The EMBO Journal Vol.16 No.4 pp.866–879, 1997

De novo telomere addition by *Tetrahymena* telomerase *in vitro*

He Wang and Elizabeth H.Blackburn[1]

Department of Microbiology and Immunology, Box 0414, University of California San Francisco, CA 94143, USA

[1]Corresponding author

Previous molecular genetic studies have shown that during programmed chromosomal healing, telomerase adds telomeric repeats directly to non-telomeric sequences in *Tetrahymena*, forming *de novo* telomeres. However, the biochemical mechanism underlying this process is not well understood. Here, we show for the first time that telomerase activity is capable *in vitro* of efficiently elongating completely non-telomeric DNA oligonucleotide primers, consisting of natural telomere-adjacent or random sequences, at low primer concentrations. Telomerase activity isolated from mated or vegetative cells had indistinguishable specificities for non-telomeric and telomeric primers. Consistent with *in vivo* results, the sequence GGGGT... was the predominant initial DNA sequence added by telomerase *in vitro* onto the 3′ end of the non-telomeric primers. The 3′ and 5′ sequences of the primer both influenced the efficiency and pattern of *de novo* telomeric DNA addition. Priming of telomerase by double-stranded primers with overhangs of various lengths showed a requirement for a minimal 3′ overhang of 20 nucleotides. With fully single-stranded non-telomeric primers, primer length up to ~30 nucleotides strongly affected the efficiency of telomeric DNA addition. We propose a model for the primer binding site of telomerase for non-telomeric primers to account for these length and structural requirements. We also propose that programmed *de novo* telomere addition *in vivo* is achieved through a hitherto undetected intrinsic ability of telomerase to elongate completely non-telomeric sequences.

Keywords: chromosome healing/*de novo* telomere addition/telomerase/telomerase–primer interaction/*Tetrahymena*

Introduction

In most organisms, telomeres, the ends of linear eukaryotic chromosomes, consist of short, G-rich sequence repeats complexed with telomere binding proteins (reviewed in Fang and Cech, 1995; Henderson, 1995). These telomere–protein complexes are essential for the integrity of eukaryotic genomes and are thought to play important roles in numerous biological processes (Blackburn, 1994; Zakian, 1995). A major telomeric function is to provide a cap to protect the chromosome from instability and to distinguish telomeres from double-stranded DNA breaks. Such functions of telomeres are most obvious by their loss, which can occur randomly, be induced experimentally or, in certain eukaryotes, occur as a developmentally programmed event through site-specific breakage of chromosomes. As a consequence, chromosomes lacking a telomere, or subchromosomal fragments, can form end-to-end fusions, cause cell cycle arrest, and are easily lost during mitotic division (McClintock, 1941; Zakian, 1989; Sandell and Zakian, 1993).

Chromosomal or subchromosomal DNA without telomeres can regain stability via chromosomal healing, a developmentally controlled process allowing *de novo* addition of telomere sequences to the broken DNA ends (Blackburn, 1995). Healing of randomly broken ends has been observed in many organisms including yeasts and humans (Pologe and Ravetch, 1988; Wilkie *et al.*, 1990; Müller *et al.*, 1991; Scherf *et al.*, 1992; Kramer and Haber, 1993; Lamb *et al.*, 1993). In ciliated protozoa and certain nematodes, a highly efficient chromosomal healing process is a developmentally programmed pathway for stabilizing subchromosomal fragments after chromosomal fragmentation (Blackburn, 1995). Telomerase, a ribonucleoprotein terminal transferase, has been implicated in programmed chromosomal healing. In particular, previous molecular genetic work showed that during *Tetrahymena* development telomerase adds marked telomeric sequences, copied from its mutated RNA template, directly to the ends of non-telomeric DNA created by chromosomal fragmentation (Yu and Blackburn, 1991).

Telomerase, initially discovered by its ability to extend DNA primers with telomeric sequences, copies one telomeric DNA strand from a template region of the RNA moiety of the enzyme. The characterization of reactions in which telomerase elongates telomeric primers *in vitro* has contributed to our understanding of the other important role of telomerase: elongating pre-existing telomeres to maintain length and hence chromosomal stability (Blackburn, 1992; Greider, 1995). In this reaction, it appears that two functional domains, the template region of the telomerase RNA which can base-pair with telomeric primer ends, and a second primer binding site specific to G-rich primers, are important for the precision and efficiency of elongation by telomerase (Collins and Greider, 1993; Lee and Blackburn, 1993).

Telomerase-mediated chromosomal healing has also been proposed for yeast and humans (Murray *et al.*, 1989; Morin, 1991; Kramer and Haber, 1993; Melek *et al.*, 1996). In these cases, *de novo* telomere addition occurs at sites containing sequence elements which, like those in a telomeric primer, can potentially interact with telomerase. In these non-programmed events, telomeric DNA addition occurs either directly onto a telomere-like seed sequence (e.g. Shampay *et al.*, 1984; Prescott and Blackburn, 1997), or onto non-telomeric sequences located at variable distances from an internal telomere-like sequence

(Murray *et al.*, 1989; Shampay and Blackburn, 1989; Kramer and Haber, 1993). Interactions of telomerase with these telomere-like sequences at or near the target site for addition may help account for the healing of this type of DNA target. In the healed regions of α-thalassemia patients, the target sites for telomere addition often contain several 3′-ending nucleotides (usually more than two) that can potentially base-pair with the telomerase RNA template. In addition, these sites also contain an upstream G-rich sequence that can potentially interact with the second primer binding site (Wilkie *et al.*, 1990; Flint *et al.*, 1994). DNA oligonucleotides derived from these target sites are competent primers for human and *Tetrahymena* telomerase activities *in vitro* (Harrington and Greider, 1991; Morin, 1991). Chimeric DNA primers containing a non-telomeric 3′ end and telomeric or G-rich 5′ end, similar to such *in vivo* target sites, in general can efficiently prime ciliate telomerase (Harrington and Greider, 1991; Melek *et al.*, 1996).

Previous efforts, however, have failed to elucidate the underlying mechanisms for telomere addition onto DNA target sequences containing no apparent telomerase-interacting sequence. This is the type of target DNA site commonly found in ciliates, and which perhaps is used at lower frequencies in other organisms such as the malarial parasite *Plasmodium* (Yao *et al.*, 1987; Pologe and Ravetch, 1988; Baird and Klobutcher, 1989; Yu and Blackburn, 1991; Scherf *et al.*, 1992). These target sequences have minimal base-pairing potential between their 3′ end nucleotides and the telomerase RNA template, ranging from two to no possible base pairs. Yet *in vivo*, these sequences are the targets for direct addition of a specific permutation of the telomeric repeat sequence, which in ciliates invariably begins with GGGG (Klobutcher *et al.*, 1981; Yao *et al.*, 1987; Yu and Blackburn, 1991; Fan and Yao, 1996). The inability to detect telomere synthesis *in vitro* using non-telomeric DNA substrates has been the primary barrier to understanding this type of *de novo* telomere addition. In contrast to the high specificity of telomerase for a telomeric primer ($K_m \approx$ 5–10 nM), it was reported previously that primers with various random sequences were poorly used by telomerase, even at concentrations >10 μM (Spangler *et al.*, 1988; Harrington and Greider, 1991). These reports led to speculations that potential auxiliary factor(s) might bind DNA and work *in trans* to recruit telomerase to non-telomeric DNA substrates (Fan and Yao, 1996; Melek and Shippen, 1996).

Here, we report efficient priming of telomerase by non-telomeric DNA primers devoid of any G-rich sequence *in vitro*. The efficiency of this *de novo* telomere addition has allowed dissection of the mechanism of this biologically significant reaction of telomerase. We show that the telomere addition reaction onto these primers begins predominantly by adding the sequence GGGGT. Telomerase purified to varying degrees, and over a variety of column chromatographic procedures, from cells undergoing developmentally programmed healing and cells dividing vegetatively, behaved similarly in all assays, suggesting that no developmentally controlled, dissociable factor is required for the healing reaction. The efficiency of the telomere addition reaction was strongly influenced by both the 3′ end nucleotide(s) and the 5′ sequence backbone of the non-telomeric primers. In addition, priming of telomerase by a double-stranded non-telomeric DNA primer required a single-stranded 3′ overhang of at least 20 nucleotides. Lengthening of a single-stranded non-telomeric primer increased its substrate specificity for telomerase. These results strongly suggest that *de novo* telomere addition to non-telomeric DNA is an intrinsic reaction of telomerase itself, and we propose a new model for a bipartite binding site of telomerase that allows productive interaction with a completely non-telomeric primer.

Table I. Sequence of DNA oligonucleotides

Primer	Sequence
T1(20)	**5′-TTGGGGTTGGGGTTGGGGTT-3′**
T1(12)	GGGGTTGGGGTT
T′(24)	AACCCCAACCCCAACCCCAACCCC
PBR(24)	**AGCCACTATCGACTACGCGATCAT**
N1(20)	TTTATTTTTTATAAAAATTA
N1(20)a	TTTATTTTTTATAAAAATTAA
N1(20)t	TTTATTTTTTATAAAAATTAT
N1(19)	TTTATTTTTTATAAAAATT
N1(30)	TCATTATTAATTTATTTTTTATAAAAATTA
N3(21)	**AAATGTTAGAAAAAATAAATA**
N3(30)	TCATTATTAAAATGTTAGAAAAAATAAATA
N2(16)	AATTTAAGAAAATAAT
N2(20)	**GTTTAATTTAAGAAAATAAT**
N2(25)	AGTTTGTTTAATTTAAGAAAATAAT
N2(30)	TAAATAGTTTGTTTAATTTAAGAAAATAAT
N2(32)	AATAAATAGTTTGTTTAATTTAAGAAAATAAT
N2(37)	TGAACAATAAATAGTTTGTTTAATTTAAGAAAATAAT
N2(20)g	GTTTAATTTAAGAAAATAATG
N2(20)t	GTTTAATTTAAGAAAATAATT
N2(20)a	GTTTAATTTAAGAAAATAATA
N2′a	3′-ATCAAACAAATTAAATTCTTTTATTAAAAG-5′
N2′b	TCAAACAAATTAAATTCTTTTATTA
N2′c	TCAAACAAATTAAATTCTTTT
N2′d	TCAAACAAATTAAATTC
N2′e	TCAAACAAATTAAAT
N2′f	TTTATCAAACAAATTAA
N2′g	TATTTATCAAACAAATT
N2′h	CTTGTTATTTATCAAAC
N2′i	CTTGTTATTTATCAAA
N2′j	CTTGTTATTTATCAA

Results

Telomerase efficiently elongates non-telomeric DNA primers

We first evaluated the ability of telomerase to elongate non-telomeric DNA oligonucleotide primer substrates in conventional *in vitro* telomerase assays. These substrates included three *Tetrahymena* telomere-adjacent sequences, onto which telomerase directly adds telomere sequence *in vivo* during *de novo* telomere addition (Yao *et al.*, 1987; Yu and Blackburn, 1991; Table I). At the initial stages of this study, a total of 19 telomere-adjacent sequences from macronuclear DNA ends had been identified (King and Yao; 1982; Yokoyama and Yao, 1986; Yao *et al.*, 1987; Spangler *et al.*, 1988; Yu and Blackburn, 1991). Other than the rDNA, with a $T_2G_4T_2$ sequence at the site of telomere addition, all 18 other sequences contain no identifiable sequence motif except for a highly AT-rich

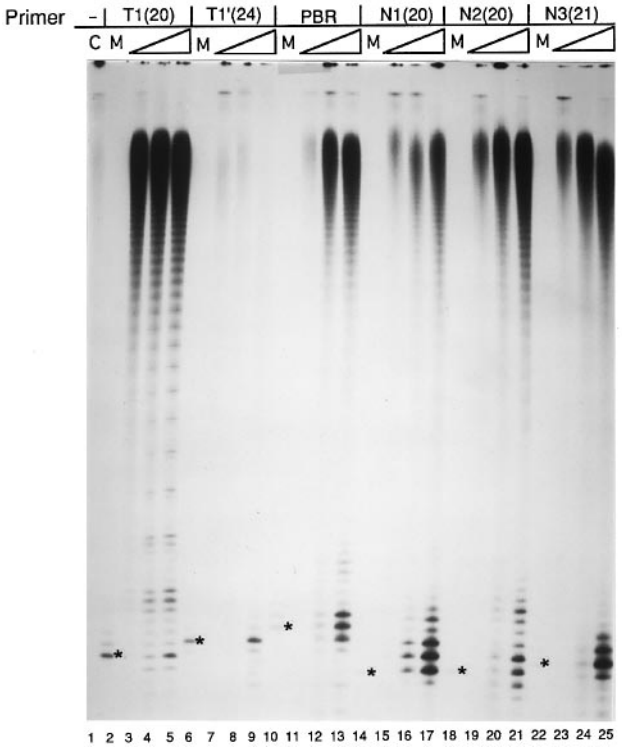


Fig. 1. Elongation of non-telomeric primers by telomerase. Standard telomerase assays were carried out as in Materials and methods. ^{32}P-labeled reaction products were separated on a 6% denaturing polyacrylamide gel and an X-ray film exposure of the gel is presented. Each primer substrate was assayed at three concentrations (2.5, 25 and 250 nM) from left to right, indicated by a triangle. Lane 1, control reaction (C) with no DNA primer substrate. Primer sequences were: T1(20), 5′-TTGGGGTTGG GGTTGGGGTT-3′; T1′(24), 5′-AACCC-CAACCCCAACCCCAA CCCC-3′; PBR, 5′-AGCCACTATC GAC-TACGCGA TCAT-3′; N1(20), 5′-TTTATTTTTT ATAAAAATTA-3′; N2(20), 5′-GTTTAATTTA AGAAAATAAT-3′; N3(21), 5′-AAAT-GTTAGA AAAAATAAAT A-3′. Lanes 2, 6, 10, 14, 18, 22 were size makers (M) derived from primer labeled with [α-^{32}P]dGTP by deoxynucleotidyl terminal transferase. Marker of primer +1 is indicated by the asterisks.

content and a lack of any cluster of G residues. Furthermore, of these 18 sequences, the precise telomere addition target sites are known for four, which were also identified in the progenitor micronuclear genomic DNA (Yao *et al.*, 1987; Yu and Blackburn, 1991). We randomly chose three of these four sequences for making the DNA oligonucleotide substrates in this work. A pBR322 sequence oligonucleotide and a control telomeric G-rich strand repeat sequence were also tested (Harrington and Greider, 1991). The telomerase reactions contained the DNA primer, partially purified telomerase fraction and nucleotide substrates [α-^{32}P]dGTP and unlabeled dTTP. The products of telomere synthesis were visualized as the characteristic repetitive ladders of ^{32}P-labeled products, resolved by denaturing gel electrophoresis.

Unexpectedly, four out of the five non-telomeric primers were remarkably competent in priming telomerase for telomere synthesis (Figure 1). Three non-telomeric primers, N1(20), N2(20) and N3(21), each consisted of a naturally occurring A-T-rich telomere-adjacent sequence (where N denotes non-telomeric sequence, and the number in parentheses the primer length in nucleotides). These primed telomere synthesis efficiently at concentrations as low as 25 nM (Figure 1, lanes 15–17, 19–21 and 23–25). The primer PBR had a random bacterial plasmid sequence, and in contrast to previous reports (Harrington and Greider, 1991; Melek *et al.*, 1996), elicited telomeric DNA synthesis with a similar high level of efficiency (Figure 1, lanes 11–13). The one inactive primer was T1′(24) (T1′ denotes an A_2C_4 repeat sequence complementary to the telomeric G_4T_2 repeats, lanes 7–9). By using chimeric primers containing both C-rich and N2(20) primer sequences, we have found that a cluster of C residues at either the 3′ or 5′ end of a primer is sufficient to strongly inhibit *de novo* telomeric DNA addition (data not shown). The control telomeric primer T1(20) primed telomere synthesis efficiently at primer concentrations as low as 2.5 nM, as expected (Figure 1, lane 3–5).

The priming of telomeric DNA addition by these completely non-telomeric primers at relatively low concentration provided us with the first opportunity to examine the mechanism of *de novo* telomere addition *in vitro*. Two predominant types of products were synthesized from non-telomeric primers: short multiple turnover products consisting of primer plus a few nucleotides, and long products hundreds of nucleotides in length. This product distribution can be explained by a high product dissociation rate of the short elongation products, and a low dissociation rate for the fraction of those nascent, elongated products that remain associated with telomerase, and which, once further elongated with telomeric sequence, acquire a higher affinity for telomerase. A similar distribution of products has been seen with short (9–10 nt) telomeric DNA primers (Lee and Blackburn, 1993), but with a much higher ratio of long to short products than in these non-telomere-primed reactions.

Elongation of non-telomeric primers by telomerase was markedly more sensitive to reaction conditions than that of telomeric primers (Figure 2). This may in part explain previous failures to detect efficient telomere synthesis using non-telomeric primer substrates. Reducing the Mg^{2+} concentration from the standard 1.25 mM to 0.5 mM led to an ~80% reduction in synthesis of the long DNA products primed by primer N2(20) (compare lanes 14–16 with lanes 17–19 in Figure 2A; also quantitation of the total reaction products in each gel lane in Figure 2B). This sensitivity of telomere synthesis to Mg^{2+} was primarily seen for the longer elongation products. In comparison, the same reduction of Mg^{2+} concentration caused ⩽50% inhibition of addition to a telomeric primer (compare lanes 1–3 with lanes 4–6 in Figure 2A, also in Figure 2B). Increasing the Mg^{2+} concentration to 10 mM had no significant effect (data not shown). Inclusion of the monovalent salt sodium acetate at 100 mM, or potassium glutamate at 80 mM, resulted in a 50–70% inhibition of synthesis of both long and short products primed by N2(20) (compare lanes 19 with 22 in Figure 2A and B). However, priming by the telomeric primer T1(20) was not significantly affected (compare lanes 4–6 with 7–9 in Figure 2A and data not shown). Supplementation with the polycation spermidine, as used in previous reports (Greider and Blackburn, 1987; Harrington and Greider, 1991), did not restore synthesis in the presence of NaOAc and lower Mg^{2+} concentration.

Under some reaction conditions, certain non-telomeric primers also caused incorporation of label into bands the

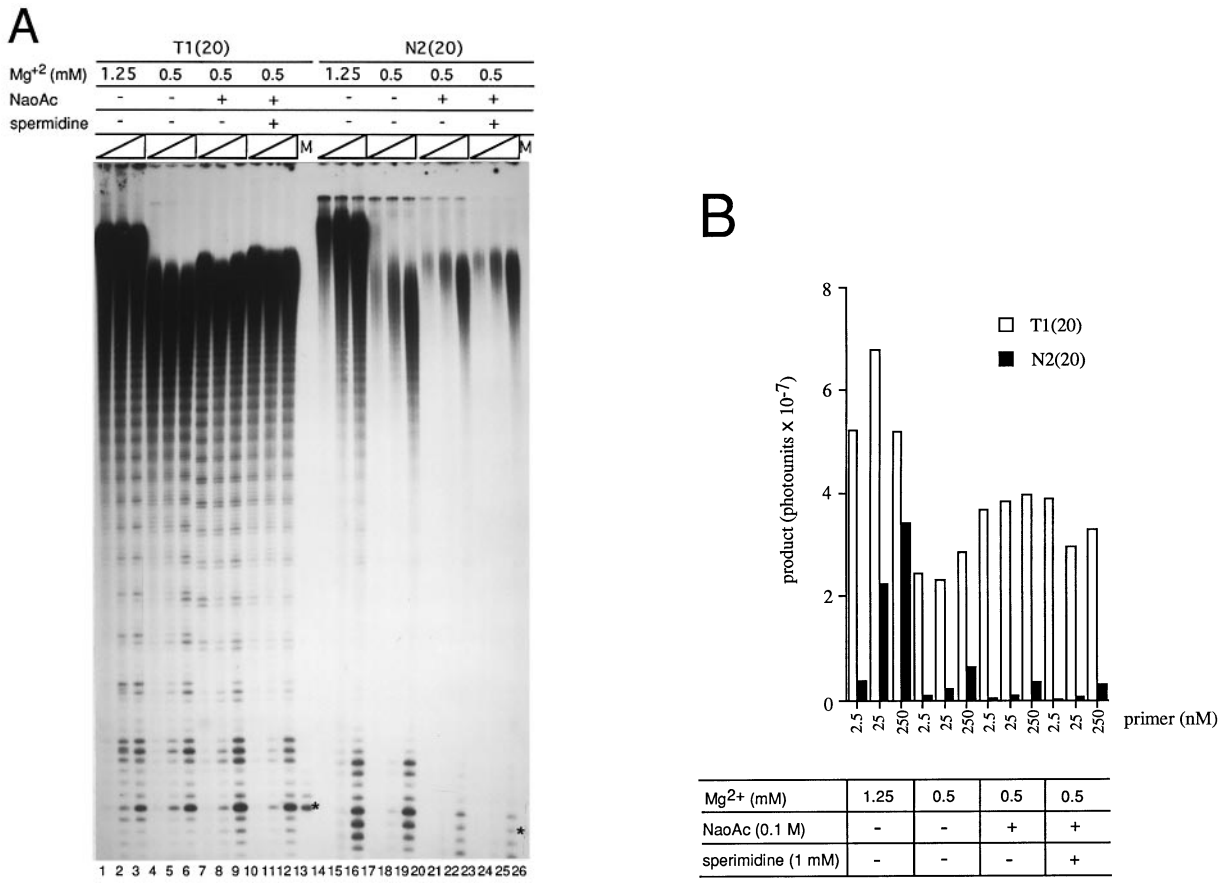


Fig. 2. Sensitivity of complete telomerase reaction to reaction conditions. (**A**) Effects of salt and spermidine on telomere synthesis. Telomerase assays were carried out as in standard conditions except for the addition of reagents as indicated on top of each gel lane. In reactions indicated, the supplied NaOAc was 0.1 M and spermidine was 1 mM. Reaction products were analyzed as in Figure 1. Migration of primer +1 band for each primer in marker lane (M) is indicated by asterisks. In each set of conditions, three primer concentrations, 2.5, 25, and 250 nM from left to right, were assayed as indicated by a triangle. (**B**) Histogram of phosphorimaging quantitation of the total radiolabeled reaction products in each gel lane in (A); corresponding reaction conditions are indicated at the bottom.

same size or shorter than the input primer [e.g. lane 25 in Figure 1; labeled bands shorter than primer+1 (indicated by the asterisk)]. These products may result from nucleotide addition to primers shortened by a telomerase-intrinsic cleavage activity, as described previously (Collins and Greider, 1993). Alternatively, contaminating nucleases might shorten the primer, which is then elongated by telomerase. A contribution from this source is suggested by the observation that certain more purified telomerase fractions produced less labeled shortened products.

Telomerase from mated and vegetative cells behaves as a single-sized complex and has similar specificity for non-telomeric primers

The results described above were obtained with telomerase from mated cells at the developmental stage at which *de novo* telomere addition takes place. We therefore wished to test whether the priming ability of the non-telomeric primers was a result of some developmentally specific modification of telomerase itself, or the presence of an auxiliary factor at this stage. We also wished to test whether such a putative mated cell-specific factor could have escaped detection due to its dissociation from telomerase during the standard multi-column fractionation procedures used in this study.

As a first step, an S100 extract from mated cells was fractionated directly on an analytical sizing column, Superose-6, to determine whether any potential telomerase complexes existed that differed in size or ability to utilize telomeric and non-telomeric DNA primers. However, in the column elution profile, only one telomerase activity peak was observed in assays using both a telomeric primer T1(20) and a non-telomeric primer N2(20) (Figure 3A). The peak of activity for both primers coincided, and had an estimated molecular weight of 500 kDa (Figure 3B), which is substantially larger than the calculated molecular weight of 230 kDa (Collins *et al.*, 1995). In addition, telomerase activity in S100 cellular extract was also fractionated by a combination of several chromatographic procedures. These procedures included sequential fractionations on either: (i) DEAE and octyl–Sepharose; (ii) Heparin, DEAE and octyl–Sepharose; (iii) Heparin, DEAE, octyl–Sepharose and Superose-6 sizing column; and (iv) DEAE and spermidine–agarose. Analyses of the activity profiles of these column fractions gave several interesting results. First, the elution size of heparin/DEAE/octyl active fractions on Superose-6 was the same as that of crude cellular S100 extract (data not shown). Furthermore, active fractions prepared by both procedures showed very similar specificity for telomeric versus non-telomeric primers in primer mixing experiments (see below; data not shown). Telomerase prepared from mated

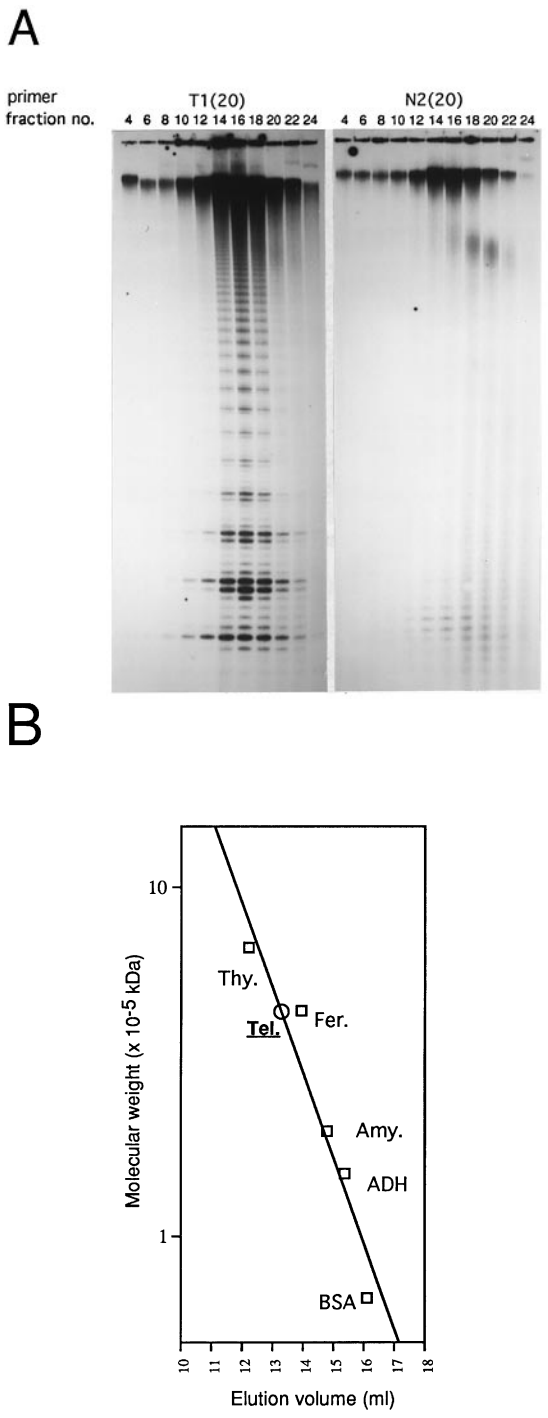


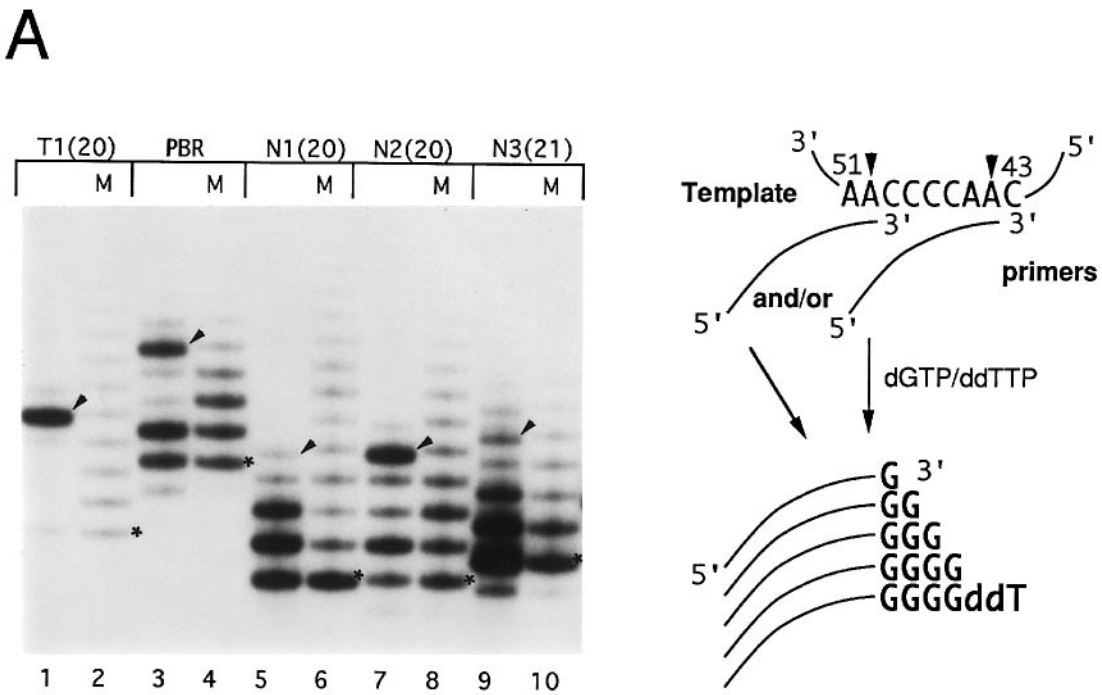
Fig. 3. Fractionation of telomerase from mated cells on a Superose-6 sizing column. Fractionation of S100 on a Superose-6 column and telomerase assays were as described in Materials and methods. ^{32}P-labeled assay products were separated on a 10% denaturing polyacrylamide gel. (**A**) Column profile of telomerase activity. Each fraction was 0.4 ml in volume starting from fraction # 4 (7.7 ml) to # 24 (16.1 ml). (**B**) Molecular weight plot of telomerase activity on the Superose-6 column against molecular weight size standards on a log scale. Protein standards (Sigma) were thyroglobulin (Thy., 670 kDa), apoferritin (Fer., 443 kDa), amylase (Amy., 200 kDa), alcohol dehydrogenase (ADH, 150 kDa) and bovine serum albumin (BSA, 66 kDa).

and log phase vegetative cells had identical elution profiles and size on the sizing column. Lastly, in primer specificity experiments (see below) telomerase from mated cells was indistinguishable from that of vegetative cells (data not shown). In conclusion, we have detected only one form of telomerase activity and have been unable to detect any significant difference between mated and vegetative cell telomerase.

Elongation of non-telomeric primers begins predominantly with dGGGGT

Recognition and elongation of a DNA primer substrate by telomerase has been best understood at the level of canonical base-pairing between two or more bases at the 3′ end of a telomeric DNA substrate and the RNA template of telomerase (Blackburn *et al.*, 1989; Greider, 1995). Yet no recognizable common sequence features that can form such base-pairs with the template are found at the target sites of *de novo* telomere addition in ciliates, other than occasionally a cluster of T residues (Yao *et al.*, 1987; Yu and Blackburn, 1991). Therefore, a base-pairing-independent mechanism appears necessary for telomerase to accomplish this role *in vivo*. As the first step toward understanding this process, we examined the initial nucleotides added onto a non-telomeric primer by telomerase in the *in vitro* reaction. We used combinations of deoxyribonucleotide(s) (dNTP) and a chain-terminating dideoxyribonucleotide (ddNTP). The sequence of added nucleotides was inferred from the specific termination pattern caused by incorporation of the chain-terminating nucleotide, as described previously (Gilley and Blackburn, 1996).

Significantly, with each non-telomeric primer, in reactions containing [α-^{32}P]dGTP and ddTTP, the latter effectively blocked extension beyond the primer+5 nucleotide product, consistent with addition of the sequence dGGGGddT-3′ (G4ddT in Figure 4A, lanes 3, 5, 7 and 9, arrowheads). This was seen as a strong band with primers PBR and N2(20) (arrowheads in lanes 3 and 7, respectively). A similar stop product, although at lower intensity, was synthesized in the reactions with the other two non-telomeric primers, N1(20) and N3(21) (Figure 4A, lanes 5 and 9, respectively). The sequences of the products were inferred from their mobility compared with a primer-plus-Gs marker ladder. In each reaction, migration of the first four bands matched the marker bands consisting of primer plus up to four dG residues (even lanes in Figure 4A). The faster migration of the +5 product, compared with the size marker consisting of primer plus five dG residues, is consistent with incorporation of four dG residues and a ddT residue at the fifth nucleotide position. In a control reaction with telomeric primer T1(20), which has a -GGGGTT-3′ end, primer extension resulted in essentially only one prominent product at the primer+5 nucleotide position, again corresponding to addition of dGGGGddT (Figure 4A, lane 1). This extension is consistent with alignment of the 3′ end T of this primer at position 50 and/or 44 in the template region (schematic in Figure 4A; also Gilley and Blackburn, 1996). The low amount of partial extension products with up to four added G residues was expected with the T1(20) primer, since the elongation of a telomeric primer of this length (20 nucleotides) by telomerase is known to be processive *in vitro* (Greider, 1991; Gilley and Blackburn, 1996). Extension of the non-telomeric primers with dGTP and ddTTP was much more distributive, with strong +1 to



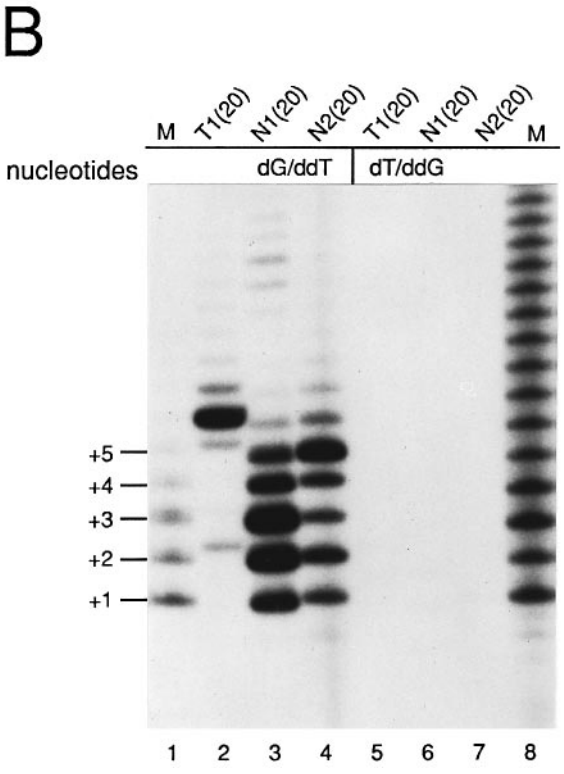


Fig. 4. Identification of initial nucleotides added to primer 3′ end by telomerase. Telomerase assays were carried out under standard conditions and labeled reaction products were analyzed as in Figure 3. (**A**) Reaction with [α-^{32}P]dGTP and ddTTP. Reaction of each primer and primer size markers (M) are indicated on top of each gel lane. Primer +5 product of each reaction is indicated by an arrowhead and primer +1 size marker is indicated by an asterisk. Schematic on the right shows the inferred alignment of the 3′ end of a primer on the RNA template of telomerase and the elongation products. (**B**) Telomerase assays with either [α-^{32}P]dGTP/ddTTP or with [α-^{32}P]dTTP/ddGTP. Marker (M) lanes were derived from primer, N2(20), labeled with dGTP (lane 1) or dTTP (lane 8) at the 3′ end by deoxynucleotidyl terminal transferase. Migration of primer +1 G to +5 Gs in lane 1 are indicated at the left.

+4 stop products. With primers N1(20) and N3(21), extension up to the full five nucleotides was significantly less efficient (Figure 4A, lanes 5 and 9).

In the reactions described above, any elongation products that had initiated with a T residue would not have been visible, as any ddTMP incorporated first would prevent further elongation. To test for any such initiation, we used labeled [α-^{32}P]dTTP and non-radiolabeled ('cold') ddGTP in telomerase assays with the non-telomeric primers. These elongation reactions were very inefficient compared with the reactions carried out in parallel with labeled dGTP plus ddTTP (Figure 4B, compare lanes 5–7 with lanes 2–4). The inefficient dTTP incorporation was not due to a high K_m of telomerase for dTTP, since additional experiments with the same labeled dTTP and cold dGTP and appropriate non-telomeric primers led to the successful labeling of long extension products (data not shown). Therefore, the defined sequen-

tial extension product dGGGGddT did not result from a bias of incorporation imposed by the nucleotide used for the ^{32}P label. When initiation by a T residue did occur, the reactions were largely limited to the addition of a single T residue (data not shown). These results support a model in which the major pathway of *de novo* telomere addition *in vitro* initiates by addition of an initial dGGGGT, rather than dT. We also confirmed that this reaction was carried out by telomerase itself, by using telomerase with template mutations in the telomerase RNA. As shown previously for telomeric primers, a rG mutation in the template sequence at position 49 of the RNA (Gilley and Blackburn, 1996) caused addition of the predicted templated dC residue, followed by three dGs and dT, to a non-telomeric primer (to be reported elsewhere).

Taken together, these data are consistent with a mechanism in which priming of non-telomeric primers initiates at a defined position(s) on the RNA template, either 50 and/or 44 (Figure 4A, schematic). Furthermore, utilization of ddTTP instead of TTP does not change the kinetics of formation of the short products, as indicated by the similarity of each primer-characteristic pattern of +1 to +5 products in both the complete and chain-terminated reactions (compare Figure 4 with Figure 1). In addition, a fraction of these primer+dG to primer+dGGGG products could be chased into primer+dGGGGddT product with longer reaction times (data not shown). Hence at least this fraction of these strong stop products were intermediates in the formation of primer+dGGGGddT product. This defined, sequential addition of dGGGGddT is reminiscent of the *de novo* telomere addition on broken chromosomal ends *in vivo*, during which, in almost every case examined, the initial sequence added to a non-telomeric sequence is G4T2 (Yao *et al*., 1987; Yu and Blackburn, 1991; Fan and Yao, 1996). Hence, the ddT-terminated reaction was chosen for further characterization of the *de novo* telomere addition reaction *in vitro*.

Effect of non-telomeric primer sequence on addition of dGGGGddT

Conceivably, addition of dGGGGddT can occur if the 3′ end nucleotide of a non-telomeric primer first interacts with nt 50 and/or 44 of the telomerase RNA template, positioning the 3′ end for addition of dGGGG by copying 49 to 46, or 43 then 48 to 46, as described for telomeric primers (see schematic in Figure 4A; also Gilley and Blackburn, 1996). Hence, the 3′ nucleotide(s) of the non-telomeric primer is expected to be one determinant of the efficiency of dGGGG addition. This was shown to be the case by comparing: (i) primers with the same two nucleotides at the 3′ end but different 5′ backbone sequences; and (ii) primers with the same 5′ backbone but carrying a different 3′ end nucleotide(s). As shown in Figure 5, the two 3′ bases of the primer had a major influence on the strong stop pattern leading up to dGGGGddT.

Primers ending with -TT 3′, N2(20)t and N1(19), showed strong +4 and +5 bands, and overall formation of the full-length +5 band was very efficient (Figure 5, lanes 3 and 7). Primers ending with -AT 3′, N2(20) and N1(20)t, showed a characteristic relatively strong +2 band [G4ddT in Figure 5, lanes 1 and 6; see also PBR (-AT 3′) reactions in Figure 4A]. This pattern was not greatly

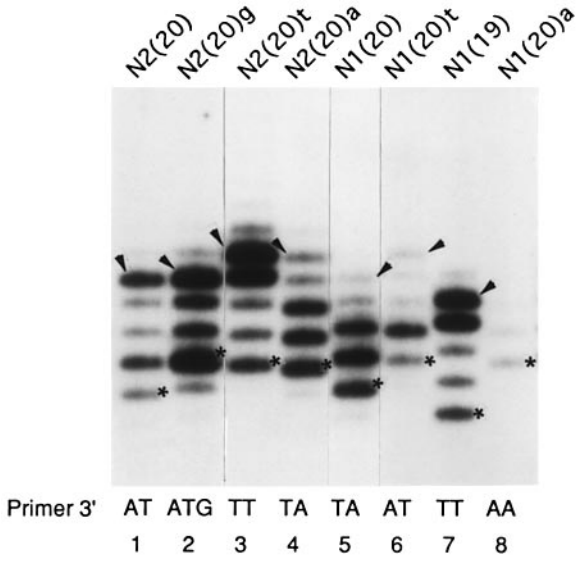


Fig. 5. Effect of the 3′ end nucleotide(s) and 5′ sequences of non-telomeric primers on the telomere addition. Telomerase reactions were carried out with [α-^{32}P]dGTP and ddTTP and products were analyzed as in Figure 3. The sequence of the 3′ ending two [three for N2(20)g] nucleotides of each primer substrate is shown at the bottom. Primer+dGGGGddT product of each reaction (except primer+dGGGddT in the reaction with primer N2(20)g) is indicated by an arrowhead and primer +1 size product by an asterisk.

changed when a G residue was already present at the primer 3′ end (primer N2(20)g in Figure 5, lane 2). With primer N2(20), with an -AT 3′, dGGGGddT comprised 17% of the total molar amount of products (Figure 5, lane 1). When the primer ended with -TA 3′, the +1, +2 and +3 bands were very strong relative to the amount of +4 and +5 products, and the overall efficiency of full-length +dGGGGddT product was very low (Figure 5, lanes 4 and 5). For example, only 1% of the total label in elongation products from N1(20) was in dGGGGddT (Figure 5, lane 5). Only primer N1(20)a, with an -AA 3′ end, failed to prime synthesis strongly enough for detection of the dGGGGddT product (Figure 5, lane 8). Taken together, these results are consistent with a model in which base-pairing of the 3′ nucleotides with positions 50 and 51, or 44 and 45, causes formation of the full-length product with highest processivity.

The 5′ backbone as well as the 3′ end of the primer affected processivity and efficiency of +dGGGGddT formation. Two primers with the same -AT 3′ end (N2(20) and N1(20)t) had very different ratios of dGGGGddT to intermediate stops, although both still produced the characteristically strong +2 band (Figure 5, compare lanes 1 and 6). Likewise, the primers N2(20)a and N1(20), with a common -TA 3′ end, both had strong stops up to +3 but different relative amounts of full-length +dGGGGddT product (Figure 5, lanes 4 and 5). Primers with the N2 5′ backbone produced significantly more +dGGGGddT product than did those with the N1 backbone (Figure 5, compare lane 1 with 6, and lane 4 with 5). Therefore, as shown previously for primers with extensive complementarity at the 3′ end with the template (Lee and Blackburn, 1993), sequence-specific interactions of telomerase with the 5′ region of a non-telomeric primer also have a significant impact on the efficiency and processivity of telomeric DNA addition.

Table II. Elongation of double-stranded DNA substrates by telomerase

Testing primer	Duplexed with	Test primer 3′ end	Extension
N2(25)	N2′a	5′ overhang (4 nt)	−
N2(25)	N2′b	blunt end (0)	−
N2(25)	N2′c	3′ overhang 4	−
N2(25)	N2′d	8	−
N2(25)	N2′e	10	−
N2(30)	N2′f	12	−
N2(32)	N2′g	14	−
N2(25)	–	(25)[a]	+
N2(30)	–	(30)[a]	+
N2(32)	–	(32)[a]	+

[a]Parentheses denote fully single-stranded oligonucleotides.

A 3′ overhang longer than 19 nt is required for telomerase to elongate a double-stranded non-telomeric DNA substrate

To date, the structure of the *in vivo* target sites for *de novo* telomere addition is unknown. Cleavage of the phosphodiester backbone of the target DNA must first create a 3′-OH end to allow addition by telomerase. However, whether this non-telomeric end is utilized by telomerase in 5′ overhanging, blunt-ended, or 3′ overhanging form is unknown. To address the potential of telomerase to use such ends as substrates *in vitro*, various duplex forms of primer substrates were made and tested for their priming efficiencies. A set of test non-telomeric primers, N2(25), N2(30), N2(32), and N2(37), was made, each consisting of primer N2(20) with varying numbers of nucleotides added to the 5′ end to make the total length indicated. The nucleotides added to the 5′ end were the naturally occurring sequence from the telomere addition region (Yao *et al.*, 1987). These were converted to duplex form by hybridizing each with one of a set of complementary DNA oligonucleotides (oligos). Each complementary oligo was blocked at the 3′ end by a dideoxyribonucleotide using terminal transferase prior to the hybridization. The resulting test duplex substrates, with various forms of 3′ ends, recessed, blunt, or in a 3′ overhang of variable length (see Tables I and II), were assayed for their ability to prime dGGGGddT addition. Reactions were carried out with labeled dGTP and ddTTP at 20°C in the presence of 60 mM potassium glutamate, to ensure that the duplex form was maintained in the telomerase reaction. All these substrates were first analyzed by native gel electrophoresis under similar temperature and salt conditions to verify that they could form a stable duplex. For every substrate used, >95% of the test primer was in duplex form (see Materials and methods).

Non-telomeric DNA duplexes with a 3′ end recessed by 4 nt, a blunt end, or a 3′ overhang of up to 14 nt were not significantly elongated (results summarized in Table II). Only when a 3′ overhang longer than 14 nt was exposed was elongation of the test primer observed. In the experiment shown in Figure 6, the test non-telomeric primer was N2(37). Four complementary oligonucleotides, N2′g to N2′j, added in molar excess, were hybridized with N2(37) (at two concentrations, 25 nM or 75 nM) to leave a 14 to 21 nt 3′ single-stranded region. A trace amount (<1%) of each primer was ^{32}P-labeled at the 5′ end. This internal marker migrated faster than the primer +1 elongation product [see 5′p-N2(37) in Figure 6A]. Quantitation of total reaction products (+1 to +5) is presented in Figure 6B. Control assays with the test primer are shown in Figure 6A, lanes 1 and 2. With a 14 nt 3′ overhang [N2(37)/N2′g], priming ability of the partially duplex test primer was diminished to ~20–30% of that of the test primer N2(37) alone (Figure 6A, lanes 9 and 10). Priming ability was partially restored (up to ~35–45% of the test primer alone) by increasing the 3′ overhang to 19 nt (Figure 6A, lanes 5 and 6), and to ~60–85% and ~80–95% respectively when the single-stranded 3′ overhang was increased to 20 and 21 nt [Figure 6A, N2(37)/N2′i in lanes 7 and 8, N2(37)/N2′j in lanes 3 and 4]. As shown in Figure 6B, there was a break point in the elongation efficiency between substrates with 19 and 20 nt overhangs, suggesting that the critical length for a 3′ overhang is 19 or 20 nt. Inclusion of 1 mM ATP had no effect on the low priming ability of any of these duplex primers as shown for N2(37)/N2′g (Figure 6A, lane 11, and data not shown), suggesting that telomerase contained no detectable inherent ATP-dependent helicase activity which might unwind a bound partially complementary primer.

One explanation for the requirement of telomerase for a single-stranded non-telomeric 3′ overhang is that only single-stranded DNA can bind telomerase productively, and a minimal length of 20 nt is required for this binding to be strong enough to lead to productive elongation. To test this hypothesis, we compared the 20 and 16 nt single-stranded DNA primers N2(20) and N2(16) in the telomerase assay. Quantifying the products of reactions with one or both primers present showed that N2(20) is ~3-fold more efficient than N2(16) (data not shown). Together with results presented in the next section, these analyses support a model for length-dependent single-stranded DNA binding, in which productive telomerase binding to a non-telomeric primer requires a minimally 20 nt single-stranded region.

Specificity of telomerase for a non-telomeric primer increases with primer length

The minimal length requirement for a single-stranded overhang in a double-stranded non-telomeric substrate suggested that, besides sequence, the total length of the non-telomeric primer substrate available for interaction with telomerase is a significant factor determining its ability to prime elongation. To address the primer specificity of non-telomeric primers more quantitatively, we compared the substrate specificities of telomerase for various non-telomeric primers with that for a telomeric primer. We measured the reaction rate for each primer substrate at a time within the linear range of the reaction. For each pair of competing substrate primers in a reaction, the concentration-independent relative specificity factor (ratio of K_{cat}/K_m for each substrate) was computed (see Materials and methods).

As shown in Figure 7A, the rate of elongation of a non-telomeric primer, present alone at 250 nM, closely resembled that of telomeric primers at 12.5 and 25 nM for primer T1(20) and T1(12) respectively (Figure 7A, compare lane 1 with lanes 2 and 3, for primer+dGGGGddT). However, when the non-telomeric primer N2(20) was mixed

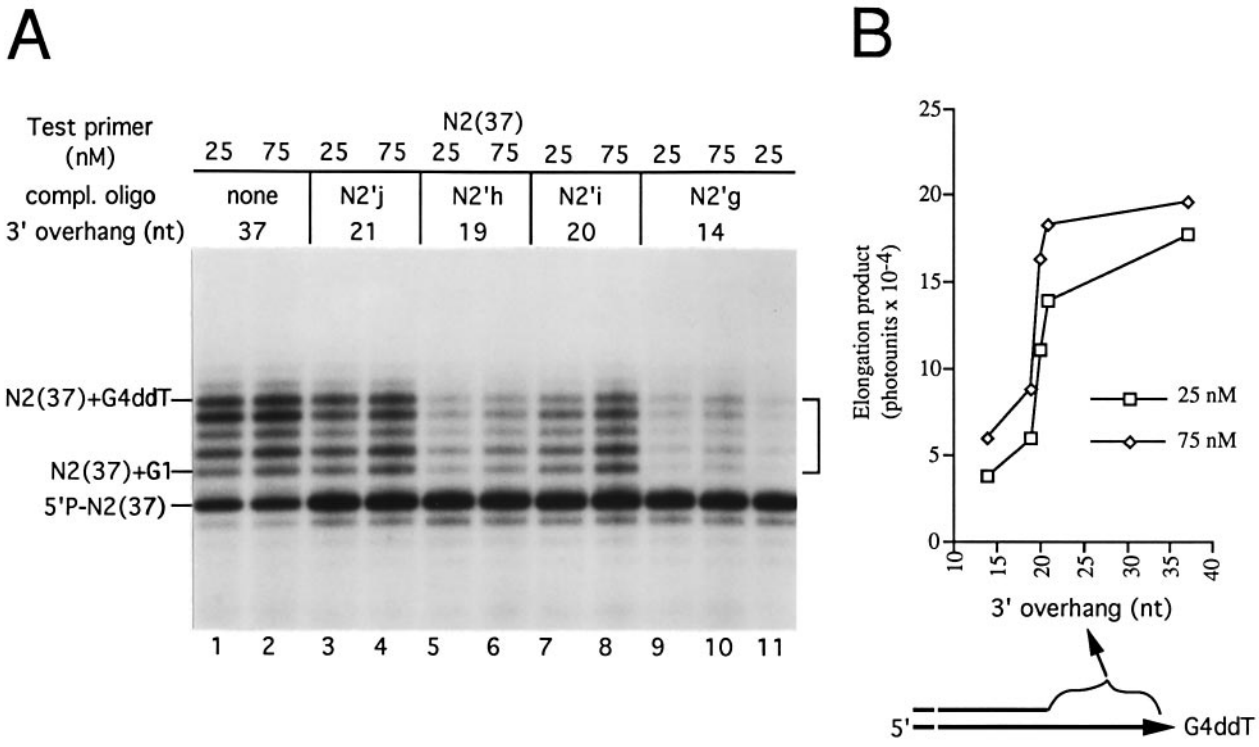


Fig. 6. Priming of telomerase by double-stranded DNA substrates. (**A**) Telomerase assays of double-stranded DNA primers. Reaction products were analyzed as in Figure 3. Each set of primers contained a fixed concentration of a complementary DNA oligonucleotide (compl. oligo) at 200 nM and test primer at two different concentration as indicated. 5′P-N2(37) indicates the migration of a 5′-labeled N2(37) primer (<1% of total primer input) in each reaction (used for monitoring the percentage of double strand formation in each substrate). N2(37)+G1 and N2(37)+G4ddT on the left indicate reaction products of primer+dG and primer+dGGGGddT respectively. The reaction in lane 11 was the same as in other assays except it contained 1 mM ATP-Mg^{2+}. A bracket on the right indicates the products quantitated in (B). (**B**) Quantitation of elongation products in (A) with a schematic of the 3′ overhang of the double-stranded substrates at the bottom.

at the same concentration with the telomeric primer T1(12), present at a 10-fold lower concentration (25 nM), elongation from N2(20) was greatly diminished (Figure 7A, compare lane 5 with lane 1), even though elongation from T1(12) was not significantly affected (Figure 7A, compare lane 5 with lane 3). The calculated specificity of telomerase for N2(20) was 200-fold lower than that for T1(12). The same specificity factor was obtained from a second reaction using a 2.5-fold lower T1(12) concentration (10 nM, Figure 7A, lane 6), verifying this method. In contrast, mixing of two telomeric primers, T1(12), at 25 nM, and T1(20), at 12.5 nM, which differ by T1(20) having an extra 5′ $T_2G_4T_2$, showed that the specificity of telomerase for primer T1(12) is within 2-fold of that for T1(20) (Figure 7A, lane 4).

Using the same approach, we analyzed the effect of length of the non-telomeric primer on its substrate specificity for telomerase. The natural telomere-adjacent sequence, non-telomeric primers N1, N2 and N3 were used in the form of single-stranded 20, 25 and 30 nt primers. The extra primer sequences consisted of natural, non-telomeric nucleotides at the 5′ of the primer. Significantly, when each non-telomeric primer was lengthened from 20 to 30 nt [e.g. from N1(20) to N1(30)], the specificity was enhanced by 3- to 12-fold [Figure 7B, compare lanes 3 with 4, and lanes 5 with 6 for N3(21) and N3(30)]. Lengthening N2(20) at its 5′ end by 5 nt, to make primer N2(25), increased the primer specificity >4-fold (Figure 7B, compare lane 2 with lane 1; see also Figure 7C and D). This contrasted with the situation for the telomeric primers, in which primer lengthening by 8 nt had little effect on primer specificity in this assay (Figure 7A, lane 4). Additional lengthening by 5 nt [N2(30)] resulted in a further 3-fold increase in specificity (Figure 7C, compare lane 3 with lane 2). The specificity for N2(30) was within an order of magnitude of that of the telomeric primer T1(12). However, further lengthening of the primer to 37 nt, N2(37), resulted in only a 10% increase in primer specificity (Figure 7C, compare lane 4 with lane 3). These results, plotted in Figure 7D, suggest that the strong effects of non-telomeric primer length on specificity might have a limit of ~30 nt.

The same specificities for non-telomeric versus telomeric primers were found with telomerase before or after fractionation over the various column fractionation procedures described above. In addition, in these primer specificity experiments, telomerase from mated cells behaved indistinguishably from telomerase prepared from vegetative cells (data not shown). Together, these results support the conclusion that, up to a limit of ~30 nt, increasing primer length enhances its substrate specificity.

Discussion

Here, we have demonstrated correct, efficient *de novo* telomere addition by *Tetrahymena* telomerase *in vitro* to primers representing the authentic telomere addition targets of *Tetrahymena*, and to other random sequence DNA primers devoid of G-rich or telomeric sequences. This efficient elongation of non-telomeric DNA primer substrates was observed even at low (nanomolar) primer

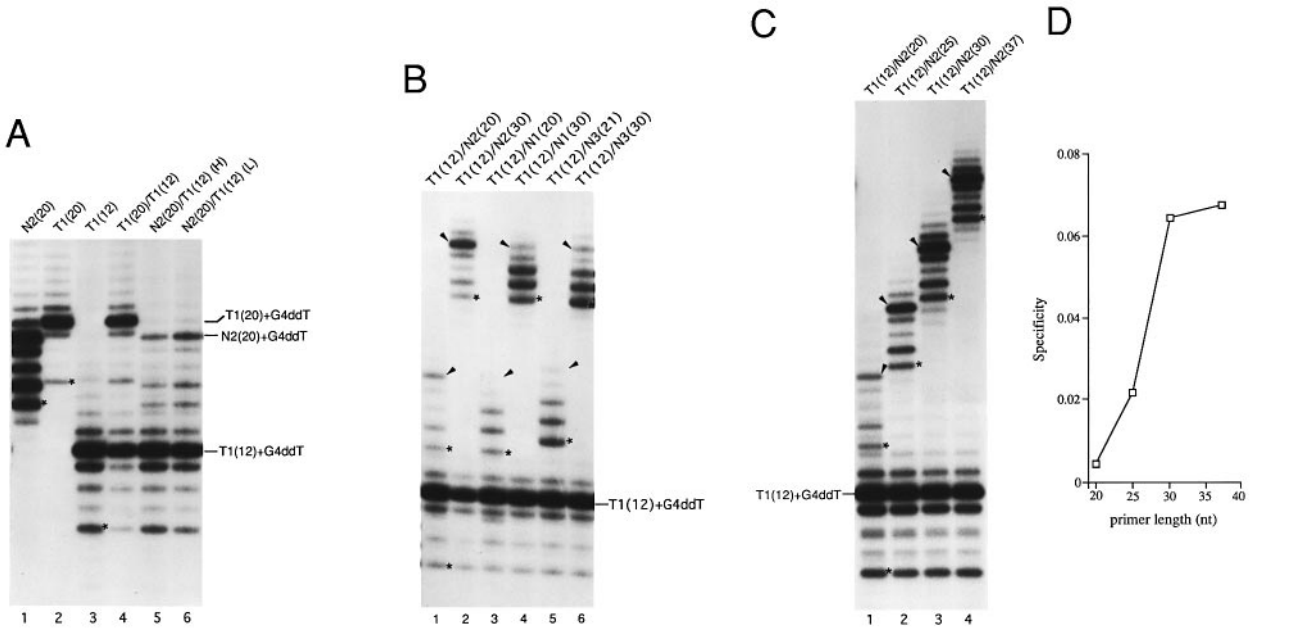


Fig. 7. Specificity of telomerase for non-telomeric primers. Telomerase assays were carried out under standard conditions with dGTP and ddTTP. Labeled reaction products were analyzed as in Figure 3. (**A**) Priming efficiency of telomeric and non-telomeric primers in one-primer versus two-primer reactions. Primer +1 product for each primer is indicated by an asterisk. The concentrations of primer substrates were: 250 nM N2(20) for reactions in lanes 1, 5 and 6; 12.5 nM T1(20) for reactions in lanes 2 and 4; 25 nM T1(12) for reactions in lanes 3, 4 and 5; 10 nM T1(12) for reaction in lane 6. (**B** and **C**) Effect of length on primer specificity for non-telomeric primer versus a telomeric primer. The concentrations of primer substrates were 25 nM for T1(12), 250 nM for N1, N2, and N3 primer series. Primer+dGGGGddT product in each reaction is indicated by an arrowhead and primer +1 product is indicated by an asterisk. (**D**) Quantitation of results in (C). The plot is drawn using the ratio of primer+dGGGGddT product from N2 primers to that from T1(12).

concentrations. Elongation of a non-telomeric primer by telomerase, in addition to being RNase-sensitive (data not shown), was especially sensitive to reaction conditions, including mono- and divalent ion concentrations. This sensitivity might have contributed to the previous failure to detect telomeric DNA addition to completely non-telomeric DNA primers *in vitro*, even using μM primer concentrations (Greider and Blackburn, 1987; Spangler *et al.*, 1988; Blackburn *et al.*, 1989; Harrington and Greider, 1991). Therefore, until now, the biochemical mechanism underlying *de novo* telomere addition onto non-telomeric DNA target sites has not been directly analyzable *in vitro*. Consistent with *in vivo* healing events, telomere addition to non-telomeric primers began specifically with dGGGGT. This highly preferential initial addition of dGGGGT supports the notion that this *in vitro* telomerase reaction reflects *de novo* telomere addition *in vivo*. Furthermore, this uniform product of the *de novo* telomeric addition suggests that non-specific base interactions occurring between the 3′ end of the primer and telomerase can lead to productive elongation. In work to be described elsewhere (H.Wang, D.Gilley and E.Blackburn, in preparation) we have used telomerase with template mutations to analyze further the telomerase RNA nucleotides utilized as the template in the elongation of non-telomeric primer.

Successful *de novo* telomere addition onto a non-telomeric 3′ end has been observed with *Tetrahymena* and *Euplotes* telomerases, using artificial chimeric primers containing a 5′ telomeric sequence (Harrington and Greider, 1991; Melek *et al.*, 1996). It was also shown that *Euplotes* telomerase uses a ‘default’ mechanism to add a precise dGGGGddT sequence to the 3′ end of such chimeric primers (Melek *et al.*, 1996). The strong binding and kinetic effects induced by interactions of telomerase with the telomeric sequences within such partially telomeric primers are likely to have masked the effects of the weaker interactions involving non-telomeric sequences. However, these weaker interactions should reflect more closely the normal requirements of the *de novo* telomere addition reaction, because in ciliates, most DNA targets for *de novo* telomere addition contain no apparent telomerase-interacting sequences.

Telomerase is unique among DNA polymerases in that its substrate specificity is not based on a thermodynamically stable stretch of duplex between the 3′ end of the primer and the template (e.g. Nevinsky *et al.*, 1990). The 3′ end nucleotides and the 5′ backbone sequence of the primer both strongly affected the efficiency of *de novo* telomere addition to non-telomeric primers. The impact of the 3′ end nucleotide of the primer, and to a lesser degree the 3′ penultimate nucleotide, is readily explainable by the influence of even a single canonical base-pair formed between the primer 3′ end and the template domain of the telomerase RNA. It has been demonstrated previously that DNA primers with two to four bases at the 3′ end complementary to the telomerase RNA template can direct telomeric DNA addition at under 10 μM primer concentrations (Morin, 1991).

It was proposed previously that telomerase possesses a primer–product binding site, independent from the telomerase RNA template, that interacts with the 5′ region of the primer. This second site model accounts for the strong effects of the 5′ sequence of both telomeric and non-telomeric primers on the reaction kinetics of telomere synthesis. In addition, a second primer binding site can

also account for the finding that increasing the ionic strength, which might have been predicted to stabilize an RNA–template DNA–primer helix, had little effect on the processivity of telomere synthesis (Lee and Blackburn, 1993; Lee *et al.*, 1993). A similar model was proposed to account for the productive elongation of chimeric primers with 5′ telomeric and 3′ non-telomeric sequences as well as the kinetic differences between telomerase reactions with telomeric primers of different length (Harrington and Greider, 1991; Morin, 1991; Collins and Greider, 1993). A candidate subunit for this second site has been identified as the p95 protein subunit of telomerase, since it shows specificity for binding telomeric primers (Collins *et al.*, 1995).

The available evidence suggests that the ability to elongate non-telomeric primers is a property intrinsic to telomerase. First, the specificities for non-telomeric primers were indistinguishable for telomerase isolated from mated cells and telomerase from vegetative cells. These results were obtained both with telomerase fractionated on a sizing column directly from an S100 extract, to avoid potential loss of protein factors during standard purification procedures, and with telomerase after various sequential column fractionations. Secondly, the impact of the 5′ sequence of the non-telomeric primer was consistent with the proposed second site model of telomerase. Furthermore, the efficient elongation of non-telomeric primers and the unchanged pattern of products made by telomerase in 60 mM potassium glutamate (compare results in Figure 4A with those in Figure 6A) lead us to conclude that the elongation of non-telomeric primers is not artifactually induced by hydrophobic interactions between these primers and telomerase under low salt conditions.

It has been shown previously that telomerase RNA levels are developmentally up-regulated (Avilion *et al.*, 1992; Price *et al.*, 1994) at the time of *de novo* telomere addition. Although putative transacting factor(s) for telomerase might have escaped our detection, our results indicate that the intrinsic specificity of *Tetrahymena thermophila* telomerase for non-telomeric DNA substrate is not developmentally regulated, since telomerase from mated and vegetative cells showed no difference in specificity for non-telomeric primers. These observations have expanded the types and the complexity of the temporal relationship between developmentally regulated chromosome cleavage and telomere addition. In one model (Yao *et al.*, 1990), it has been proposed that, during the process of macronuclei development from a progenitor micronuclei in *Tetrahymena*, chromosome cleavage and *de novo* telomere addition might occur as a concerted (or coupled) reaction. Specifically, it was proposed that the chromosome cleavage machinery might recruit telomerase to the chromosome cleavage site for telomere addition. This model was proposed largely based on two kinds of experimental observations. One was the failure in transformation of DNAs lacking a chromosome cleavage signal sequence, which is deleted in the process of chromosome cleavage (Yao *et al.*, 1990; Fan and Yao, 1996). The other was the previous reported failures to detect telomere addition to non-telomeric substrate by telomerase *in vitro* (Spangler *et al.*, 1988; Harrington and Greider, 1991). In light of the results presented here, it is necessary to consider other possible connections between chromosome cleavage and telomere addition. For example, the chromosome cleavage machinery might create substrates with the correct structure for telomerase, i.e. double strand with 20 nt single-stranded 3′ overhang (discussed below), and up-regulated telomerase might be sufficient for *de novo* telomere addition. In another model, other factor(s), besides the cleavage activity, may be required to create a DNA end with the correct structure for telomerase after the cleavage event. Alternately, instead of playing an active role by recruiting telomerase as suggested previously (Yao *et al.*, 1990), the cleavage activity may stabilize the cleavage site long enough for telomerase to act on it. In this case, cleavage and *de novo* telomere addition could still be functionally coupled despite the absence of direct physical interactions between the cleavage machinery and telomerase.

In contrast to telomeric primers (Lee *et al.*, 1993), efficient elongation of completely non-telomeric primers required a double-stranded non-telomeric substrate to have a single-stranded 3′ overhang of $\geqslant$20 nt. In addition, priming efficiency from fully single-stranded primers increased as a function of their length, up to ~30 nt. These results suggest that, *in vivo*, the chromosomal substrate for *de novo* telomere addition may also require a long 3′ overhang, which could be generated in various ways. The direct product of DNA cleavage formed during programmed chromosomal fragmentation is unknown. DNA ends with long single-stranded 3′ overhangs might be directly generated by a DNA cleavage activity that makes widely staggered cuts, although some form of helicase may be required to unwind these overhangs. Alternately, an auxiliary factor *in vivo* may convert a blunt-ended product of DNA cleavage into a telomerase substrate. This could be a helicase, or a 3′–5′ exonuclease which exposes a long, single-stranded 3′ overhang, similar to the single-stranded 3′ overhangs generated *in vivo* in *Saccharomyces cerevisiae* after double-strand break formation (White and Haber, 1990).

We propose the working model shown in Figure 8 for the molecular basis of the non-telomeric primer requirements of telomerase. In this model most, if not all, nucleotides in the primer cumulatively contribute to primer–telomerase interactions. Primer length effects fell off at ~30 nt, suggesting a possible maximal size for the total zones of interaction. However, the breakpoint between 19 and 20 nt seen with partially double-stranded substrates suggests that double-stranded DNA within a fixed distance from the 3′ end sterically hinders primer binding. Hence, we suggest that the primer binding site of telomerase has two zones: the zone nearest the template domain is a narrow groove, long enough to accommodate ~20 nt of single-stranded DNA (Figure 8A), but too narrow for duplex DNA. Therefore, duplex DNA intruding further than 20 nt into the 3′ overhang forces it partly out of the groove, interfering with the weak primer–telomerase interactions necessary to position the 3′ end nucleotides (-NN 3′) by the templating domain for productive elongation (Figure 8B and C). With partially double-stranded telomeric primers, only a 13-base (and possibly shorter) 3′ single-strand overhang is required *in vitro* for elongation by *Tetrahymena* telomerase (Lee *et al.*, 1993). Similarly, telomerase from the ciliate *Euplotes aediculatus* can

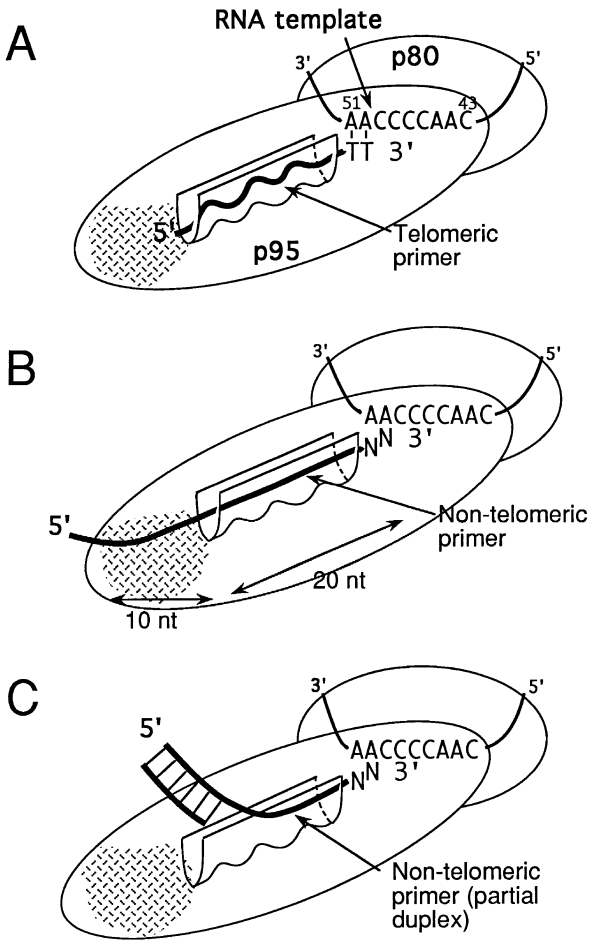


Fig. 8. A model of primer binding site of telomerase. *Tetrahymena* telomerase RNP; consisting of two protein subunits, p95 and p80, and one RNA subunit, is schematically drawn based on (Collins *et al.*, 1995). A groove in p95 indicates a proposed primer binding site specific for G-rich telomeric sequence. The hatched area on p95 indicates a proposed domain that can interact non-specifically with single-stranded DNA primer substrate. (**A**) Telomeric primer–telomerase complex. (**B**) Non-telomeric primer-telomerase complex. (**C**) Partially duplex non-telomeric primer–telomerase complex; 3′ overhang is <20 nt long.

elongate a 6-base 3′ overhang of a telomeric duplex primer (Lingner and Cech, 1996). We propose that for such telomeric primers a long region of contact with the second site is not necessary, because of the combined strong interactions between the 3′ nucleotides of these primers and the template (base-pairing), and between the 5′ telomeric sequences and the second site (G-rich specific interactions, Figure 8A). For simplicity, we show the template-proximal portion of the second site as being the same for the telomere sequence-specific and non-telomeric interactions with telomerase, although they could be different sites on telomerase. The other zone of the primer-binding second site of telomerase, located next to the groove, consists of a surface (hatched area in Figure 8) which can interact with a further 10 bases of single-stranded primer DNA. Interaction of non-telomeric primers with the second site, like that of telomeric primers, are likely to be with the p95 protein subunit. Such interactions might involve a variety of chemical as well as structural features of both the primer and the primer-binding site of telomerase.

In summary, *in vitro* telomerase reactions primed by completely non-telomeric DNA primers have allowed us to dissect the requirements for the relatively weak interactions between telomerase and the DNA sequences at the sites used *in vivo* for *de novo* telomere addition. These DNA target sites frequently contain no G-rich sequences capable of interacting specifically with the second site of telomerase, nor 3′ end nucleotides capable of pairing with the telomerase RNA template. This system will be a useful basis for examining factors that may modulate chromosome healing.

Materials and methods

Tetrahymena thermophila strains

CU427, CU428, SB2086 were kindly provided by Dr Ed Orias, University of California at Santa Barbara. Liquid culture of *T.thermophila* in PPYS medium was as described (Gilley *et al.*, 1995). Mating of *Tetrahymena* from starved cells of two different mating types was as described (Martindale *et al.*, 1982); mating efficiency was >80%.

DNA oligonucleotides

All synthetic DNA oligonucleotides (Cruachem, Dulles, VA) were purified by denaturing gel electrophoresis and Sep-Pak column (Waters, Milford, MA). The concentration of purified oligonucleotides was calculated based on 1 OD_{260} = 20 μg of DNA and molecular weight of the individual oligonucleotide. For preparation of double-stranded primer substrates, two complementary DNA oligonucleotides were mixed and incubated in 50 mM Tris–HCl, pH 8.0, 60 mM potassium glutamate at 90°C for 5 min and slowly cooled to room temperature.

Partial purification of telomerase

S100 cell extracts from log phase vegetative cells, 12–24 h starved vegetative cells, and 9–11 h mated cells were made as described (Greider and Blackburn, 1987). Chromatographic procedures were carried out at 4°C and all buffers contained protease inhibitors (2 μg/ml E64, 1 μg/ml leupeptin, 0.1 mM pefabloc; Boehringer-Mannheim, Indianapolis, IN). For DEAE–agarose (Bio-Rad, Hercules, CA) chromatography, 1 ml of S100 (prepared from 50–100 ml culture cells) was loaded onto a 1 ml pre-equilibrated DEAE column, in TMG (20 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$, 10% glycerol). The column was washed with TMG and 0.2 M NaOAc/TMG and telomerase eluted by 0.3 M NaOAc/TMG. After adjusting to 0.5 M NaOAc, telomerase fraction was loaded onto a 0.3 ml pre-equilibrated octyl–Sepharose (Pharmacia, Uppsala, Sweden) column in 0.5 M NaOAc/TMG. After this column had been washed with TMG, telomerase was eluted with 1% Triton-X100/TMG and stored at –80°C. For heparin–agarose (Bio-Rad) chromatography, 1 ml S100 was loaded onto a 1 ml pre-equilibrated heparin–agarose column with TMG. The column was washed with TMG and telomerase eluted with 0.2 M NaOAc/TMG. This fraction was loaded onto a 0.5–1 ml DEAE–agarose column in 0.2 M NaOAc/TMG. Elution of telomerase from DEAE and octyl–Sepharose was the same as above. For spermidine–agarose (Sigma, St Louis, MO) chromatography, telomerase from the DEAE column was loaded onto a 1 ml pre-equilibrated spermidine–agarose column in 0.4 M NaOAc/TMG. The column was washed with 0.4 M NaOAc/TMG and telomerase eluted with 0.6 M NaOAc/TMG. Telomerase fractions from this column were buffer-exchanged with a Biospin-6 column (Bio-Rad) into 1% Triton-X100/TMG before use.

Superose-6 column chromatography was as follows: S100 or other telomerase-active fraction was buffer-exchanged to 0.125 KGlu/TMG (twice for S100) and then loaded onto a pre-equilibrated Superose-6 column (Pharmacia) in 0.125 M KGlu/TMG. Fractionation was performed by either FPLC (Pharmacia) or HPLC (Waters) at 0.25 ml/min flow rate. Fractions were collected starting from 6.5 ml after the sample injection and up to a total of 12 ml.

Telomerase assay

Unless stated specifically, all telomerase reactions were carried out in a 20 μl reaction mix containing 50 mM Tris–HCl, pH 8.3, 1.25 mM $MgCl_2$, 5 mM DTT, 100 μM dTTP (ddTTP, or ddGTP), 1.25–2.5 μM [α-^{32}P]dGTP (or [α-^{32}P]dTTP, Amersham, Arlington Heights, IL, or Dupont NEN, Boston, MA, 800 mCi/mmol) and DNA primers (100–250 nM or otherwise specified). Reactions were initiated by addition of

5 µl of enzyme fractions and incubated at 30°C for 10 min (reactions presented in Figure 1 were incubated for 30 min). Incubation of reaction mixes containing double-stranded DNA primer were performed at 20°C. All reactions were terminated with addition of 80 µl TES (50 mM Tris–Cl, pH 8.0, 20 mM EDTA, 0.2% SDS). Reaction products were precipitated by 2.5 volumes ethanol in the presence of 2.5 M ammonium acetate and 20 ng glycogen, and separated on 6–12% denaturing polyacrylamide gels. When required, gels were exposed and quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). DEAE/octyl–Sepharose telomerase fraction was used in assays presented in Figures 1 and 2. Heparin/DEAE/octyl–Sepharose telomerase fraction was used in experiments presented in Figures 4, 5 and 7. S100/Superose fraction was used in the experiments presented in Figure 6.

Analysis of primer specificity

In these analyses, a non-telomeric primer was mixed with a telomeric primer, so both were at a defined concentration in a single telomerase reaction with [α-^{32}P]dGTP and ddTTP. Assays were performed for 10 min, which is within the linear range of the reaction (data not shown). Extension products were separated on a denaturing gel, identified and quantified (as a measure of the velocity of each reaction), to obtain a ratio of products (primer+dGGGGddT), the reaction rate, from each primer. After adjusting this ratio to take into account the concentration of each primer used, a number was obtained for the relative specificity (ratio of K_{cat}/K_m from two substrates) of the two competing substrates at equal concentration (Fersht, 1985).

Native gel analysis of duplex DNA substrates

After hybridization, each set of two complementary DNA oligonucleotides was analyzed by electrophoresis in a 12% polyacrylamide gel in TBM (50 mM Tris–Borate, pH 8.3, 1 mM $MgCl_2$) at 60 V for 10–12 h, at 20°C.

Acknowledgements

We thank Anat Krauskopf, Jagoree Roy and David Gilley for helpful comments on the manuscript. H.W. was supported by a National Research Service Award from the NIH. This work was supported by grant GM26259 from the NIH to E.H.B.

References

Avilion,A.A., Harrington,L.A. and Greider,C.W. (1992) *Tetrahymena* telomerase RNA levels increase during macronuclear development. *Dev. Genet.*, **13**, 80–86.

Baird,S.E. and Klobutcher,L.A. (1989) Characterization of chromosome fragmentation in two protozoans and identification of a candidate fragmentation sequence in *Euplotes crassus*. *Genes Dev.*, **3**, 585–597.

Blackburn,E.H. (1992) Telomerases. *Annu. Rev. Biochem.*, **61**, 113–129.

Blackburn,E.H. (1994) Telomeres: no end in sight. *Cell*, **77**, 621–623.

Blackburn,E.H. (1995) Developmentally programmed healing of chromosomes. In Blackburn,E.H. and Greider,C.W. (eds), *Telomeres*. Cold Spring Harbor Laboratory Press, Plainview, New York, pp. 193–218.

Blackburn,E.H., Greider,C.W., Henderson,E., Lee,M.S., Shampay,J. and Shippen-Lentz,D. (1989) Recognition and elongation of telomeres by telomerase. *Genome*, **31**, 553–560.

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.

Fan,Q.C. and Yao,M.-C. (1996) New telomere formation coupled with site-specific chromosome breakage in *Tetrahymena thermophila*. *Mol. Cell. Biol.*, **16**, 1267–1274.

Fang,G. and Cech,T.R. (1995) Telomere proteins. In Blackburn,E.H. and Greider,C.W. (eds), *Telomeres*. Cold Spring Harbor Laboratory Press, Plainview, New York, pp. 69–105.

Fersht,A. (1985) *Enzyme Structure and Mechanism*. W.H.Freeman, New York.

Flint,J. (1994) Healing of broken human chromosomes by the addition of telomeric repeats. *Am. J. Hum. Genet.*, **55**, 505–512.

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., 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, Plainview, New York, pp. 35–68.

Greider,C.W., and Blackburn,E.H. (1987) The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell*, **51**, 887–898.

Harrington,L.A. and Greider,C.W. (1991) Telomerase primer specificity and chromosome healing. *Nature*, **353**, 451–454.

Henderson,E. (1995) Telomere DNA structure. In Blackburn,E.H. and Greider,C.W. (eds), *Telomeres*. Cold Spring Harbor Laboratory Press, Plainview, New York, pp. 11–34.

King,B.O. and Yao,M.-C. (1982) Tandemly repeated hexanucleotide at *Tetrahymena* rDNA free end is generated from a single copy during development. *Cell*, **31**, 177–182.

Klobutcher,L.A., Swanton,M.T., Donini,P. and Prescott,D.M. (1981) All gene sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3′ terminus. *Proc. Natl Acad. Sci. USA*, **78**, 3015–3019.

Kramer,K.M. and Haber,J.E. (1993) New telomeres in yeast are initiated with a highly selected subset of $TG_{1–3}$ repeats. *Genes Dev.*, **7**, 2345–2356.

Lamb,J., Harris,P.C., Wilkie,A.O.M., Wood,W.G., Dauwerse,J.G. and Higgs,D.R. (1993) De novo truncation of chromosome 16p and healing with (TTAGGG)n in the α-thalassemia/mental retardation syndrome (ATR-16). *Am. J. Hum. Genet.*, **52**, 668–676.

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., and Gallagher,R.C., Bradley,J. and Blackburn,E.H. (1993) In vivo and *in vitro* studies of telomeres and telomerase. *Cold Spring Harbor Symp. Quant. Biol.*, **58**, 707–718.

Lingner,J. and Cech,T.R. (1996) Purification of telomerase from *Euplotes aediculatus*: requirement of a primer 3′ overhang. *Proc. Natl Acad. Sci. USA*, in press.

Martindale,D.W., Allis,C.D. and Bruns,P.J. (1982) Conjugation in *Tetrahymena thermophila*. *Exp. Cell Res.*, **140**, 227–236.

McClintock,B. (1941) The stability of broken ends of chromosomes in *Zea mays*. *Genetics*, **26**, 234–282.

Melek,M. and Shippen,D.E. (1996) Chromosome healing: spontaneous and programmed *de novo* telomere formation by telomerase. *BioEssays*, **18**, 301–308.

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. (1991) Recognition of a chromosome truncation site associated with α-thalassaemia by human telomerase. *Nature*, **353**, 454–456.

Müller,F., Wicky,C., Spicher,A. and Tobler,H. (1991) New telomere formation after developmentally regulated chromosomal breakage during the process of chromatin diminution in *Ascaris lumbricoides*. *Cell*, **67**, 815–822.

Murray,A.W., Claus,T.E. and Szostak,J.W. (1989) Characterization of two telomeric DNA processing reactions in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **8**, 4642–4650.

Nevinsky,G.A., Veniaminova,A.G., Levina,A.S., Podust,V.N., Lavrik,O.I. and Holler,E. (1990) Structure-function analysis of mononucleotides and short oligonucleotides in the priming of enzymatic DNA synthesis. *Biochemistry*, **29**, 1200–1207.

Pologe,L.G. and Ravetch,J.V. (1988) Large deletions result from breakage and healing of *P. falciparum* chromosomes. *Cell*, **55**, 869–874.

Prescott,J. and Blackburn,E.H. (1997) Telomerase RNA mutations in *Saccharomyces cerevisiae* alter telomerase action and reveal non-processivity *in vivo* and *in vitro*. *Genes Dev.*, in press.

Price,C.M., Adams,A.K. and Vermeesch,J.R. (1994) Accumulation of telomerase RNA and telomere protein transcripts during telomere synthesis in *Euplotes*. *J. Eukaryotic Microbiol.*, **41**, 267–275.

Sandell,L.L. and Zakian,V.A. (1993) Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell*, **75**, 729–739.

Scherf,A., Carter,R., Petersen,C., Alano,P., Nelson,R., Aikawa,M., Mattei,D., de Silva,L.P. and Leech,J. (1992) Gene inactivation of PF11-1 of *Plasmodium falciparum* by chromosome breakage and

healing: identification of a gametocyte-specific protein with a potential role in gametogenesis. *EMBO J.*, **11**, 2293–2301.
Shampay,J. and Blackburn,E.H. (1989) *Tetrahymena* micronuclear sequences that function as telomeres in yeast. *Nucleic Acids Res.*, **17**, 3247–3260.
Shampay,J., Szostak,J.W. and Blackburn,E.H. (1984) DNA sequences of telomeres maintained in yeast. *Nature*, **310**, 154–157.
Spangler,E.A., Ryan,T. and Blackburn,E.H. (1988) Developmentally regulated telomere addition in *Tetrahymena thermophila*. *Nucleic Acids Res.*, **16**, 5569–5585.
White,C.I. and Haber,J.E. (1990) Intermediates of recombination during mating type switching *Saccharomyces cerevisiae*. *EMBO J.*, **9**, 663–674.
Wilkie,A.D.M., Lamb,J., Harris,P.C., Finney,R.D. and Higgs,D.R. (1990) A truncated human chromosome 16 associated with α-thalassaemia is stabilized by addition of telomeric repeat (TTAGGG)n. *Nature*, **346**, 868–871.
Yao,M.-C., Zheng,K. and Yao,C.-H. (1987) A conserved nucleotide sequence at the sites of developmentally regulated chromosomal breakage in *Tetrahymena*. *Cell*, **48**, 779–788.
Yao,M.-C., Yao,C.-H. and Monks,B. (1990) The controlling sequence for site-specific chromosome breakage in *Tetrahymena*. *Cell*, **63**, 763–772.
Yokoyama,R. and Yao,M.-C. (1986) Sequence characterization of *Tetrahymena* macronuclear DNA ends. *Nucleic Acids Res.*, **14**, 2109–2122.
Yu,G.L. and Blackburn,E.H. (1991) Developmentally programmed healing of chromosomes by telomerase in *Tetrahymena*. *Cell*, **67**, 823–832.
Zakian,V.A. (1989) Structure and function of telomeres. *Annu. Rev. Genet.*, **23**, 579–604.
Zakian,V.A. (1995) Telomeres: beginning to understand the end. *Science*, **270**, 1601–1607.

Received on August 8, 1996; revised on September 27, 1996