric repeats synthesized *in vivo* were 99% G_3T_3 . A duplicate experiment demonstrated the dosage dependence of this phenomenon. When 88% of telomerase RNAs in transformants were C52A, 85% of *de novo* telomeric repeats synthesized were G_3T_3 .

De novo telomeric DNA in C49A transformants included a high percentage of the 7 nt repeat G_4T_3 (~74%),

Fig. 4. Southern blot analysis of a defined telomeric fragment from transformants expressing *P. caudatum* telomerase RNA. A Southern blot of total DNA (1 µg), isolated from transformants and digested with *SacI*, was probed with the radiolabeled *P.tetraulera* telomerase RNA gene. *SacI* digestion generates a telomeric fragment ~1.2 kb in length that includes the telomerase RNA gene (black arrow; see Figures 1B and 3). Hybridization and wash conditions were at low stringency to ensure cross-hybridization of the *P.tetraulera* and *P. caudatum* telomerase RNA genes. pPXVI, pPXVI transformant; P.t. w.t., pPXVI-WT transformant; P.c. w.t., pPXVI-WTC transformant; P.t. C52A, pPXVI-C52A transformant; P.c. C52A, pPXVI-C52AC transformant.

as well as two novel 8 bp repeats (G_4T_4 and G_5T_3). It has been previously reported that the analogous template substitution in the *T. thermophila* telomerase RNA (C43A) leads to G_4T_3 telomeric repeat synthesis (Gilley *et al.*, 1995). The effect of the G57A substitution was the somewhat unexpected increase in G_3T_3 repeat synthesis, from ~30% in the wild-type controls to nearly 50%. Finally, the A50G mutated RNA contributed to telomeric repeat synthesis, albeit very poorly. Only two repeats (G_4TC and G_3TC) were detected from 10 rescued plasmids (data not shown). There was an apparent dominant effect with the A50G template mutation, as the contribution of the endogenous telomerase RNA (up to 13% of the total complement of telomerase RNA in transformants) to *de novo* telomere addition was much reduced (see Table I and Figure 3).

Only a fraction of the 30 bp distal to the 'seed' telomeric repeats of linearized plasmids was detected in 'rescued' plasmids, if at all. The junction between the pre-existing and *de novo* telomeric repeats ranged between 0 and 23 bp for the >70 transformant telomeres sequenced, consistent with degradation of non-telomeric DNA ends prior to *de novo* telomere addition (data not shown). In contrast, a previous study demonstrated that the ends of microinjected, linearized plasmids were generally not removed prior to telomere addition, as measured by the retention of restriction sites very near the linearized plasmid ends (Gilley *et al.*, 1988). Nucleolytic trimming of the distal 30 bp, perhaps by a nuclease activity associated with telomerase (Collins and Greider, 1993; Melek *et al.*, 1996), can apparently precede telomere addition to the linearized plasmids used in this study. It has been shown that developmentally controlled telomere addition at a single locus in *P. tetraulera* is specific to a region rather than to a specific site (Forney and Blackburn, 1988). It would appear that the determinants for *Paramecium* telomerase substrate recognition, during either vegetative growth or macronuclear development, are ill defined at this time.

Table II. The sequence of *de novo* telomeric repeats from transformants expressing telomerase RNAs from microinjected plasmids

Construct	Telomeric repeats (%) ^a								No. of repeats
	G ₅ T ₃	G ₄ T ₄	G ₅ T ₂	G ₃ T ₄	G ₄ T ₃	G ₄ T ₂	G ₃ T ₃	G ₃ T ₂	
pPXVI (1)					1.4	65.2	30.4	2.9	69
pPXVI (2)			3.3			70.0	26.7		30
Wild-type						69.3	29.3	1.3	75
C52A (1)						1.4	98.6		70
C52A (2)				2.5		12.5	85.0		80
C49A (1)	0.9	3.7			73.1	14.8	7.4		108
C49A (2)					74.5	12.7	11.8	1.0	102
G57A (1)						50.0	44.6	5.4	74
G57A (2)						45.5	54.5		44

^aTotal of two microinjections.

Telomeric repeats were cloned and sequenced from transformant DNA ~20–22 generations after microinjection, as described in Materials and methods. The percentages of the various telomeric repeats are as indicated. The total number of *de novo* repeats sequenced from duplicate microinjection experiments is shown on the right. Single nucleotide substitutions in the telomerase RNA are as indicated in Figure 1A. The plasmid pPXVI lacks the telomerase RNA gene (Figure 1B).

Table III. The sequence of *de novo* telomeric repeats from transformants expressing *P. caudatum* telomerase RNAs

Construct	TER ratio ^a		RNA ^b (%)	Telomeric repeats (%) ^c		No. of repeats
	DNA	RNA		G ₄ T ₂	G ₃ T ₃	
P.c. C52A (1)	4	25	96.1	8.0	92.0	75
	1	4	80.0			
P.c. C52A (2)	11	10	90.0	4.7	95.3	43
	19	14	93.3			
P.c. wild-type	7	25	96.1	76.7	23.3	90
	2	8	88.8			

^aRatio (plasmid/endogenous) for each microinjected clonal line.

^bPercent plasmid-encoded telomerase RNA.

^cTotal of two microinjections.

Both wild-type and C52A *P. caudatum* telomerase RNAs were expressed in *P. tetraurelia* transformants as described in the text. The abundance of *P. caudatum* telomerase RNA genes and transcripts, relative to those of the *P. tetraurelia* host, were determined as described in Table I. The percentages of G₃T₃ and G₄T₂ *de novo* telomeric repeats cloned and sequenced from duplicate microinjections are as indicated.

P. caudatum telomerase RNA expression in *P. tetraurelia*

Given the effect of various template mutations on *P. tetraurelia* telomerase fidelity, it is conceivable that a region of the telomerase RNA other than the template accounts for the apparent high fidelity of the *P. caudatum* enzyme. To test this possibility, a chimeric gene was constructed, consisting of the wild-type *P. caudatum* telomerase RNA coding sequence flanked by the upstream and downstream non-transcribed regions of the *P. tetraurelia* gene. The chimeric gene was cloned into the expression vector pPXVI and subsequently microinjected into *P. tetraurelia* as described above. The C52A template mutation, which directs the efficient synthesis of G₃T₃ repeats in *P. tetraurelia*, was introduced as a marker to determine if the *P. caudatum* telomerase RNA can contribute to *P. tetraurelia* telomere synthesis *in vivo*. Such a telomerase would have to function despite a primary sequence divergence of 24% between the two species' telomerase RNAs (McCormick-Graham and Romero, 1996).

Stable maintenance and transcription of the *P. caudatum* plasmid constructs following microinjection were confirmed and quantitated by Southern and Northern analyses as described earlier (Table III). Figure 4 indicates that telomere extension was similar in transformants expressing either *P. tetraurelia* or *P. caudatum* telomerase RNA. As shown previously, *de novo* telomeres from transformants

expressing the C52A telomerase RNA were consistently longer than those expressing the wild-type RNA.

The sequence of *de novo* telomeric repeats from the *P. caudatum* C52A transformants demonstrates that the *P. caudatum* telomerase RNA can form a functional telomerase enzyme with the *P. tetraurelia* telomerase proteins. Consistent with the result from the *P. tetraurelia* C52A telomerase RNA, 94% of *de novo* telomeric repeats from cells expressing the *P. caudatum* homolog were G₃T₃ (compare Tables II and III). However, the G₄T₂ content of telomeres from transformants expressing the *P. caudatum* wild-type RNA (76%) was similar to that seen for the *P. tetraurelia* wild-type controls (70%). These data suggest that the high fidelity of the *P. caudatum* telomerase does not reside solely in the telomerase RNA from that species.

Discussion

Paramecium tetraurelia telomerase is inherently imprecise, in contrast to telomerase activities from other ciliates that synthesize telomeric repeats with a very low error frequency. In addition to the G₄T₂ repeats predicted from *Paramecium* spp. telomerase RNA templates (McCormick-Graham and Romero, 1996), G₄T₃ and G₃T₂ repeats are also detected, with G₃T₃ constituting the vast majority of variable repeats (Baroin *et al.*, 1987; Forney and Blackburn, 1988).

How are variable telomeric repeats synthesized by *Paramecium* telomerase? Three error mechanisms, stuttering, stalling and misincorporation, can account for variable telomeric repeats in other organisms. *In vitro* characterization of telomerase activities from the yeasts *Saccharomyces cerevisiae* and *S.castelli* suggests that the normal variations seen in their telomeres are due to premature dissociation from and stuttering along the template, respectively (Cohn and Blackburn, 1995). Insertion of an additional C residue in the *T.thermophila* telomerase RNA template can lead to dramatic stuttering errors, with multiple dG residues added sequentially, both *in vivo* and *in vitro* (Yu and Blackburn, 1991; Gilley *et al.*, 1995). Misincorporation errors (addition of a non-cognate dNTP opposite a templating nucleotide) have been documented for a C→U transition in the *T.thermophila* telomerase RNA. A consequence of the C48U mutation is misincorporation of dA at adjacent templating rC residues *in vitro* (Gilley *et al.*, 1995). These data indicate that the templating nucleotides are involved in the active site of the enzyme, with contributions to telomerase activity beyond that of a passive template. We have shown that *Paramecium* spp. telomerase RNA template mutations also affect the fidelity of the enzyme *in vivo*. However, in contrast to the *Tetrahymena* enzyme, template substitutions in *Paramecium* can dramatically increase telomerase fidelity.

Based on the sequence of *de novo* telomeric repeats presented in Table II, we propose that the primary mechanism for variable telomeric repeat synthesis by wild-type *P.tetraurelia* telomerase is via misincorporation of dT residues, with the majority of errors occurring at templating nucleotide C52. Such errors should result in a *de novo* telomeric repeat of 5'-GGGTTTG-3'. This free 3'-end can be properly positioned at the alignment region immediately 3' of the template for subsequent telomeric repeat synthesis (Figure 5). Misincorporation of dT at C52 would account for the high frequency of G₃T₃ in *Paramecium* telomeres (Baroin *et al.*, 1987; Forney and Blackburn, 1988) and in our transformant controls (an average of 45 and 30% respectively). The C52A substitution apparently decreases the incidence of errors by accommodating a presumed tendency of *P.tetraurelia* telomerase to incorporate dT at this templating position. Alternatively, the three T residues in G₃T₃ could be generated by stuttering at template positions A50 and/or A51. A minor set of variable repeats, such as G₅T₂ and G₅T₃ (Table II), are best explained by stuttering. However, we consider misincorporation rather than stuttering as a more likely mechanism for the majority of G₃T₃ repeat synthesis by wild-type telomerase, given our results with the C49A template mutation (see below).

The *P.tetraurelia* C49A template substitution is analogous to the C43A mutation described previously for the *T.thermophila* telomerase RNA (Gilley *et al.*, 1995). A consequence of the *T.thermophila* C43A substitution was the synthesis of G₄T₃ telomeric repeats, a phenomenon also seen *in vivo* with the *Paramecium* C49A substitution (Table II). The most straightforward interpretation of these data is that nucleotide C55, normally a member of the alignment region (Autexier and Greider, 1995), is 'recruited' as a templating nucleotide. Alignment of a *de novo* 3'-end from the C49A enzyme (5'-TTT-3') for

Fig. 5. Model for telomere synthesis with mutated telomerase RNAs. Templating nucleotides are shown boxed, with the alignment region underlined (Autexier and Greider, 1995). Extension of a primer (lower case) by a single telomeric repeat (upper case) is shown for the wild-type and various mutated RNA genes. The degree of misincorporation at the shaded nucleotide for wild-type and G57A is indicated by the size of the nucleotide added at that position. Increased fidelity of telomerase with C49A and C52A substitutions is represented by the absence of shading at position 52. Alignment position C55 is utilized as a templating nucleotide in telomerase RNAs with a C49A substitution. See text for discussion.

a subsequent round of polymerization would occur at nucleotide positions 3'-A₅₈GA₅₆-5', allowing the 7 nt from positions 55 to 49 to serve as the template (Figure 5). Our interpretation is in concordance with that drawn by Gilley and Blackburn (1995) with regard to the *Tetrahymena* mutation. Infrequent dT misincorporation at template position C49 may account for the occasional G₄T₃ repeats detected in *Paramecium* telomeres (Baroin *et al.*, 1987; Forney and Blackburn, 1988) and in the *de novo* telomeres from wild-type transformants described in this study (1.5 and 1.4% respectively). An alternative explanation for the occurrence of 5'-TTT-3' at the end of a telomeric repeat is an occasional stutter by the wild-type telomerase at templating nucleotides A50 or A51, followed by dissociation from the enzyme prior to dG addition at C49.

Another consequence of the C49A template change is an increase in the *Paramecium* telomerase fidelity *in vivo*. When 72% of the *P.tetraurelia* telomerase RNA molecules in transformants include this substitution, 74% of *de novo* telomeric repeats synthesized are G₄T₃ (Table II). Continued misincorporation of dT at C52 in the C49A transformants should result in up to 30% of the *de novo* repeats consisting of G₄T₄. However, only 3.7% of *de*

*nov*o telomeric repeats in these transformants are G₄T₄ (Table II). It is not clear how the C49A mutation increases telomerase fidelity. Perhaps the C49A mutation itself, or the utilization of C55 as a templating nucleotide instead of participation in 3'-end alignment, alters the configuration of the active site such that dT misincorporation at C52 is much reduced.

The putative alignment region of wild-type *Paramecium* telomerase RNA includes a G residue (G57), with the potential to form a rG-dT wobble base pair during continued telomeric repeat synthesis (Figure 5). This is in contrast to all other ciliate RNAs, which include an A residue at the homologous position. We explored the effect of a G57A substitution on the error rate of *Paramecium* telomerase. Somewhat surprisingly, we found that the G57A mutation leads to a 20% increase in G₃T₃ errors, from 30 to 50%. Chemical and enzymatic probing of the *Tetrahymena* telomerase RNA indicate that both the template and alignment regions are in an accessible and highly ordered structure (Bhattacharyya and Blackburn, 1994; Zaug and Cech, 1995). The 20% increase in G₃T₃ errors with the G57A mutation may be due to a subtle perturbation of this structure, consequently increasing dT misincorporation at C52. Altering the geometry of the enzyme's templating domain with the G57A substitution paradoxically has the opposite effect of the C49A mutation on fidelity. Interestingly, substitution of the homologous nucleotide in the *Tetrahymena* RNA (A51) with a G residue leads to a different sort of error during polymerization: premature product dissociation *in vitro* (Gilley and Blackburn, 1996).

Telomeres extended primarily by the addition of G₄T₃ or G₃T₃ repeats (in the C49A and C52A transformants respectively) were consistently longer than those from wild-type controls (Figures 3 and 4). This loss of telomere length regulation resembles a similar phenomenon in *T.thermophila* and *Kluvermyces lactis* transformants synthesizing mutated telomeres, although not to the same degree (Yu *et al.*, 1990; McEachern and Blackburn, 1995; Romero and Blackburn, 1995). It has been determined that diminished binding of the sequence-specific DNA-binding protein RAP1 to mutated telomeres is responsible for the loss of telomere length control in *K.lactis* (Krauskopf and Blackburn, 1996). A similar defect in the recognition of altered telomeric DNA is the most likely mechanism for the apparent loss of telomere length regulation in this study.

Inefficient telomere addition in cells expressing the A50G telomerase RNA (Figure 3) appears to be a dominant effect, as *de novo* telomere synthesis was reduced in these transformants, despite the presence of the endogenous, wild-type RNA (up to 13%; Table II). This phenotype may be due to diminished binding and/or recognition of the *de novo* 3'-end (5'-GT $\overline{\text{C}}$ G-3') by telomerase. A critical Watson-Crick base pair between the 3'-end of a telomeric primer and nucleotide A50 in the *Tetrahymena* RNA (the homolog to the *Paramecium* A56 alignment nucleotide) has been demonstrated *in vitro* (Gilley and Blackburn, 1996). Successful extension of a 5'-GT $\overline{\text{C}}$ G-3' telomeric end by either wild-type or A50G *Paramecium* telomerase would necessarily have to tolerate a rA-dC mismatch at alignment nucleotide A56 (Figure 5). Given our results with A50G transformants, this is an apparently inefficient

process *in vivo* in *Paramecium*. Somewhat paradoxically, *in vivo* synthesis of G₄TC repeats by *T.thermophila* is quite efficient when the A44G telomerase RNA gene (analogous to *Paramecium* A50G) is introduced into cells by microinjection (Yu *et al.*, 1990; Romero and Blackburn, 1995).

It is remarkable that wild-type *P.tetraurelia* telomerase misincorporates dT residues at the same template position at a fairly high frequency and to the exclusion of non-cognate dA or dC residues. It is conceivable that a post-transcriptional modification of C52 could promote pairing to dTTP rather than dGTP at that templating position during telomere synthesis. Alternatively, the observed bias for dT incorporation at C52 may indicate greater binding efficiency for dTTP than the other dNTPs (excluding the cognate dGTP) at that point in the polymerization cycle. Competition and chain termination studies with nucleoside analogs indicate that the nucleotide-binding properties of *Tetrahymena* telomerase differ at various positions along the template (Strahl and Blackburn, 1994). X-ray crystallographic studies of nucleic acid structures have demonstrated the potential for pyrimidine-pyrimidine base pair formation (reviewed by Wahl and Sundaralingam, 1995). A mismatched C-U pair can form with a single base-base hydrogen bond with a water molecule bridging the bases, accommodated by minor distortion of the helix (Cruse *et al.*, 1994). A novel Hoogsteen-like U-U base pair has been described, demonstrating the possibility of unusual pyrimidine-pyrimidine interactions (Wahl *et al.*, 1996). On the other hand, misincorporation of dT juxtaposed to C52 may be more a function of the *P.tetraurelia* enzyme's active site conformation than the ability of a C residue to form a base pair with dTTP. It is conceivable that substitution of C52 with either a G or U residue may result in continued dT misincorporation at that position.

What factors determine the error rate of *Paramecium* telomerase? We have established the involvement of the RNA moiety in fidelity, as a variety of nucleotide substitutions can either increase or decrease the percentage of *de novo* G₃T₃ repeats. Mutagenic experiments with *Tetrahymena* telomerase also indicate that the templating nucleotides contribute more to the active site than simply serving as a template (Gilley *et al.*, 1995). However, our data indicate that the telomerase RNA is not solely responsible for the observed variability.

Greater than 95% of *P.caudatum* telomeres are comprised of G₄T₂ repeats (McCormick-Graham and Romero, 1996), consistent with a low error rate for telomerase from this species. Despite the substantial primary sequence divergence of the RNAs from the two species, a chimeric enzyme composed of C52A *P.caudatum* telomerase RNA and *P.tetraurelia* proteins functions *in vivo*, synthesizing a high percentage of G₃T₃ repeats (Figure 4 and Table III). However, the parallel experiment with wild-type *P.caudatum* telomerase RNA expressed in transformants does not lead to a chimeric telomerase with high fidelity (compare Tables II and III). Our data indicate that additional factors, most likely telomerase proteins, contribute to telomerase fidelity. Our data also support the contention of Gilley and Blackburn (1996) that telomerase RNA is a component of a telomerase active site, working in concert with telomerase protein(s) to catalyze the synthesis of telomeric repeats.

Altering the sequence of telomeric DNA in organisms that normally synthesize invariant telomeric repeats can have drastic effects on cell division and morphology, ultimately leading to senescence (Yu *et al.*, 1990; McEachern and Blackburn, 1995). In contrast, the only phenotype detected in *P.tetraulera* transformants synthesizing a reduced percentage of G₄T₂ repeats is a moderate loss of telomere length regulation. Perhaps this is not too surprising, given the naturally occurring variability in wild-type *P.tetraulera* telomeres. It would be interesting to determine whether telomere sequence variability is so readily tolerated in *P. caudatum*, a species whose telomerase has evolved to precisely synthesize G₄T₂ repeats. A comparison of *in vitro* telomerase activities from these two species may reveal whether *P. caudatum* telomerase is inherently more precise at templating nucleotide C52 or if there is a specific proof-reading mechanism during telomere addition, perhaps involving the exonucleolytic activity associated with telomerase, that can correct the mistakes of an error-prone enzyme.

Materials and methods

Materials

Paramecium tetraulera (nd6/nd6 and cam²/cam²; Lefort-Tran *et al.*, 1981; Kanabrocki *et al.*, 1991) were maintained at room temperature in a monoxenic wheat grass infusion, containing *Enterobacter aerogenes* (Sonneborn, 1970). Restriction enzymes and other molecular biology reagents were purchased from New England Biolabs (Beverly, MA). The antibiotic G-418 was purchased from Sigma (St Louis, MO).

General methods

Genomic DNA was isolated and total RNA extracted *P.tetraulera* as previously described (McCormick-Graham and Romero, 1996). Oligonucleotides were radiolabeled at the 5'-end with T4 polynucleotide kinase and [γ -³²P]ATP (7000 Ci/mmol; ICN) as described (Sambrook, 1989). Radiolabeled telomerase RNA gene probe was generated by a PCR strategy as previously described (McCormick-Graham and Romero, 1996). Conventional PCR protocols (Saiki *et al.*, 1988) and molecular techniques (Sambrook *et al.*, 1989) were used for plasmid construction. DNA sequencing was performed with either SequenaseTM (US Biochemical, Cleveland, OH) or the CircumVent Thermal Cycle DNA sequencing kit (New England Biolabs, Beverly, MA).

Oligonucleotides

Utilization of the following oligonucleotides (Amitof Biotech, Boston, MA) is included in the text. PCR primers (P), mutagenic oligonucleotides (M), antisense probes (A) and sequencing primers (S) are as indicated. All oligonucleotides are written 5'→3': PIT, CGC CAG CTG CTT TGG AAC ATG GGC AC; P2T, CGC CAG CTG CTT GAA TTA CGA; PIC, CGC GTA TAC TGA CAA TTG CTG TAT TCA; P2C, CCG CAG ATC TAA AAA TAA CCA TTC AGA G; M1, TTT CTG GGG TCG TAT GAC TT; M2, GTA ATG GTT TTT GGG GTT GT; M3, GGT TTC TGG GTT TGT ATG AC; M4, TTC TGG GGT TTT ATG ACT TCC; M5, ATT AAT GAA AAA GAT CTA AAA ATA AC; M6, ATT AAT GAA AAA AAT ATA AAA ATA AC; S1, TAA CTT TTA CTC AAT GTC AAA G; S2, GCG TCT AGA AAT AAC TAT TTA GA GC; A1, GGT TTC TGG GGT TGT ATG AC; A2, GGT TTC TGG GGT TTT ATG AC; A3, CAC TTA TGA CTC GTC; A4, TAC TTT TAG GTT CGT.

Transformation vector modification

Plasmid pPXVI was constructed by modification of pPXV-NEO (Haynes *et al.*, 1995). The APH-3'-II expression cassette (2.0 kb) was removed from pPXV-NEO by digestion with *PvuII*. A 260 bp *PvuII*-*SmaI* fragment from the cloning vector pBluescript II SK (Stratagene, La Jolla, CA) was ligated to the large 3.5 kb fragment from the pPXV-NEO *PvuII* digest. Restriction digests confirmed the orientation of pPXVI polylinker sites shown in Figure 1B.

Paramecium tetraulera telomerase RNA gene mutagenesis

The *P.tetraulera* telomerase RNA gene was amplified from genomic DNA by a standard PCR reaction, utilizing oligonucleotides PIT and

P2T. The resultant 565 bp amplified product includes 240 bp upstream and 110 bp downstream of the transcribed RNA (McCormick-Graham and Romero, 1996). This PCR product was cloned into the *SmaI* site of pUC118 and screened by restriction digests for the correct orientation (designated pPTER). Oligonucleotide, site-directed mutagenesis of the telomerase RNA gene in pPTER was by the method of Kunkel *et al.* (1987). Mutagenic oligonucleotides M1, M2, M3 and M4 were used to introduce A50G, G57A, C52A and C49A substitutions respectively (Figure 1A), which were subsequently confirmed by single-strand DNA sequencing. The wild-type and mutated telomerase RNA genes were cloned into the vector pPXVI by virtue of unique *SacI* and *XbaI* restriction sites. The telomerase RNA genes of the resultant pPXVI-TER constructs (Figure 1B) were confirmed by double-stranded DNA sequencing.

Chimeric *P. caudatum*/*P. tetraulera* telomerase RNA gene construct

A unique *BglIII* restriction site was introduced at nucleotide position +209 of the *P.tetraulera* telomerase RNA gene in pPTER by site-directed mutagenesis (oligonucleotide M5). The resultant plasmid (pPTERb) includes a unique *BstI*1109I site at nucleotide position -4 (McCormick-Graham and Romero, 1996) and a singular *BglIII* site at nucleotide position +209. The cloned *P. caudatum* telomerase RNA gene was amplified by PCR with oligonucleotides PIC and P2C, which were designed to ensure unique *BstI*1109I and *BglIII* restriction sites at the termini of the 218 bp PCR product. Both pPTERb and the *P. caudatum* PCR product were digested with *BstI*1109I and *BglIII*, ligated and recombinant molecules screened for an *EcoRI* site present in the *P. caudatum* RNA gene sequence (pPCTERb). Site-directed mutagenesis with oligonucleotide M6 removed the unique *BglIII* site, restoring nucleotide positions +209 to +214 of pPCTERb to the wild-type sequence (pPCTER). Further mutagenesis of pPCTER with M3 introduced a C52A templating nucleotide substitution (pPCTER-C52A). The chimeric wild-type and C52A telomerase RNA genes were cloned into the vector pPXVI at *SacI* and *XbaI* restriction sites as described above. Telomerase RNA gene sequences of the resultant pPXVI-WTC and pPXVI-C52AC constructs were confirmed by direct sequencing.

Transformation of *P. tetraulera* by microinjection

Plasmids suitable for microinjection were prepared with the WizardTM DNA purification system (Promega, Madison, WI). The plasmids pPXV-NEO, pPXVI and various pPXVI-TER constructs were linearized by *SfiI* digestion (Figure 1B). Preparation and monitoring of post-autogamous cells prior to microinjection were as previously described (Haynes *et al.*, 1995). Macronuclei were injected with 10–20 pl each DNA sample (1 μ g/ μ l, $\sim 2 \times 10^6$ molecules/pl) between the third and sixth post-autogamous division (Haynes *et al.*, 1995). Following microinjection, clonal lines were allowed to expand to 100 cells (3–4 days at room temperature). Approximately 20 cells of potentially transformed cultures were challenged with G-418 (50 μ g/ml). Successfully transformed cells were resistant to the antibiotic 2 days after the initial challenge. Transformant cultures were carefully maintained at a density of 200 cells/ml and expanded for 20–22 generations following microinjection.

In vitro transcription of mutated telomerase RNAs

A plasmid (pPTER-T7) designed for *in vitro* transcription of the *P.tetraulera* telomerase RNA has been described (McCormick-Graham and Romero, 1996). Mutagenic oligonucleotides M1–M4 were used to introduce A50G, G57A, C52A and C49A substitutions into pPTER-T7 as described above. Linearization of these plasmids with *XbaI* followed by *in vitro* transcription with T7 RNA polymerase and rNTPs (Promega Transcription *in vitro* System) results in run-off transcription of both wild-type and mutant *P.tetraulera* telomerase RNAs.

Northern blot allele-specific hybridizations

Total RNA (0.2–2 μ g) isolated from transformants and telomerase RNA *in vitro* transcripts (50 ng) were electrophoresed through an 8% polyacrylamide–7.5 M urea gel and electroblotted to Nytran filters (Schleicher & Schuell). The specificity of ³²P-labeled oligonucleotide binding to Northern blots was determined empirically by varying the washing temperatures (52–58°C) and monitoring hybridization to the *in vitro* transcripts as previously described (McCormick-Graham and Romero, 1996).

Northern blots were probed sequentially with the appropriate oligonucleotides radiolabeled to a specific activity of 7000 Ci/mmol. Wash temperatures, buffer volumes and specific activities of hybridization solutions (0.4 μ Ci/ml) were carefully monitored to ensure an accurate,

quantitative comparison. A Molecular Dynamics PhosphorImager was used to quantitate the relative ratio of the plasmid-encoded to endogenous telomerase RNA isolated from transformants. Exposure times to the phosphorimager were adjusted to correct for decay of the ^{32}P radiolabel.

Southern blot hybridizations

DNA transfer from agarose gels onto Nytran membranes, hybridization of radiolabeled DNA probes and washing conditions were as previously described (McCormick-Graham and Romero, 1996). A Molecular Dynamics PhosphorImager was used to quantitate the relative ratio of the plasmid-encoded to endogenous telomerase RNA genes present in total DNA from transformants.

Cloning de novo telomeres from *P.tetraurelia* transformants

Total DNA (5 μg) extracted from transformants 20–22 fissions after microinjection was digested with the restriction enzyme *Xba*I in a 50 μl reaction, effectively removing one telomeric end from the microinjected pPXVI-TER plasmids (Figure 1B). The *Xba*I-digested DNA was treated with 6 U T4 DNA polymerase (New England Biolabs) in a standard reaction buffer (0.1 mM dNTPs) at 12°C for 15 min to generate blunt ends suitable for ligation. Following phenol:chloroform extraction and ethanol precipitation, 0.5 μg DNA were incubated overnight at 16°C with T4 DNA ligase (100 μl reaction volume). Competent *E.coli* (strain DH10B) were transformed by electroporation with the ligation products. DNA prepared from ampicillin-resistant clones was screened for the telomerase RNA gene by Southern blot hybridization. The C-strands of cloned, *de novo* telomeric repeats were sequenced, utilizing radiolabeled oligonucleotide S1 as a sequencing primer. The identities of telomerase RNA genes in 'rescued' plasmids were also confirmed by sequencing through the plasmid-encoded genes with oligonucleotide primer S2 (complementary to nucleotide positions +209 to +191 inclusive of the telomerase RNA gene).

Acknowledgements

We thank Karin Musier-Forsyth and Greg Connell for critical reading of and helpful comments on the manuscript. This research was supported by NIH grant GM50861.

References

Agard,D.A. and Sedat,J.W. (1983) Three-dimensional architecture of a polytene nucleus. *Nature*, **302**, 676–681.

Autexier,C. and Greider,C.W. (1994) Functional reconstitution of wild-type and mutant *Tetrahymena* telomerase. *Genes Dev.*, **8**, 563–575.

Autexier,C. and Greider,C.W. (1995) Boundary elements of the *Tetrahymena* telomerase RNA template and alignment domains. *Genes Dev.*, **9**, 2227–2239.

Baroin,A., Prat,A. and Caron,F. (1987) Telomeric site position heterogeneity in macronuclear DNA of *Paramecium primaurelia*. *Nucleic Acids Res.*, **15**, 1717–1728.

Bhattacharyya,A. and Blackburn,E.H. (1994) Architecture of telomerase RNA. *EMBO J.*, **13**, 5721–5731.

Biessmann,H. and Mason,J.W. (1992) Genetics and molecular biology of telomeres. *Adv. Genet.*, **30**, 185–249.

Blasco,M.A., Funk,W., Villeponteau,B. and Greider,C.W. (1995) Functional characterization and developmental regulation of mouse telomerase RNA. *Science*, **269**, 1267–1270.

Bourgain,F.M. and Katinka,M.D. (1991) Telomeres inhibit end to end fusion and enhance maintenance of linear DNA molecules injected into the *Paramecium primaurelia* macronucleus. *Nucleic Acids Res.*, **19**, 1541–1547.

Cohn,M. and Blackburn,E.H. (1995) Telomerase in yeast. *Science*, **269**, 396–400.

Collins,K. and Greider,C.W. (1993) *Tetrahymena* telomerase catalyzes nucleolytic cleavage and nonprocessive elongation. *Genes Dev.*, **7**, 1364–1376.

Collins,K., Kobayashi,R. and Greider,C.W. (1995) Purification of *Tetrahymena* telomerase and cloning of genes encoding the two protein components of the enzyme. *Cell*, **81**, 677–686.

Cruse,W.B., Saludjian,P., Biala,E., Strazewski,P., Prange,T. and Kennard,O. (1994) Structure of a mispaired RNA double helix at 1.6-Å resolution and implications for the prediction of RNA secondary structure. *Proc. Natl Acad. Sci. USA*, **91**, 4160–4164.

de Lange,T. (1992) Human telomeres are attached to the nuclear matrix. *EMBO J.*, **11**, 717–724.

Feng,J. *et al.* (1995) The RNA component of human telomerase. *Science*, **269**, 1236–1241.

Forney,J.D. and Blackburn,E.H. (1988) Developmentally controlled telomere addition in wild-type and mutant *Paramecia*. *Mol. Cell. Biol.*, **8**, 251–258.

Gilley,D. and Blackburn,E.H. (1996) Specific RNA residue interactions required for enzymatic functions of *Tetrahymena* telomerase. *Mol. Cell. Biol.*, **16**, 66–75.

Gilley,D., Preer,J.J., Aufderheide,K.J. and Polisky,B. (1988) Autonomous replication and addition of telomeric sequences to DNA microinjected into *Paramecium tetraurelia* macronuclei. *Mol. Cell. Biol.*, **8**, 4765–4772.

Gilley,D., Lee,M.S. and Blackburn,E.H. (1995) Altering specific telomerase RNA template residues affects active site function. *Genes Dev.*, **9**, 2214–2226.

Greider,C.W. and Blackburn,E.H. (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature*, **337**, 331–337.

Haynes,W.J., Ling,K.Y., Saimi,Y. and Kung,C. (1995) Induction of antibiotic resistance in *Paramecium tetraurelia* by the bacterial gene *APH-3'-II*. *J. Eukaryote Microbiol.*, **42**, 83–91.

Kanabrocki,J.A., Saimi,Y., Preston,R.R., Haynes,W.J. and Kung,C. (1991) Efficient transformation of *cam*², a behavioral mutant of *Paramecium tetraurelia*, with the calmodulin gene. *Proc. Natl Acad. Sci. USA*, **88**, 10845–10849.

Krauskopf,A. and Blackburn,E.H. (1996) Control of telomere growth by interactions of *RAP1* with the most distal telomeric repeats. *Nature*, **383**, 354–357.

Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) Rapid and efficient site-directed mutagenesis without phenotypic selection. *Methods Enzymol.*, **155**, 166–178.

Lefort-Tran,M., Aufderheide,K., Pouphile,M., Rossignol,M. and Beisson,J. (1981) Control of exocytotic processes: cytological and physiological studies of trichocyst mutants in *Paramecium tetraurelia*. *J. Cell Biol.*, **88**, 301–311.

Lingner,J. and Cech,T.R. (1996) Purification of telomerase from *Euplotes aediculatus*: requirement of a primer 3' overhang. *Proc. Natl Acad. Sci. USA*, **93**, 10712–10717.

Lingner,J., Hendrick,L.L. and Cech,T.R. (1994) Telomerase RNAs of different ciliates have a common secondary structure and a permuted template. *Genes Dev.*, **8**, 1984–1998.

McCormick-Graham,M. and Romero,D.P. (1995) Ciliate telomerase RNA structural features. *Nucleic Acids Res.*, **23**, 1091–1097.

McCormick-Graham,M. and Romero,D.P. (1996) A single telomerase RNA is sufficient for the synthesis of variable telomeric DNA repeats in ciliates of the genus *Paramecium*. *Mol. Cell. Biol.*, **16**, 1871–1879.

McEachern,M.J. and Blackburn,E.H. (1995) Runaway telomere elongation caused by telomerase RNA gene mutations. *Nature*, **376**, 403–409.

Melek,M., Greene,E.C. and Shippen,D.E. (1996) Processing of nontelomeric 3' ends by telomerase—default template alignment and endonucleolytic cleavage. *Mol. Cell. Biol.*, **16**, 3437–3445.

Romero,D.P. and Blackburn,E.H. (1991) A conserved secondary structure for telomerase RNA. *Cell*, **67**, 343–353.

Romero,D.P. and Blackburn,E.H. (1995) Circular rDNA replicons persist in *Tetrahymena thermophila* transformants synthesizing GGGGTC telomeric repeats. *J. Eukaryote Microbiol.*, **42**, 32–43.

Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487–491.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shippen-Lentz,D. and Blackburn,E.H. (1990) Functional evidence for an RNA template in telomerase. *Science*, **247**, 546–552.

Singer,M.S. and Gottschling,D.E. (1994) *TLCl*: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science*, **266**, 404–409.

Sonneborn,T.M. (1970) Methods in *Paramecium* research. *Methods Cell Physiol.*, **4**, 242–339.

Strahl,C. and Blackburn,E.H. (1994) The effects of nucleoside analogs on telomerase and telomeres in *Tetrahymena*. *Nucleic Acids Res.*, **22**, 893–900.

Wahl,M.C. and Sundaralingam,M. (1995) New crystal structures of nucleic acids and their complexes. *Curr. Opin. Struct. Biol.*, **5**, 282–95.

- Wahl,M.C., Rao,S.T. and Sundaralingam,M. (1996) The structure of r(UUCGCG) has a 5'-UU-overhang exhibiting Hoogsteen-like trans U-U base pairs. *Nature Struct. Biol.*, **3**, 24–31.
- Yu,G.-L. and Blackburn,E.H. (1991) Developmentally programmed healing of chromosomes by telomerase in *Tetrahymena*. *Cell*, **67**, 823–832.
- Yu,G.-L., Bradley,J.D., Attardi,L.D. and Blackburn,E.H. (1990) *In vivo* alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature*, **344**, 126–132.
- Zakian,V.A. (1989) Structure and function of telomeres. *Annu. Rev. Genet.*, **23**, 579–604.
- Zaug,A.J. and Cech,T.R. (1995) Analysis of the structure of *Tetrahymena* nuclear RNAs *in vivo*—telomerase RNA, the self-splicing rRNA intron, and U2 snRNA. *RNA*, **1**, 363–374.

Received on December 9, 1996; revised on February 10, 1997