Three telomerases with completely non-telomeric template replacements are catalytically active

Tracy L. Ware¹, He Wang^{2,3}
and Elizabeth H. Blackburn^{1,2,4}

¹Department of Biochemistry and Biophysics and ²Department of Microbiology and Immunology, Box 0414, University of California, San Francisco, CA 94143, USA

³Present address: Department of Microbiology and Cancer Center, Michigan State University, 426 Giltner Hall, East Lansing, MI 48824, USA

⁴Corresponding author
e-mail: telomer@itsa.ucsf.edu

Telomerase is a reverse transcriptase minimally composed of a reverse transcriptase protein subunit and an internal RNA component that contains the templating region. Point mutations of template RNA bases can cause loss of enzymatic activity, reduced processivity and misincorporation *in vitro*. Here we report the first complete replacement of the nine base *Tetrahymena thermophila* telomerase templating region *in vivo* with non-telomeric sequences. Rather than ablating telomerase activity, three such replaced telomerases (U9, AUN and AU4) were effective in polymerization *in vitro*. *In vivo*, the AU4 and AUN genes caused telomere shortening. We demonstrated the fidelity of the AUN and U9 telomerases *in vitro* and utilized AUN telomerase to demonstrate that 5' end primer recognition by telomerase is independent of template base pairing. However, the mutant AUN template telomerase catalyzed an abnormal DNA cleavage reaction. For these U-only and AU-substituted templates, we conclude that base-specific interactions between the telomerase template and protein (or distant parts of the RNA) are not absolutely required for the minimal core telomerase functions of nucleotide addition and base discrimination.

Keywords: anchor site/reverse transcriptase/telomerase/*Tetrahymena*

Introduction

Telomerase has been identified in many organisms as the cellular reverse transcriptase that counterbalances chromosome shortening (for reviews see Greider, 1995, 1996). The cellular DNA polymerases replicate in a 5' to 3' direction, initiating from an RNA primer that is later removed, leaving the 3' terminus of the parental DNA strand uncopied, thereby shortening the chromosome over successive cell divisions (for reviews see Blackburn, 1991; Lingner and Cech, 1998). Telomerase synthesizes multiple copies of a non-coding DNA sequence by direct addition onto the chromosome 3' end, thereby extending chromosome length and effectively creating a buffer against the

future loss of genetic information. While telomerase is similar to other DNA polymerases in its 5' to 3' direction of DNA synthesis, it differs in using the chromosome 3' end as a primer and in carrying an internal RNA template, a small portion of which (3'-AACCCCAAC-5' in the ciliate *Tetrahymena thermophila*) is used for making complementary telomeric repeats (TTGGGG, repeated ~50 times at each chromosome end). The telomeric repeats and several specifically bound proteins together comprise the telomere.

Telomerase synthesizes DNA by copying an RNA template, making it a reverse transcriptase (Greider and Blackburn, 1989; Shippen-Lentz and Blackburn, 1990). In its most minimal active form, telomerase is composed of one protein called TERT or TP2 (Beattie *et al.*, 1998; Collins and Gandhi, 1998) and an intrinsic RNA molecule called TER, sized 0.15–1.4 kb (for review see Nugent and Lundblad, 1998). The telomerase protein TERT, identified in many organisms, has conserved reverse transcriptase amino acid motifs, including motifs A and C (Bryan *et al.*, 1998) that contain the three invariant aspartates necessary for catalytic activity (Harrington *et al.*, 1997; Lingner *et al.*, 1997; Collins and Gandhi, 1998). *Tetrahymena* telomerase activity, the best characterized *in vitro*, differs significantly from retroviral reverse transcriptases in several ways. Whereas retroviral reverse transcriptases can utilize a variety of RNA molecules as templates for DNA synthesis, telomerase is dedicated to its integral RNA (Collins and Gandhi, 1998). Furthermore, retroviral reverse transcriptases have the ability to transcribe long stretches of RNA, whereas copying by telomerase is restricted to only the short template region of its RNA, defined by specific borders encoded in the telomerase RNA (Autexier and Greider, 1995; Tzfati *et al.*, 2000). Telomerase requires structures present in the entire RNA molecule for proper enzymatic activity (Bhattacharyya and Blackburn, 1997; Roy *et al.*, 1998; Gilley and Blackburn, 1999; Ware, 1999) and remains stably associated with its RNA, which is an intrinsic part of the telomerase particle (Prescott and Blackburn, 1997; Licht and Collins, 1999). Lastly, during the *in vitro* synthesis reaction, *Tetrahymena* telomerase is unlike retroviral reverse transcriptases in its ability to translocate many times to the beginning of its template region, placing dozens of DNA repeats on a single primer (Greider, 1991; Lee and Blackburn, 1993).

Telomerase RNA templates from several organisms have been mutated and studied, including those from *T. thermophila* (Autexier and Greider, 1994; Gilley *et al.*, 1995; Gilley and Blackburn, 1996), *Kluyveromyces lactis* (McEachern and Blackburn, 1995), human (Marusaic *et al.*, 1997) and *Saccharomyces cerevisiae* (Prescott and Blackburn, 1997; S.Chan and E.Blackburn, unpublished data). Biochemical studies of such mutated telomerases

(assembled *in vivo* and extracted from cells) have shown that base changes in the template RNA are usually copied into DNA products. Template mutations can also cause specific changes in enzymatic properties *in vitro*. A specific triplet substitution in the telomerase of the yeast *S.cerevisiae* caused complete loss of enzymatic activity both *in vivo* and *in vitro* (Prescott and Blackburn, 1997). In *Tetrahymena*, single base substitutions in the template sequence can cause dNTP misincorporation, early dissociation of product at a particular template position and mispaired primer extension (Gilley *et al.*, 1995; Gilley and Blackburn, 1996). *In vivo* results obtained using substitutions of the templating sequence of *Paramecium* telomerase suggest that a programmed misincorporation causes synthesis of variant repeat units *in vivo* (McCormick-Graham *et al.*, 1997).

Taken together, the studies summarized above suggested that specific template RNA residues play an active role in enzymatic function, beyond providing a template for nucleotide addition (reviewed in Blackburn, 1999). These roles have been proposed to occur through base-specific interactions between the template and other RNA or amino acid residues. Therefore, we tested the effects of replacing the templating domain of *Tetrahymena* telomerase activity with a completely non-telomeric sequence. Three different non-telomeric sequences, composed entirely of rA and/or rU residues, were chosen and substituted for wild-type sequence. Thus, we eliminated the core of four C residues from the template (complementary to the preferred G-rich sequence at the 3' end of substrate primers) and forced utilization of the non-telomeric nucleotide dATP. Although *in vivo* results of a complete template replacement have been reported previously in yeast (Henning *et al.*, 1998), this study involved substituting the yeast telomeric template for the human telomeric template, thereby retaining the clusters of rC residues in the template and mutating only nine of 16 template residues.

Here we report the results of the first complete *in vivo* replacement of a telomerase RNA template with non-telomeric sequences (U9, AUN and AU4; Figure 1A). Substituting the template did not ablate *in vitro* polymerization activity or telomerase-mediated cleavage of the template-aligned primer. The template replacement telomerases faithfully copied nucleotides using the new templates *in vitro*, indicating that telomerase can perform

correct dNTP selection utilizing a fully non-telomeric sequence as a template. However, the template substitutions impaired processivity and translocation to the beginning of the template, and cleavage occurred at template positions different from wild type.

Fig. 1. AU4-substituted telomerase is active *in vitro* and dependent on a primer complementary to its new template. (A) Template sequences of wild-type and non-telomeric template substitutions, all within an otherwise wild-type telomerase RNA. (B) Telomerase assay primer g3 (used to detect wild-type activity in each extract) is the complement of the wild-type template. Primer at3 is designed to have its 3' end nucleotides complementary to the AU4 template. (C) Products of telomerase assays (see Materials and methods) using partially purified wild-type transformed (WT-t) extracts (lanes 1–3) and AU4 mutant extracts (lanes 4–8). Unlabeled dNTPs were provided at 100 μ M as indicated above each lane; radiolabeled dNTPs are provided at 1.9 μ M and indicated by *. Primers were gel purified and present at 1 μ M. Markers indicate the migration of input primer +1 product for at3 and g3 primers. Telomerase reaction products that migrate at the input primer position result from the inherent ability of telomerase to perform both endonucleolytic cleavage and polymerization on a single substrate. (D) Proposed elongation reaction by AU4 telomerase using the ata primer.

Results

We separately substituted AU4, AUN and U9 template sequences (Figure 1A) into otherwise wild-type telomerase RNA genes and transformed *Tetrahymena* using a high-copy number vector. This strategy was chosen to allow *in vivo* transcription and assembly of mutant telomerase RNAs into the telomerase ribonucleoprotein (RNP), as previously described (Yu *et al.*, 1990; Bhattacharyya and Blackburn, 1997). Clonal lines expanded from single transformant cells, or pooled transformant cells, were used to prepare genomic DNA. Pooled transformants were used to prepare nuclear extracts to assess telomerase activity. Although wild-type copies of the endogenous telomerase RNA gene are still present in the cell, previous studies have shown that mutant telomerase RNAs introduced using this high-copy number vector (~10 000) can replace the endogenous RNA in the telomerase RNP, leaving no detectable trace of wild-type activity when assayed *in vitro* (Gilley *et al.*, 1995). During normal development of mated *Tetrahymena*, cells undergo chromosome breakage and *de novo* telomere addition (reviewed in Blackburn, 1995). Since the mutant gene is introduced after this process begins (Orias, 1986), *de novo* telomere addition is performed by the maternal endogenous telomerase (Yu and Blackburn, 1991) until transformants are swamped by expression of the introduced mutant telomerase RNA (see Materials and methods). Hence, all telomeres were expected to contain wild-type ends when first encountered by newly created mutant telomerase.

To test whether telomerase was present and active in cells transformed with AU4, AUN and wild-type (control) telomerase RNA genes, we made extracts and purified activity according to standard protocols (Wang and Blackburn, 1997). Telomerase assays were performed using an unlabeled oligonucleotide primer, one α -³²P-labeled dNTP and the remaining unlabeled dNTPs. Radiolabeled products were precipitated and resolved by electrophoresis in denaturing gels. Specific primers and dNTPs used depended on the telomerase activity under study and are indicated in each figure.

AU4 telomerase, which has a 3'-(AU)4C-5' templating domain sequence, is active *in vitro*

Telomerase activity was measured first in extracts of cells transformed with either wild-type (called wild-type-transformed, abbreviated WT-t) or AU4 telomerase RNA genes (Figure 1A). Since it was not known whether cells would contain wild-type telomerase, mutant telomerase or no telomerase, we performed initial assays with three different primers. The primer 3' ends were designed for alignment (base pairing) with either the wild-type template (primer g3; Figure 1B) or AU4 template (primers at3 and ata; Figure 1B) while maintaining the preferred G-rich sequence at the 5' end. In extracts of WT-t cells, telomerase assays revealed abundant wild-type telomerase, as shown by the typical ladder of products with six-base periodicity, generated using the g3 primer (Figure 1C, lane 1). The WT-t extract activity also synthesized long products that were dependent on the at3 primer (Figure 1C, lanes 2 and 3). We have demonstrated previously that this long product formation, on non-telomeric substrates such

as primer at3, is an activity intrinsic to wild-type telomerase and a likely mechanism by which telomerase performs *de novo* telomeric repeat addition *in vivo* (Wang and Blackburn, 1997). In extracts of AU4 cells, we were successful in eliminating detectable wild-type activity, as no polymerization was observed with primer g3 as a substrate (Figure 1C, lane 4), and no long products were synthesized using the at3 primer (lanes 7–8). Instead, AU4 extracts contained a strong telomerase activity that was dependent on the at3 or ata primer, whose 3' end sequences were complementary to the AU4 template (Figure 1C, lanes 7 and 9). This result indicates that the AU4 template actively used was in the telomerase RNP assembled with the mutant template RNA. A direct demonstration that the AU4 sequence was copied faithfully as a template was obtained by adding ddATP to telomerase reactions containing primer at3. WT-t telomerase was insensitive to ddATP, while AU4 telomerase products were terminated cleanly by ddATP incorporation (Figure 1C, compare lanes 3 and 8). The appearance of labeled products at input primer size revealed that AU4 telomerase was capable of performing nucleolytic cleavage, an activity known to be intrinsic to telomerase. In this reaction, the 3' nucleotide(s) of a template-paired primer are cleaved off, normally opposite the 5' end of the template, between template positions 43 and 44 (Collins and Greider, 1993; Melek *et al.*, 1996; Bhattacharyya and Blackburn, 1997). The products in Figure 1C, lane 8 are predicted to result from cleavage of the 3'-terminal dT residue of primer at3, addition of a labeled dT residue and chain termination by addition of the ddA residue at the +1 nucleotide position. Further evidence for the correct copying of the template sequence came from results with primer ata. With α -³²P-labeled TTP and unlabeled dATP, a ladder of four products was formed starting at the primer +1 position (Figure 1C, lane 9), consistent with product synthesis as shown in Figure 1D. The stronger accumulation of bands before the position at which the rate-limiting α -³²P-labeled TTP is expected to be incorporated (the strong +2 and +4 bands in lane 9) is also consistent with correct copying of the template.

Telomerase is active *in vitro* with a fully AU-substituted template

Finding that telomerase could actively use the AU4 template, we examined the enzymatic properties of another AU-substituted telomerase, AUN, in more detail. Unlike AU4, AUN telomerase RNA has an undisrupted alignment region (bases 50–51; Gilley and Blackburn, 1996), allowing assignment of the position of pairing of the 3' end of the primer on the template. The AUN template is also completely free of rC residues in the region of the template that is copied (bases 43–49; Gilley and Blackburn 1996). Figure 2 shows the results obtained with the telomerase in AUN extracts using [α -³²P]TTP or [α -³²P]dATP as the labeled triphosphate substrate, with different primers. We tested the prediction that AUN telomerase would copy the rU residues in its template by incorporating radiolabeled dATP. Indeed, the AUN cell extract contained a telomerase activity that incorporated radiolabeled dATP using primer n2 and [α -³²P]dATP and unlabeled TTP (Figure 2A, lane 3), but n2 was not extended detectably by wild-type control extract

Fig. 2. AUN-substituted telomerase is active and incorporates more *dTTP per RNA than wild-type telomerase. (A) Telomerase assays using wild-type-transformed (WT-t) extract are shown in lanes 1 and 12–18, while AUN extract assays are shown in lanes 2–11. Primer and dNTPs supplied for each reaction are indicated on the grid above each lane. Pre-incubation with RNase or substitution of dATP for ddATP are indicated with (+) signs in the same grid. The sequences of telomerase RNA templates and assay primers are given to the right of the autoradiogram. To measure product recovery, an equal aliquot of 5'-radiolabeled tracer primer (37mer) was added after each reaction and before precipitation. The position of tracer primer (TR) is shown. The positions of input primer +1 for a2 and g4 are indicated. (B) Quantitative comparison of WT-t and AUN telomerase assay products. See the text for a description of quantitation and calculations performed.

(Figure 2A, lane 1). Primers a2, a5 and n2 were extended by the AUN extract using [α -³²P]TTP and unlabeled dATP (Figure 2A, lanes 4, 6 and 9). The activity was eliminated by pre-treatment with RNase, as expected for a telomerase activity (Figure 2A, lanes 5, 7 and 10). With primer a5, only a single labeled residue was added when ddATP was present, consistent with the expected copying of the AUN template and cessation of copying before incorporation of ddATP (Figure 2A, lane 8). Similarly weak incorporation of ddNTPs has been noted previously for other mutant template *Tetrahymena* telomerases (Gilley and Blackburn, 1996.)

The input primer sized labeled product in Figure 2A, lane 9 demonstrated that AUN telomerase, like AU4 telomerase, performs nucleolytic cleavage, although apparently between primer residues paired with positions 45 and 46, rather than 43 and 44 as observed with wild-type telomerase (see alignment in Figure 1A and further

description below). As expected (Lee and Blackburn, 1993), the wild-type control extract activity using [α -³²P]TTP and unlabeled dATP extended primer g4 by two nucleotides (the same products made in a T-only telomerase assay), and the extension was unaffected by the presence of ddATP (Figure 2A, lanes 16 and 18). The faint activity visible in the AUN extract reaction using the completely telomeric primer g4 (with the sequence GGGG at its 3' end, which is unable to pair with any bases within or next to the templating domain of the AUN enzyme) (Figure 2A, lane 11) is likely to represent endogenous wild-type telomerase, as products were RNase sensitive (data not shown) and similar to the products of WT-t telomerase seen in lanes 16 and 18.

To make a quantitative assessment of AUN telomerase activity, we compared the levels of nucleotide incorporation in extracts of both AUN and WT-t cells at saturating primer concentrations. Wild-type telomerase is known to

have a different K_m (Lee and Blackburn, 1993) for each dNTP (G<T<A). In a standard telomerase assay, the radiolabeled dNTP is provided in lower concentration than other unlabeled dNTPs needed for synthesis. Therefore, WT-t and AUN telomerase activity were compared quantitatively under identical assay conditions, using a radiolabeled dNTP that both enzymes were predicted to incorporate. We provided AUN and WT-t telomerase with the best primers tested for incorporating [α - 32 P]TTP (primers a5 and g4, respectively) and limited the products to 1–2 quantifiable bands by using ddATP in place of dATP (Figure 2A, lanes 8 and 18). Total radioactivity was measured in each lane for product bands that exceeded background by >10-fold. Sample background was subtracted for each band and product totals are shown in Figure 2B, row V. To correct for differences in product recovery, an equal aliquot of radiolabeled tracer primer (TR) was added to each reaction before phenol extraction and ethanol precipitation. TR bands were quantitated and levels are shown relative to lane 10 (Figure 2B, row TR). This number was used to normalize product totals for recovery (Figure 2B, row X). Separately, we measured total telomerase RNA in the same extracts used for telomerase assays (Figure 2B, row Y). This was achieved by dot-blot hybridization to an antisense oligonucleotide specific for telomerase RNA and complementary to a sequence outside the template region. Finally, telomerase activity levels were expressed as TTP incorporation per RNA level (Figure 2B, row Z). By this comparison of the activities of AUN telomerase and WT-t telomerase, AUN incorporated 6.7 times more [α - 32 P]TTP than WT-t (Figure 2B). This high incorporation level using a primer such as a5 or a2 may be attributable to increased turnover if product dissociation with AUN telomerase and its product (e.g. GT₂G₄T₂G₄ATATA*T for primer a2; lane 4) is faster than with wild-type telomerase and its product (T₂G₄T₂G₄T₂G₄*T*T).

The quantitative data presented in Figure 2B also allowed determination of the level of background wild-type telomerase activity present in the extracts of AUN cells (Figure 2A, lane 11 and B, row Z). Because primer g4 is extended efficiently by wild-type telomerase and primers a2 and a5 are only utilized detectably by AUN telomerase, comparing TTP incorporation using these primers allowed the relative levels of mutant and wild-type activity in the AUN extract to be assessed. The incorporation of TTP using primer g4 was ~0.3% of the level of TTP incorporated using primer a2. Taking into account the higher level of TTP incorporation seen with AUN telomerase activity versus wild type (discussed above), we conclude that <2% of the total telomerase in AUN cells is wild type. This conclusion is consistent with the observation that AUN cells have short telomeres (see below).

The sequence synthesized by the AUN enzyme was confirmed to arise from copying of the template by the experiments shown in Figure 3. First, the n1 and a5 primers were elongated to form labeled +1 and +2 products, in the presence of either [α - 32 P]dATP plus unlabeled TTP (*A + T reaction), or [α - 32 P]TTP plus unlabeled dATP (*T + A reaction) (Figure 3, lanes 1 and 8, and schematics below). A single +1 product was synthesized using primer a5 with [α - 32 P]TTP alone

Fig. 3. AUN-substituted telomerase synthesizes the predicted templated sequence with correctly aligned primers, and has a cleavage activity localized to the central region of the template. Telomerase assays were done using AUN-transformed extract and the indicated triphosphate and primer substrates (primer sequences are below the autoradiogram). Primer and dNTP usage for each reaction is indicated on the grid above each lane. Pre-incubation with RNase or substitution of dATP by ddATP are indicated with (+) signs in the grid. Tracer primer (TR) is as in Figure 2. The positions of input primer and the -1, +1, +2 and +3 nucleotide products are indicated. Schematics showing the predicted primer alignments and reaction products synthesized are in the lower portion of the figure. Cleavage of the aligned primer n2 is indicated by the vertical open arrowhead.

(lane 11). Primer g4 was not extended (lanes 5, 10 and 12), consistent with its predicted inability to pair its 3' end with the AUN template. Cleavage activity was evident with the n2 primer. Labeled input sized and input -1 sized bands appeared in the *A + T reaction (Figure 3, lane 3),

consistent with cleavage between template positions 47 and 48, and extension of the cleaved product starting with incorporation of a labeled dA residue (see top right reaction schematic in Figure 3). Similar cleavage in the *T + A reaction with n2 was consistent with the result seen in Figure 3, lane 6, in which the shortest labeled product was at input size, consistent with the scheme shown for the n2 primer in Figure 3, lower right.

We further examined the nucleotide substrate specificity of the telomerase in AUN extracts by testing different radiolabeled triphosphate substrates. With a completely non-telomeric primer ending in ...TTA at its 3' end, non-T primer (for the complete sequence of non-T see Materials and methods), correct alignment as shown in Figure 4B was predicted to allow incorporation of [α - 32 P]dATP alone into a +1 sized product, but not of [α - 32 P]TTP alone or [α - 32 P]dGTP alone, as confirmed in Figure 4A, lanes 1–3. These results and those in Figures 2 and 3 showed that the AUN enzyme only used the expected dATP and TTP substrates, and thus had apparently normal fidelity.

Processivity of the template replacement telomeres was examined. Control reactions with wild-type extract showed that radiolabel from [α - 32 P]dGTP alone was incorporated efficiently by wild-type control extract using the non-T primer (Figure 4A, lane 4; similar addition of a string of dG residues has been seen previously for wild-type telomerase when other dNTPs are omitted from the reaction). The wild-type extract also incorporated [α - 32 P]dGTP label efficiently into products using a telomeric primer (tt) or the non-T primer in the presence of ddTTP to chain terminate the reaction (*G + ddT reactions in Figure 4A, lanes 9 and 10). This pattern of incorporation has been described previously for both primers (Wang and Blackburn, 1997; Wang *et al.*, 1998) and is shown in Figure 4B, lower right schematics. In *G + ddT reactions, the AUN activity was inactive with these primers (Figure 4A, lanes 11 and 12). However, the primer tt has the sequence GGGGTT at its 3' end and can therefore align with the AA alignment sequence at positions 50 and 51 in the AUN telomerase. In an *A + T reaction, the AUN activity efficiently elongated the tt primer, adding at least 20 nucleotides in an alternating pattern of bands (Figure 4A, lane 8). As with the other primers, these products were distinct from those formed by wild-type enzyme with this primer (Figure 4A, lane 9), and thus their synthesis was not attributable to any trace wild-type activity present in the cellular extracts assayed. The alternating pattern of bands was consistent with synthesis of an alternating d(AT)_n repeat. Since the primer is in great excess, this result showed that at least 20 residues can be added to a single substrate molecule by the telomerase in AUN extracts. Therefore, the AUN enzyme exhibits limited processivity. A similar result was found for the AU4 enzyme with the same tt primer (data not shown). The AUN telomerase primarily adds 1–2 labeled dT residues to primers a2, a5 and n2 (Figure 2A, lanes 4, 6 and 9). However, a minor fraction of the products consisted of 9–12 nucleotides added to the input primer (Figure 2A, lanes 4, 6 and 9). The AUN telomerase did not form high molecular weight products (like those shown for wild-type telomerase in Figure 1C, lanes 1–3) with any primer tested.

Fig. 4. Template-substituted AUN telomerase shows high nucleotide specificity and processively synthesizes tandem alternating dAdT dinucleotide repeats. (A) Telomerase assays were done using AUN-transformed extract or wild-type-transformed (WT) extract and the indicated triphosphate and primer substrates (the sequence of primer tt is shown in Figure 2 and the non-T primer sequence is given in Materials and methods). Primer and dNTP usage and pre-incubation with RNase are indicated as in Figures 2 and 3. Tracer primer (TR) is as in Figure 2. Lanes 1–7: the indicated α - 32 P-labeled dNTP was the only triphosphate substrate present. Lanes 9–12: α - 32 P-labeled dGTP plus ddTTP were the only triphosphate substrates present. The faint bands visible below the tracer bands in lanes 6, 11 and 12 were attributable to product synthesis by the small amount of endogenous wild-type telomerase activity in the AUN cells. (B) Schematics showing primer alignments and labeled product synthesis by AUN template and wild-type telomerase using non-T and tt primers in the reactions shown in (A).

In conclusion, the AUN telomerase had robust polymerization activity and copied the template faithfully but with relatively low processivity compared with wild-type enzyme. Its cleavage activity of template-paired primers

appeared to be shifted into a more central position on the template than in the wild-type enzyme.

Telomerase recognizes the 5' region of the substrate primer independently of template base pairing: support for an independent anchor site in telomerase

Wild-type telomerase has a preference for G-rich, telomeric sequences at the 5' end of its primer substrate, even in primers that are non-telomeric at the 3' end. This preference is manifested in a lower apparent K_m for the primer, and a higher k_{cat} for polymerization (Harrington and Greider, 1991; Lee and Blackburn, 1993; Melek *et al.*, 1996; Wang and Blackburn, 1997). Models presented to account for this phenomenon include a 'bind and slide' model, which suggested that the telomerase template sequence base-pairs with the telomeric sequence anywhere in the primer substrate and slides along the primer until it reaches the 3' end and begins polymerization. Another proposal was a 'loop-out' model wherein telomerase binds the telomeric sequence at the primer 5' end by base pairing with the alignment nucleotides of the template, loops out the intervening non-telomeric sequence, and uses one nucleotide at the 3' end for alignment and extension. The 'second site' or 'anchor site' model proposes that the 5' end of the substrate (or product) is bound by telomerase at a site distinct from the base pairing and catalysis occurring at the template (Morin, 1989; Harrington and Greider, 1991; Lee and Blackburn, 1993; Lee *et al.*, 1993). Several lines of evidence support the idea that the G-rich sequence at the primer 5' end binds to an anchor site, notably the photo-cross-linking of a distal 5' region of the primer to specific parts of the *Euplotes* telomerase protein and RNA in active telomerase complexes (Hammond *et al.*, 1997). However, it has not previously been possible to rule out the involvement of the template in 5' end primer sequence recognition by *Tetrahymena* telomerase because telomeric (TTGGGG) or G-rich sequences at either the 5' or 3' end of a substrate can theoretically base-pair with the wild-type templating region (AACCCCAAC).

Our finding that AUN telomerase, containing the non-telomeric template 3'-AAUAUAUAU-5', was highly active created a new biochemical opportunity to test whether recognition of a G-rich 5' end of a primer was independent of template base pairing. To test primer utilization by AUN telomerase, two sets of primers were designed. These are shown in Figure 5A; the positions of the bases in the primer are numbered with reference to the primer 3' end. The 3' ends of these primers are AT rich and were designed to be complementary to the AUN template, since this was a requirement for proper alignment and activity of AUN telomerase (Figure 3). The sequence CACACA, which is neither G-rich nor complementary to the AUN template, was used to replace telomeric sequence in various positions in the 5' region of primers. Supporting the independent anchor site model, AUN telomerase was like wild type in most efficiently utilizing a substrate primer with maximum telomeric sequence at the 5' end (primers a and e, Figure 5A and B). A modest reduction in AUN telomerase activity was caused by replacement of telomeric sequence with CACACA at positions 6–11 from the substrate 3' end (primers d and i, Figure 5A and B). Completely replacing the 5' end telomeric sequence with

Fig. 5. AUN telomerase prefers a telomeric sequence at the 5' end of primer substrates: template base pairing does not contribute to anchor site binding. (A) AUN template RNA and primers used to test anchor site binding specificity. The AT-rich 3' end of primers complements the AUN templating domain. The 5' end of primers (indicated by negative numbers from the 3' end) is either telomeric (TTGGGG) or disrupted by blocks of CACACA sequence. + and – signs indicate relative telomerase activity as shown in (B). (B) Telomerase assays using AUN telomerase, the indicated primers and 1.9 μ M radiolabeled dTTP. The uniform band appearing at the top of the gel is tracer primer (TR), used to normalize for recovery as indicated in Figure 2. Primer +1 positions are indicated for a, e and f. Primers e and f run at different positions due to large differences in sequence composition.

the CACACA sequence caused a larger reduction in utilization by AUN telomerase (primers b and f, Figure 5A and B). Interestingly, for both 17 and 23 base primer substrates, the most significant reduction in telomerase activity occurred when the bases in positions 12–17 were replaced with CACACA, and the bases in positions 6–11 were still telomeric (primers c and h, Figure 5A and B). This difference may reflect different modes of telomerase substrate binding, telomeric and non-telomeric (Wang *et al.*, 1998). Together, these results are consistent with anchor site binding in telomerase being independent of substrate base pairing to the RNA at templating positions 49–43.

Telomerase with a completely replaced template (U9) is active and demonstrates fidelity in vitro

Since the AUN enzyme still had some templating domain bases that were not mutated from wild type (44 in the

template, and 50 and 51 in the alignment region), we constructed a telomerase with no wild-type residues in the templating domain. We chose all rU residues (U9 telomerase), reasoning that rU residues would have a minimal possibility of forming extraneous secondary or other structures in the telomerase RNA, compared with rG residues. Telomerase assays were performed with U9 and control wild-type-transformed extracts as described for AUN and AU4 extracts, using appropriate radiolabeled dNTP substrates and 3' end primer sequences (which base-pair with the template; Figure 6A). The levels of wild-type telomerase in both extracts were assayed using the telomeric primer g4 and [α - 32 P]TTP as substrates. Only the WT-t cell extract incorporated [α - 32 P]TTP using the g4 primer (Figure 6B, compare lanes 2 and 3). Conversely, when assay substrates were switched to [α - 32 P]dATP and primers carrying a 3' end complementary to the U9 template (primers u1 and u2), only the U9 extract actively incorporated [α - 32 P]dATP (Figure 6B, compare lanes 10 and 12 with lanes 7 and 9). No [α - 32 P]dATP incorporation occurred if the U9 extract was pre-treated with RNase (Figure 6B, lanes 11 and 13) or provided with a completely telomeric primer substrate (primer g4; Figure 6B, lane 14). Extracts made from WT-t controls incorporated little or no [α - 32 P]dATP with any primer tested, as expected for telomerase lacking rU residues in the template region (Figure 6B, lanes 5, 7 and 9). Using the primer u3 (Figure 6A), we saw addition of dA residues to the primer, forming products up to 12 nucleotides longer than the input primer (Figure 6B, lane 15). Therefore, this enzyme, like the AUN and AU4 enzymes, was capable of limited processivity. These results showed that although the entire U9 templating domain (bases 43–51) is devoid of wild-type sequence, U9 RNA assembles into an active telomerase RNP that can template the addition of [α - 32 P]dATP *in vitro* when provided with a primer complementary at its 3' end to the novel templating domain.

The U9 enzyme also showed a cleavage activity. This was evident from the primer-dependent production of labeled products one, two and three nucleotides shorter than the input primer (Figure 6B, lanes 10 and 15). Hence

we deduced from these results that cleavage and elongation reactions such as those shown schematically in Figure 6B had occurred.

Although high fidelity is generally observed in assays of wild-type telomerase, previous work showed that certain

Fig. 6. Complete replacement of the templating domain of telomerase (U9) does not abolish basic polymerization or fidelity *in vitro*. (A) Template sequences of U9 and WT-t telomerase RNAs with assay primers. (B) Telomerase assays of U9 extract (lanes 2 and 10–16) and WT-t extract (lanes 1 and 3–9). Substrate primers and dNTPs used are indicated by the grid, and concentrations are as described in Figure 2. The position and recovery of tracer primer are shown (TR). The position of input primer +1 is indicated for primer g4 (left side), input +1 and –1 are indicated for primers u1 and u2, and input +1 and input –2 are indicated for primer u3 (right side). The schematic below the autoradiogram shows the minimal deduced cleavages (open vertical arrow) of primer u3 needed to account for the observed synthesis of labeled products of input size and shorter than the input primer. Only one labeled addition product is shown (–2 size). (C) Fidelity tests of U9 and WT-t telomerase. All reactions contained primer u2 at 1 μ M. In lanes 2–5 and 10–13, telomerase was provided with radiolabeled 1.9 μ M dATP (*A) and challenged with additions of 100 μ M cold dNTPs as indicated. In lanes 6–8 and 14–16, telomerase was tested for its ability to incorporate the indicated radiolabeled dNTP (*) in the presence of the three remaining unlabeled dNTPs (+3). The smallest product appearing in most lanes is RNase insensitive and therefore not attributable to telomerase.

template mutations cause dNTP misincorporation, notably the point mutant 48U (Gilley *et al.*, 1995). Because the U9 template, like that of the 48U mutant, contains an rU residue at position 48 (as well as at all the other templating domain positions), we tested whether the U9 telomerase exhibited low-fidelity activity *in vitro*, by determining whether any evidence could be found for incorporation of nucleotides other than dATP. Specifically, we tested whether addition of 100 μ M cold dGTP, dCTP and TTP (separately and in combination) could support additional or altered patterns of elongation, using the best primer tested for U9 telomerase (u2). As shown in Figure 6C (lanes 1–4), U9 telomerase activity was not affected by the presence of any non-templated dNTPs. Furthermore, U9 telomerase did not misincorporate radiolabeled TTP, dGTP or dCTP (Figure 6C, lanes 6–8). Similar negative results were obtained, as expected, with WT-t telomerase using the u2 primer (Figure 6C, lanes 9–16). (Note that the short radiolabeled product seen at the bottom of many lanes was RNase insensitive and hence not attributable to telomerase; Figure 6C, lane 5.) We conclude that although the processivity of U9 telomerase is low, its fidelity is intact, in that it adds only the correct dA residues, as specified by the template sequence.

AU4 and AUN templates cause telomere shortening

We analyzed telomere length in transformants expressing the template replacement telomerases. For several different time points and independent electroporation experiments, genomic DNA was extracted from transformant cell populations or clonal lines and Southern blotting analysis was performed. Using *Pst*I digestion, the ribosomal DNA minichromosome (rDNA) telomeres and several non-rDNA telomeres are well resolved, allowing an accurate assessment of telomere length. Since these telomere fragments all contained some wild-type sequence, a single (G₄T₂)₂ telomeric probe was sufficient for detection. Representative Southern blots are shown in Figure 7. The AU4- and AUN-transformed cells reproducibly displayed mean telomere shortening of ~150 bp relative to WT-t control cells. This was the case for all repeated electroporations and time points studied (days 3–10). Included on the gel for comparison (Figure 7, left panel) is genomic DNA from cells with the previously studied template mutation, 43A (Gilley *et al.*, 1995), which is known to cause telomeres to become stably short. In contrast to the telomere shortening seen *in vivo* with AU4 and AUN template mutants, substitution of the template region with an all-U sequence (U9 template) caused no detectable difference in telomere length compared with WT-t, at any time point analyzed (Figure 7). The telomeres were analyzed over several days but no change in telomere length over time was seen for any of the mutant or wild-type transformants, even at the earliest time points (Figure 7, right panel, and data not shown).

Discussion

Here we have examined the consequences of complete and near-complete *in vivo* replacement of the *T. thermophila* telomerase RNA template. The three different non-telomeric template replacements tested each produced

Fig. 7. Template substitutions AUN and AU4 cause telomere shortening. Genomic DNAs from the indicated cell strains were restricted with *Pst*I, separated on 1.0% agarose and subjected to Southern blot analysis using the indicated telomeric probe. Left panel, control samples are shown for length comparison: untransformed parental strain CU428, the previously characterized template mutant 43A (Gilley *et al.*, 1995) and wild-type transformed (WT-t). Telomere hybridizing DNA from non-telomeric template substitutions U9, AUN and AU4 are shown. Marker fragment sizes are indicated in kb. The strong lower band indicated by the square bracket is the telomeric band of the multicopy rDNA minichromosome, previously shown to be representative of the size of all macronuclear telomeres (Larson *et al.*, 1987). The strong sharp band between the 2 and 3 kb markers is a telomere sequence-containing fragment within the high-copy, self-replicating circular plasmid used for transformation (Romero and Blackburn, 1995). Right panel, analysis of telomere length at early times after substitution of wild-type telomerase RNA with U9, control wild-type or AUN telomerase RNA. Genomic DNA in the right panel was extracted on day 4 or 5 as indicated. Only the rDNA minichromosome telomeric band is shown. After selection for transformant cells, pooled transformant cells were analyzed for the day 4 samples (see Materials and methods). Single cell clones 1–4 were analyzed on the earliest day possible, day 5. No changes in telomere length over time were seen for any transformant type under the growth conditions used (see Materials and methods).

telomerase that was catalytically active *in vitro*, although some properties of the enzymatic reaction were altered.

Preservation of nucleotide addition and dNTP selectivity in telomerases containing non-telomeric template replacements

Previous studies of telomerase have demonstrated that certain mutations in the template RNA region cause specific changes in aspects of telomerase activity *in vitro*. For example, four separate template mutations in *Tetrahymena* and one in yeast cause various forms of loss of fidelity, and a specific triplet substitution in the 17 base yeast templating domain destroys enzymatic activity (Gilley *et al.*, 1995; Gilley and Blackburn, 1996; Prescott and Blackburn, 1997). As a consequence, the templating region has been viewed as a highly specialized domain functioning not just as a blueprint for dNTP addition, but

also to provide base-specific interactions involved in catalytic activity. That the AU4, AUN and U9 telomerases catalyzed correct nucleotide addition was therefore unanticipated.

The utilization of these new templates by telomerase was confirmed in several ways. First, template-substituted enzymes each developed a biochemical dependence on a primer substrate whose 3' end was complementary to its new template (Figures 1–6). Secondly, telomerase altered its nucleotide incorporation to reflect the new templates: AUN, U9 and AU4 telomerase each gained the ability to incorporate dATP efficiently (Figures 2–4 and 6, and data not shown, respectively); U9 enzyme did not incorporate TTP, dCTP or dGTP (Figure 6B and data not shown). The AUN substitution telomerase did not incorporate dGTP or dCTP (Figure 4 and data not shown). Thirdly, AU4 and AUN, but not wild-type telomerase, abruptly terminated synthesis in the presence of ddATP (Figures 1C and 2A). Finally, the AUN enzyme correctly extended a telomeric and a non-telomeric primer without misincorporation (Figure 4).

The core function of any template-dependent polymerase is to select and insert the correct base, i.e. to perform nucleotide addition without misincorporation. The U9 template-substituted telomerase was of particular interest because the wild-type sequence is replaced at every templating domain position (51–43; see Figure 1A for positional assignments), and the U9 template sequence includes the C to U mutation at position 48 analyzed previously as a single point mutation (Gilley *et al.*, 1995). The correct incorporation of only [α - 32 P]dATP (at 1.9 μ M) in the presence of up to 100 μ M dCTP, dGTP or dTTP by U9 telomerase (Figure 6C) suggests effective discrimination between templated and non-templated incorporation. Likewise, we found no evidence for misincorporation by the AUN telomerase. Hence, at least for these U-only or AU-substituted templates, base-specific interactions between the telomerase template and protein (or other parts of the RNA) are not required for the core polymerase functions of nucleotide addition and correct base insertion. This can be contrasted with the misincorporation of dATP of the previously characterized template point mutant, 48U. In that case, although the mutant 48U in the template was copied correctly, in addition, dATP was misincorporated with high efficiency opposite the two neighboring template C residues at positions 47 and 46, even in the presence of dGTP (Gilley *et al.*, 1995).

Beyond the correct base selection and addition, completely template-substituted telomerase differed from wild type in several properties. Neither the U9 nor the AUN telomerase was as processive as the wild type. With some primers, they yielded predominant products only a few nucleotides in length, although with other primers the products extended up to ~10–20 added residues. Such addition of a tract of nucleotides longer than the template sequence indicated that AUN telomerase retains some ability to reposition products along the template. However, this differs markedly from the synthesis of dozens of repeats on a single primer characteristic of the wild-type enzyme reaction *in vitro*. In no case did we detect any long products that migrated slowly and accumulated at the top of the DNA sequencing gels used for product analysis, like those that are prominent products of wild-type telomerase.

The repositioning by the AUN and U9 telomerases did not appear to occur through a wild-type-like translocation to the beginning of the template, since, unlike wild-type telomerase, a characteristic strong band was not seen at a position corresponding to the 5' end of the template. The ~12 nucleotides added to the u3 primer by the U9 enzyme could have been synthesized by switching to a reiterative mode of dATP addition (at a single template position), as was inferred previously *in vitro*, both for repetitive dG addition during *de novo* telomere addition and with specific mutant template telomerases using telomeric primers (Gilley and Blackburn, 1996; Wang and Blackburn, 1997). In contrast, the sensitivity of AUN telomerase to ddATP was not consistent with this enzyme exhibiting slippage or reiterative addition of only dATP or only TTP. In addition, the pattern of alternating strong and light bands resulting from processive synthesis by the AUN enzyme was consistent with templated synthesis of alternating dAT dinucleotide repeats rather than slippage utilizing a single triphosphate substrate.

Another property of wild-type telomerase is the ability to cleave substrate primers, potentially to allow their extension. Replacement of the template in the AU4, AUN or U9 telomerases did not abolish this nucleolytic cleavage activity (Figures 1C, 2A, 3 and 6B). With the U9 enzyme and primer u3, assuming the alignment with the template takes place on the U9 template as shown in Figure 6B (schematic), minimally a cleavage appears to occur at the phosphodiester bond of the primer positioned between positions 49 and 50 on the template. Further non-random cleavage also appears to take place, since a labeled product was not seen at the position of the input primer with primer u2, or at input –1 with primer u3 (Figure 6B, lanes 10 and 15). The absence of these particular products suggests that, since up to 12 bases could be added to the u3 primer, after cleavage only one or two bases could be added. With the AUN enzyme, alignment of the primer with the complementary bases in the templating domain indicated that the position of cleavage was moved to the middle of the template, rather than corresponding to the 5' end of the template, as in wild-type telomerase. An apparently similar displacement of the position of cleavage of primers base-paired to the template was seen previously when the *T.thermophila* telomerase RNA was replaced by that of a different ciliate species (Bhattacharyya and Blackburn, 1997). However, in contrast to the template substitution enzymes analyzed in the present work, in that case a 23 nucleotide sequence comprised of the template and surrounding nucleotides was identical to wild type, and only bases outside the template differed from wild type. Together, these studies show that the cleavage position depends on features of the telomerase RNA, both in and out of the template region.

The AU4, AUN and U9 mutants each efficiently replaced the endogenous wild-type telomerase RNA in the cells, with the level of wild-type activity in AUN cells being <2% of that found in control WT-t cells. Telomere length regulation is influenced by a complex interaction between factors including the telomeric sequences added to the ends and overall telomerase activity levels (McEachern and Blackburn, 1995; Krauskopf and Blackburn, 1996; Kirk *et al.*, 1997; Roy *et al.*, 1998). Shortened telomeres are consistent with addition of mutant

repeats *in vivo* and/or diminished telomerase activity caused by the template mutations, as has been described for yeast telomerase template mutants (Prescott and Blackburn, 1997). The AU-substituted telomerases caused telomere shortening *in vivo* (Figure 7) and impaired cell growth and enlarged nuclei. These phenotypes are similar to those reported for the 43AA mutant telomerase of *Tetrahymena*, in which mutant repeats were added to telomeres, which also became shortened overall (Kirk *et al.*, 1997). The U9 substitution allowed apparently normal telomere length maintenance and cell growth. Although it is catalytically competent *in vitro*, it is not known whether U9 telomerase is active in the cell or whether other processes (McEachern and Blackburn, 1996; Le *et al.*, 1999) maintain the telomeres in U9 transformants. Notably, U9 telomerase has a disrupted alignment region (bases 50 and 51) while AUN telomerase has a wild-type sequence at these positions. Our *in vitro* results using primers with wild-type telomeric 3' ends predict that the wild-type telomere ends *in vivo* (created after chromosome fragmentation by the maternal endogenous telomerase during *de novo* telomere formation) would be poor substrates for U9 telomerase. However, they are potentially utilized by AUN telomerase, since the AUN enzyme was able to elongate a telomeric primer with a wild-type sequence at its 3' end (Figure 4A). This may be a key difference in whether these template-substituted telomerases could act on the pre-existing wild-type sequences at the telomere ends in the cells in these experiments.

Telomerase as a specialized DNA polymerase

All DNA polymerases, including telomerase, must be able to select the correct nucleotide substrate from a pool of similar substrates. Our biochemical data demonstrate that telomerase can perform nucleotidyl transfer with fidelity, using three novel template sequences within the context of otherwise wild-type RNA. The fact that seven different TERT genes contain conserved reverse transcriptase sequence motifs (alignment shown in Bryan *et al.*, 1998) suggests that telomerase and other DNA polymerases share a similar catalytic mechanism. Conserved motifs A and C of four different DNA polymerase families (Pol I, Pol α , reverse transcriptase and terminal transferase) are also found in TERT. These motifs contain three strictly conserved carboxylates that are believed to participate directly in catalysis. Abundant biochemical data (reviewed in Echols, 1991; Joyce and Steitz, 1994) and the crystal structures of polymerase ternary complexes (reviewed in Brautigam and Steitz, 1998) provide evidence for a conserved two-metal ion mechanism of nucleotidyl transfer wherein the conserved carboxylates participate in the chemical step. Furthermore, steric hindrance has been proposed, on the basis of biochemical data and the fact that all correctly paired DNA bases share similar overall geometry, as a mechanism of maintaining the fidelity of DNA polymerases (reviewed in Echols, 1991). Recent studies of four different polymerase complexes (crystallized with nucleotide substrate, template and primer) now provide a structural basis for steric hindrance of template-dNTP mispairing (Pelletier *et al.*, 1996; Doublet *et al.*, 1998; Huang *et al.*, 1998; Kiefer *et al.*, 1998). Within the context of the narrow space available in the active site,

only a correct nascent base pair is proposed to permit an essential conformational change preceding the chemical step (Johnson, 1993).

While further analyses are required to define the mechanism of base selection and insertion by telomerase fully, the data presented here are consistent with the steric hindrance model. We therefore propose that telomerase resembles other DNA polymerases in its mechanism for achieving nucleotide addition with fidelity, but relies on the sequence or structure of the RNA and/or additional protein components to confer the specialized properties of telomerase. Such properties include stable presentation of template to the catalytic subunit, restriction of templating to a confined region of the RNA, coordination of product movement such as translocation (to the beginning of the template) and shifting of product from active site to anchor site.

Materials and methods

Site-directed mutagenesis

The template sequence of the wild-type telomerase RNA gene was substituted using prTER1 template, gene-flanking primers with *Xho*I sites and mutagenic primers (according to the template created; Figure 1A) in a PCR essentially as described (Higuchi, 1989). The reactions were placed in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 30 cycles (94, 55 and 72°C, 30 s each). The 0.5 kb products were cloned into the vector prD4-1 (Yu and Blackburn, 1990) at the *Xho*I site, checked for orientation, and sequenced (Sequenase 2.0, United States Biochemical).

Strains and electroporation

Fresh *T. thermophila* strains CU428 and CU427 were kindly provided by Jacek Gaertig, University of Georgia. Cells were grown in 2% PPYS (2% proteose peptone, 0.2% yeast extract, 0.003% Sequestrene) plus 1× Antibiotic/Antimycotic (Pen Strep Fungizone) with shaking at 100 r.p.m., 30°C, to a density of 5×10^5 . Cells were starved in 10 mM Tris pH 8.0 for 5 h then adjusted to a density of 4×10^5 . The two strains were mixed (50 ml each in a 2 l flask) and shaken at 160 r.p.m. to prevent mating until starvation had continued for a total of 18 h. Pairing efficiency was assessed at +4 h (70%) after cessation of shaking. Electroporation of wild-type and mutant telomerase RNA genes (prTER1, prTER-au4, prTER-aun and prTR-u9) was performed at +10.5 h as described (Gaertig and Gorovsky, 1992). The electroporator (Bio-Rad Gene Pulser) was set to 0.44 kV, 200 Ω and 25 μ F with 0.4 cm gap cuvettes. Cells were transferred immediately to 50 ml of 2% PPYS with shaking at 30°C. Dilutions of this culture were plated in 96-well dishes to assess transformation efficiency and to obtain single transformants. At 12 h after electroporation, the selection drug was applied to individual wells (paromomycin, final concentration 100 μ g/ml) and the 50 ml culture was diluted into 0.25–0.5 l of 2% PPYS + paromomycin for growth of the pooled transformant cells to a density of $2\text{--}5 \times 10^5$ (usually 3 days). Clonal transformant lines were followed for telomere length, cell growth and nuclear morphology changes. All transformants were cultured in log phase with continued paromomycin selection unless indicated otherwise. The persistence of maternal, endogenous telomerase for some period after electroporation is supported by the fact that telomeres cloned from cells transformed in this manner contained abundant wild-type repeats in a centromere-proximal position (Bhattacharyya and Blackburn, 1997; Kirk *et al.*, 1997). A previous study also demonstrated that telomerase is not destroyed with each vegetative fission and persists after the addition of new genes via microinjection (Yu and Blackburn, 1991).

Partial purification of telomerase

The 0.5 l transformant cultures (described above) were starved (on day 5 after electroporation) for 16 h, washed in T2MG++ [20 mM Tris pH 8.0, 1 mM MgCl₂, 10% glycerol, plus phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT)] and concentrated to 1–2 ml. Throughout the purification, all extracts and buffers were kept at 4°C with 0.1 mM PMSF and 10 mM DTT added immediately before use. NP-40 detergent was added to a final concentration of 0.1% and medium vortexing (setting 5) was performed for 30 min at 4°C. Lysed cells were spun in a TL-100 rotor at 48 000 r.p.m. for 60 min. The supernatant was applied to a 1.2 ml

DEAE-agarose (Bio-Rad) column, pre-equilibrated with T2MG. Flow-through was re-loaded and subsequently washed with 6 ml of 0.2 M NaOAc in T2MG. Telomerase was eluted with 2 ml of 0.3 M NaOAc in T2MG, eluate adjusted to a concentration of 0.5 M NaOAc in T2MG, and loaded onto a 0.5 ml octyl-Sepharose (Pharmacia) column (pre-equilibrated with 0.5 M NaOAc in T2MG). Telomerase was desalted with 6 ml of T2MG, eluted with 1% Triton X-100/T2MG into bovine serum albumin (BSA), 0.5 mg/ml final (New England Biolabs), and stored in 0.3 ml fractions at -80°C .

Telomerase reactions and RNA quantitation

All DNA primers were purified by denaturing PAGE, elution in dH_2O , and EtOH precipitation in 0.3 M NaOAc. Primer concentrations were based on OD_{260} readings and an approximate molar extinction coefficient (No. of bases $\times 10$). Reactions were carried out with 5–10 μl of extract for 10–30 min at 30°C . The final reaction mix contained 60 mM Tris-HCl pH 8.0, 1 mM MgCl_2 , 1 mM spermidine, 3 mM DTT, 1 μM primer, 100 μM unlabeled dNTP or ddNTP (where indicated) and 1.88 μM [$\alpha\text{-}^{32}\text{P}$]dATP or [$\alpha\text{-}^{32}\text{P}$]dATP. Primer non-T had the sequence 5'-TCATTATTAATTTATTTTATAAAAAATTA-3'. The sequences of the other primers used are shown in the figures. Reactions were stopped with the addition of 80 μl of TES (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.2% SDS), containing a 5'- ^{32}P -labeled 37mer tracer primer (to normalize for subsequent recovery), and extracted with an equal volume of phenol/chloroform. Products were precipitated in 2.75 M NH_4OAc , 10 μg glycogen and 3 vols of EtOH, and resolved on denaturing 15% polyacrylamide gels. The telomerase RNA levels in purified fractions were determined by dot-blotting RNA. An equal volume of each telomerase fraction was subjected to acidic phenol extraction and EtOH/0.3 M NaOAc precipitation using glycogen as a carrier. The pellet was resuspended in denaturation buffer (50% TE, 6 $\times$ SSC, 20% formaldehyde) and heated to 55°C for 15 min. Samples were applied by vacuum to Hybond N+ (Amersham) soaked in 6 $\times$ SSC (Sambrook *et al.*, 1989) and clamped within a dot-blot apparatus. After six SSC washes, the membrane was UV cross-linked and hybridization was performed (Church and Gilbert, 1984) using the ^{32}P -labeled oligonucleotide 5'-GAAGGTTATATCAGCACTAG-3'. Quantitation was performed for reaction products, tracer primer and RNA levels of the extract fractions using a PhosphorImager (Molecular Dynamics).

Telomere blots

Genomic DNA (gDNA) was extracted from *Tetrahymena* as previously described (Romero and Blackburn, 1995) on days 3, 4, 5 and 7 after transformation. The representative telomere blot shown in Figure 7, left panel, contains gDNA extracted on day 5; DNA in the right panel of Figure 7 was extracted on day 4 or 5 as indicated. The gDNA was restricted with *Pst*I for 2 h at 37°C and run overnight at 25 V on 1.0% agarose in 1 $\times$ TBE. Southern blotting was performed as described (Church and Gilbert, 1984) with hybridization to a 5'- ^{32}P -labeled oligonucleotide at 33°C (polynucleotide kinase, New England Biolabs). Overnight hybridization was followed by two 5 min 33°C washes and subsequent exposure to autoradiography film.

Acknowledgements

We thank the Orias laboratory and Jacek Gaertig for donating strains and patient advice on conjugation and electroporation, Carol Gross for helpful feedback on the manuscript, and Iain Murray for enzymology discussions and manuscript review. We thank Jeff Kapler for suggesting the template 'knockout' experiment many years ago. We are grateful to members of the ciliate biologist community (FASEB meeting, 1995) for sharing essential, often unrecorded information about *Tetrahymena* biology. This work was supported by NIH grant GM26259 to E.H.B.

References

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.
 Beattie,T.L., Zhou,W., Robinson,M.O. and Harrington,L. (1998) Reconstitution of human telomerase activity *in vitro*. *Curr. Biol.*, **8**, 177–180.
 Bhattacharyya,A. and Blackburn,E.H. (1997) A functional telomerase

RNA swap *in vivo* reveals the importance of nontemplate RNA domains. *Proc. Natl Acad. Sci. USA*, **94**, 2823–2827.
 Blackburn,E.H. (1991) Structure and function of telomeres. *Nature*, **350**, 569–573.
 Blackburn,E.H. (1995) Developmentally programmed healing of chromosomes. In Blackburn,E.H. and Gredier,C.W. (eds), *Telomeres*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 193–218.
 Blackburn,E.H. (1999) Telomerase. In Gesteland,R.F. and Atkins,J.F. (eds.), *The RNA World II*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 609–635.
 Brautigam,C.A. and Steitz,T.A. (1998) Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes. *Curr. Opin. Struct. Biol.*, **8**, 54–63.
 Bryan,T.M., Sperger,J.M., Chapman,K.B. and Cech,T.R. (1998) Telomerase reverse transcriptase genes identified in *Tetrahymena thermophila* and *Oxytricha trifallax*. *Proc. Natl Acad. Sci. USA*, **95**, 8479–8484.
 Church,G.M. and Gilbert,W. (1984) Genomic sequencing. *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.
 Collins,K. and Gandhi,L. (1998) The reverse transcriptase component of the *Tetrahymena* telomerase ribonucleoprotein complex. *Proc. Natl Acad. Sci. USA*, **95**, 8485–8490.
 Collins,K. and Greider,C.W. (1993) *Tetrahymena* telomerase catalyzes nucleolytic cleavage and nonprocessive elongation. *Genes Dev.*, **7**, 1364–1376.
 Double,S., Tabor,S., Long,A.M., Richardson,C.C. and Ellenberger,T. (1998) Crystal structure of a bacteriophage T7 DNA replication complex at 2.2Å resolution. *Nature*, **391**, 251–258.
 Echols,H. (1991) Fidelity mechanisms in DNA replication. *Annu. Rev. Biochem.*, **60**, 477–511.
 Gaertig,J. and Gorovsky,M.A. (1992) Efficient mass transformation of *Tetrahymena thermophila* by electroporation of conjugants. *Proc. Natl Acad. Sci. USA*, **89**, 9196–9200.
 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. and Blackburn,E.H. (1999) The telomerase RNA pseudoknot is critical for the stable assembly of catalytically active telomerase. *Proc. Natl Acad. Sci. USA*, **96**, 6621–6625.
 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. (1991) Telomerase is processive. *Mol. Cell. Biol.*, **11**, 4572–4580.
 Greider,C.W. (1995) Telomerase biochemistry and regulation. In Blackburn,E.H. and Greider,C.W. (eds), *Telomeres*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 35–68.
 Greider,C.W. (1996) Telomere length regulation. *Annu. Rev. Biochem.*, **65**, 337–365.
 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.
 Hammond,P.W., Lively,T.N. and Cech,T.R. (1997) The anchor site of telomerase from *Euplotes aediculatus* revealed by photo-cross-linking to single- and double-stranded DNA primers. *Mol. Cell. Biol.*, **17**, 296–308.
 Harrington,L., Zhou,W., McPhail,T., Oulton,R., Yeung,D.S., Mar,V., Bass,M.B. and Robinson,M.O. (1997) Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev.*, **11**, 3109–3115.
 Harrington,L.A. and Greider,C.W. (1991) Telomerase primer specificity and chromosome healing. *Nature*, **353**, 451–454.
 Henning,K.A., Moskowitz,N., Ashlock,M.A. and Liu,P.P. (1998) Humanizing the yeast telomerase template. *Proc. Natl Acad. Sci. USA*, **95**, 5667–5671.
 Higuchi,R. (1989) Using PCR to engineer DNA. In Erlich,H.A. (ed.), *PCR Technology*. Stockton Press, New York, NY, pp. 61–70.
 Huang,H., Chopra,R., Verdine,G.L. and Harrison,S.C. (1998) Structure of a covalently trapped catalytic complex of HIV-1 RT reverse transcriptase: implications for drug resistance. *Science*, **282**, 1669–1675.
 Johnson,K.A. (1993) Conformational coupling in DNA polymerase fidelity. *Annu. Rev. Biochem.*, **62**, 685–713.
 Joyce,C.M. and Steitz,T.A. (1994) Function and structure relationships in DNA polymerases. *Annu. Rev. Biochem.*, **63**, 777–822.
 Kiefer,J.R., Mao,C., Braman,J.C. and Beese,L.S. (1998) Visualizing

- DNA replication in a catalytically active *Bacillus* DNA polymerase crystal. *Nature*, **391**, 304–307.
- Kirk, K.E., Harmon, B.P., Reichardt, I.K., Sedat, J.W. and Blackburn, E.H. (1997) Block in anaphase chromosome separation caused by a telomerase template mutation. *Science*, **275**, 1478–1481.
- 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.
- Larson, D.D., Spangler, E.A. and Blackburn, E.H. (1987). Dynamics of telomere length variation in *Tetrahymena thermophila*. *Cell*, **50**, 477–483.
- Le, S., Moore, J.K., Haber, J.E. and Greider, C.W. (1999) RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase. *Genetics*, **152**, 143–152.
- Lee, M.S. and Blackburn, E.H. (1993) Sequence-specific DNA primer effects on telomerase polymerization activity. *Mol. Cell. Biol.*, **13**, 6586–6599.
- Lee, M.S., Gallagher, R.C., Bradley, J. and Blackburn, E.H. (1993) *In vivo* and *in vitro* studies of telomeres and telomerase. *Cold Spring Harb. Symp. Quant. Biol.*, **58**, 707–718.
- Licht, J.D. and Collins, K. (1999) Telomerase RNA function in recombinant *Tetrahymena* telomerase. *Genes Dev.*, **13**, 1116–1125.
- Lingner, J. and Cech, T.R. (1998) Telomerase and chromosome end maintenance. *Curr. Opin. Genet. Dev.*, **8**, 226–232.
- Lingner, J., Hughes, T.R., Shevchenko, A., Mann, M., Lundblad, V. and Cech, T.R. (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science*, **276**, 561–567.
- Marusaic, L., Anton, M., Tidy, A., Wang, P., Villeponteau, B. and Bacchetti, S. (1997) Reprogramming of telomerase by expression of mutant telomerase RNA template in human cells leads to altered telomeres that correlate with reduced cell viability. *Mol. Cell. Biol.*, **17**, 6394–6401.
- McCormick-Graham, M., Haynes, W.J. and Romero, D.P. (1997) Variable telomeric repeat synthesis in *Paramecium tetraurelia* is consistent with misincorporation by telomerase. *EMBO J.*, **16**, 3233–3242.
- McEachern, M.J. and Blackburn, E.H. (1995) Runaway telomere elongation caused by telomerase RNA gene mutations. *Nature*, **376**, 403–409.
- McEachern, M.J. and Blackburn, E.H. (1996) Cap-prevented recombination between terminal telomeric repeat arrays (telomere CPR) maintains telomeres in *Kluyveromyces lactis* lacking telomerase. *Genes Dev.*, **10**, 1822–1834.
- 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.
- Morin, G.B. (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell*, **59**, 521–529.
- Nugent, C.I. and Lundblad, V. (1998) The telomerase reverse transcriptase: components and regulation. *Genes Dev.*, **12**, 1073–1085.
- Orias, E. (1986) Ciliate conjugation. In Gall, J.G. (ed.), *The Molecular Biology of Ciliated Protozoa*. Academic Press, Orlando, FL, pp. 45–80.
- Pelletier, H., Sawaya, M.R., Wolffe, W., Wilson, S.H. and Kraut, J. (1996) Crystal structures of human DNA polymerase β complexed with DNA: implications for catalytic mechanism, processivity and fidelity. *Biochemistry*, **35**, 12742–12761.
- Prescott, J. and Blackburn, E.H. (1997) Telomerase RNA mutations in *Saccharomyces cerevisiae* alter telomerase action and reveal nonprocessivity *in vivo* and *in vitro*. *Genes Dev.*, **11**, 528–540.
- Romero, D.P. and Blackburn, E.H. (1995) Circular rDNA replicons persist in *Tetrahymena thermophila* transformants synthesizing GGGGTC telomeric repeats. *J. Eukaryot. Microbiol.*, **42**, 32–43.
- Roy, J., Fulton, T.B. and Blackburn, E.H. (1998) Specific telomerase RNA residues distant from the template are essential for telomerase function. *Genes Dev.*, **12**, 3286–3300.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. 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.
- Tzfati, Y., Fulton, T.B., Roy, J. and Blackburn, E.H. (2000) Template boundary in a yeast telomerase specified by RNA structure. *Science*, **288**, 863–867.
- Wang, H. and Blackburn, E.H. (1997) *De novo* telomere addition by *Tetrahymena* telomerase *in vitro*. *EMBO J.*, **16**, 866–879.
- Wang, H., Gilley, D. and Blackburn, E.H. (1998) A novel specificity for the primer-template pairing requirement in *Tetrahymena* telomerase. *EMBO J.*, **17**, 1152–1160.
- Ware, T.L. (1999) The role of template and non-template RNA bases in *Tetrahymena* telomerase. PhD thesis. University of California, San Francisco, CA.
- Yu, G.L. and Blackburn, E.H. (1990) Amplification of tandemly repeated origin control sequences confers a replication advantage on rDNA replicons in *Tetrahymena thermophila*. *Mol. Cell. Biol.*, **10**, 2070–2080.
- 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.

Received January 13, 2000; revised and accepted April 25, 2000