21.7.2 Recognition and elongation of telomeres by telomerase

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Telomeres stabilize chromosomal ends and allow their complete replication *in vivo*. In diverse eukaryotes, the essential telomeric DNA sequence consists of variable numbers of tandem repeats of simple, G+C rich sequences, with a strong strand bias of G residues on the strand oriented 5′ to 3′ toward the chromosomal terminus. This strand forms a protruding 3′ overhang at the chromosomal terminus in three different eukaryotes analyzed. Analysis of yeast and protozoan telomeres showed that telomeres are dynamic structures *in vivo*, being acted on by shortening and lengthening activities. We previously identified and partially purified an enzymatic activity, telomere terminal transferase, or telomerase, from the ciliate *Tetrahymena*. Telomerase is a ribonucleoprotein enzyme with essential RNA and protein components. This activity adds repeats of the *Tetrahymena* telomeric sequence, TTGGGG, onto the 3′ end of a single-stranded DNA primer consisting of a few repeats of the G-rich strand of known telomeric, and telomere-like, sequences. The shortest oligonucleotide active as a primer was the decamer $G_4T_2G_4$. Structural analysis of synthetic DNA oligonucleotides that are active as primers showed that they all formed discrete intramolecular foldback structures at temperatures below 40°C. Addition of TTGGGG repeats occurs one nucleotide at a time by *de novo* synthesis, which is not templated by the DNA primer. Up to 8000 nucleotides of G_4T_2 repeats were added to the primer *in vitro*. We discuss the implications of this finding for regulation of telomerase *in vivo* and a model for telomere elongation by telomerase.

Key words: chromosome telomeres, telomerase, oligonucleotide repeats.

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Les télomères stabilisent les extrémités des chromosomes et permettent leur reproduction complète *in vivo*. Chez divers eucaryotes, la séquence d'ADN télomérique est essentiellement constituée d'un nombre variable de répétitions en tandem de séquences simples, riches en G + C, avec un brin fortement biaisé constitué de résidus G sur le brin orienté en 5′–3′, vers l'extrémité chromosomique. Ce brin forme une protubérance en surplomb de 3′ à l'extrémité chromosomique chez trois eucaryotes différents soumis à l'analyse. L'analyse de télomères de protozoaires et de levures a montré que les télomères sont des structures dynamiques *in vivo*, étant l'objet d'activités de racourcissement et d'allongement. Nous avons antérieurement identifié une activité enzymatique et partiellement purifié la transférase du télomère terminal ou télomérase chez des *Tetrahymena* ciliés. La télomérase est une enzyme ribonucléoprotéique, avec des composés essentiels des protéines et de l'ARN. L'activité de cette enzyme ajoute des répétitions à la séquence télomérique TTGGGG de *Tetrahymena* jusqu'à l'extrémité 3′ de l'initiateur d'un seul brin d'ADN, constituée de quelques répétitions du brin riche en G, de séquences télomériques ou de nature télomérique connues. L'oligonucléotide le plus court en tant qu'initiateur a été le décamère $G_4T_2G_4$. L'analyse de la structure des oligonucléotides synthétiques d'ADN qui sont actifs comme initiateus a montré que chacun d'eux a formé des structures intramoléculaires discrètes, repliées, à des températures inférieures à 40°C. L'addition de répétitions TTGGGG procède, un nucléotide à la fois, par synthèse *de novo* qui ne résulte pas de l'initiateur d'ADN. Jusqu'à 8000 nucléotides de répétition G_4T_2 ont été ajoutés à l'initiateur *in vitro*. Les implications de ces travaux sont discutées en fonction de la régulation de la télomérase *in vivo*, ainsi que d'un modèle pour l'élongation des télomères par la télomérase.

Mots clés : télomères chromosomiques, télomérase, répétitions des oligonucléotides.

[Traduit par la revue]

Introduction

Telomeres, the ends of eukaryotic chromosomes, are specialized structures that stabilize chromosomes by allowing their complete replication, and by preventing inappropriate recombination or loss by degradation of the chromosomal ends (reviewed in Blackburn and Szostak, 1984). The essential DNA sequences of telomeres are tandem repeats of short G+C rich sequences, which have as an evolutionarily conserved feature a strand composition bias, resulting in distinctly G- and C-rich strands. The G-rich strand is always oriented so that the 5′ to 3′ polarity is toward the chromosomal terminus (for reviews, see Blackburn, 1984; Henderson *et al.* 1987; and Forney *et al.* 1987).

We have previously analyzed the structure and function of telomeres *in vivo* and *in vitro*. *In vivo*, telomeres are dynamic structures that undergo lengthening and shortening (Bernards *et al.* 1983; Larson *et al.* 1987; Shampay and Blackburn 1988). The changes in length involve increases or decreases in the numbers of the short tandem repeats that comprise the essential telomeric DNA sequences (Walmsley and Petes 1985; Larson *et al.* 1987; Henderson *et al.* 1988). The telomeres of the ciliate *Tetrahymena thermophila* consist of tandem repeats of the hexanucleotide d(TTGGGG) · d(CCCCAA) (Blackburn and Gall 1978; Yao *et al.* 1987; Spangler *et al.* 1988), abbreviated as G_4T_2 repeats, and lengthen or shorten according to the physiological and developmental state of the cell (Larson *et al.* 1987). In yeast, which has telomeric repeats of the general formula $G_{1-3}T$ (Shampay *et al.* 1984), individual telomeres have been studied in clones of haploid cells and

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shown to exhibit microheterogeneity in length (Shampay and Blackburn 1988).

To account for the lengthening of telomeres *in vivo*, we previously proposed that a mechanism exists for nontemplated addition of telomeric repeats to telomeric DNA (Shampay *et al.* 1984). This model was prompted by the observation that *Tetrahymena* telomeres, when introduced into yeast cells, stabilize the ends of linear plasmids or chromosomes and become elongated by the addition of yeast repeats (Szostak and Blackburn 1982; Walmsley *et al.* 1983; Shampay *et al.* 1984). Similar findings were made when the telomeres of a different ciliate, *Oxytrichia*, were introduced into yeast cells (Pluta *et al.* 1984). Strong direct evidence for a nontemplated addition model came from the isolation of a novel enzyme activity, telomere terminal transferase (abbreviated telomerase) from *Tetrahymena* cell extracts (Greider and Blackburn 1985, 1987).

Telomerase is a ribonucleoprotein enzyme that elongates synthetic or purified natural telomeres *in vivo* (Greider and Blackburn 1985, 1987; Henderson and Blackburn 1989). The specificity of telomerase for DNA sequences that can prime elongation has been studied using synthetic oligonucleotide sequences (Greider and Blackburn 1985, 1987). It was found that DNA oligonucleotides with sequences consisting of two or more repeats of the G-rich strand of known telomeric sequences were active as primers in this assay, whereas control oligonucleotides were not.

While a variety of different G-rich telomere sequence primers all primed elongation, the repeated sequences added were *Tetrahymena* GGGGTT repeats, regardless of the primer sequence. These results argue that, first, telomerase recognition of active primers must reflect common structural feature(s) of the G-rich telomere sequence primers, rather than DNA sequence-specific binding. Hence, the specificity of telomerase for oligonucleotide primers of different telomeric sequences is remarkable because it does not appear to be a sequence specificity analogous to that seen, for example, when a transcription factor binds a specific sequence in duplex DNA. Second, the properties of telomerase itself must dictate the specificity of the DNA sequence that is added, rather than the sequence information being provided by the primer.

We describe here further analysis of the action of the *Tetrahymena* telomerase *in vitro*, and of the *in vivo* behavior of telomeres in yeast. In testing the telomerase of *Tetrahymena* with new primers we find additional evidence for a bipartite recognition of primers by telomerase: a sequence-specific recognition of the 3′ end sequence of the primer, and a structural recognition of the class of G-rich telomere sequences. In yeast, individual chromosomal telomeres can be analyzed, providing a situation in which the action of telomerase might be evident from direct examination of telomeres *in vivo*. The effect of the yeast genetic background on the length variability of yeast telomeres *in vivo* was investigated. The behavior of these telomeres is consistent with the notion that a yeast telomerase with similar properties plays a role in telomere maintenance in this and probably other organisms as well.

Materials and methods

DNA oligonucleotides

DNA oligonucleotides were synthesized using an Applied Biosystems automated DNA synthesizer, purified, and radiolabelled as described previously (Henderson *et al.* 1987; Henderson and Blackburn 1989). The oligonucleotide $G_4T_2G_4$ was provided by C. Hardin and I. Tinoco, Jr. For conformational studies, polyacrylamide gel electrophoresis under nondenaturing conditions was carried out as described previously (Henderson *et al.* 1987).

Telomerase preparation and assays

Fractionated *Tetrahymena* cell extracts containing telomerase activity were prepared as described previously (Greider and Blackburn 1987). Sephacryl S-500 (Pharmacia) gel filtration chromatography of S-100 fractions was followed by heparin agarose chromatography. Active fractions, eluted from the heparin agarose column, were concentrated by pelleting the telomerase by centrifugation in a Beckman TL-100 tabletop ultracentrifuge in a TLA-100 fixed angle rotor at 100 000 rpm ($\sim$400 000 $\times$ *g*) for 1 h at 4°C. Pellets were resuspended in TMG buffer (10 mM Tris–HCl, pH 8.5, 1 mM $MgCl_2$, and 10% glycerol) and stored in liquid N_2. Enzyme reactions in which different oligonucleotides were tested as primers for telomerase were done as described by Greider and Blackburn (1987), except that 0.1 μg of each oligonucleotide was heated to 95°C for 1 min and then cooled to 30°C before adding to the reaction mix (total volume, 20 μL for $G_4T_2G_4$, and 40 μL for the other oligonucleotides).

Alkaline agarose gel electrophoresis

Telomerase reaction products were fractionated by alkaline agarose gel electrophoresis as described by McDonell *et al.* (1977). Reaction products were analyzed at different time points during a standard reaction, using 0.1 μg $(T_2G_4)_4$ primer/40 μL reaction volume (Greider and Blackburn 1987).

Analysis of telomere length in yeast

Length variation of individual chromosomal telomeres in clones of haploid *Saccharomyces cerevisiae* was analyzed as described previously (Shampay and Blackburn 1988). Strains A364A and *cdc*17-1 were provided by D. Schild, Yeast Genetic Stock Center, Lawrence Berkeley Laboratory (Berkeley, CA). To measure average telomere lengths of the A364A and the *cdc*17-1 strains after prolonged growth at either 25 or 30°C, a portion of a single colony of either A364 or *cdc*17-1 cells was inoculated into YPD medium and maintained in log phase growth at 30°C for 50 generations by transfers of large aliquots into fresh YPD medium. Another portion of the same colony was grown in parallel for the same number of generations, but growth was at 25°C. DNA was prepared from cells harvested at the end of these growth periods, and telomere length analyzed as described previously (Shampay and Blackburn 1988).

Results

Ability of telomeric and telomere-like DNA sequences to prime telomerase

We showed previously that G-rich DNA oligonucleotides consisting of a few repeats of five different known telomeric sequences primed the addition of T_2G_4 repeats by the telomerase activity of *Tetrahymena in vitro* (Greider and Blackburn 1987). In these experiments, telomerase was incubated with the deoxynucleoside triphosphates dTTP and dGTP, Mg^{2+} ion, salts, and the oligonucleotide to be tested as a primer. Furthermore, we showed that these active DNA oligonucleotides, but not those inactive as primers for telomerase, assumed foldback secondary structures stabilized by non-Watson-Crick, G-G base pairing (Henderson *et al.* 1987). That these are intramolecular foldback forms rather than intermolecular associations is shown in Fig. 1 for an oligonucleotide, $(T_2G_4)_4$, which is active as a primer. The pattern of base-paired conformations seen in nondenaturing gel electrophoresis (Henderson *et al.* 1987) is independent of oligonucleotide concentration. The gel whose autoradiogram is shown in Fig. 1 was a nondenaturing 12% polyacrylamide gel run at 5°C, as described previously (Henderson *et al.* 1987). The DNA concentration in the gel shown in Fig. 1 was varied

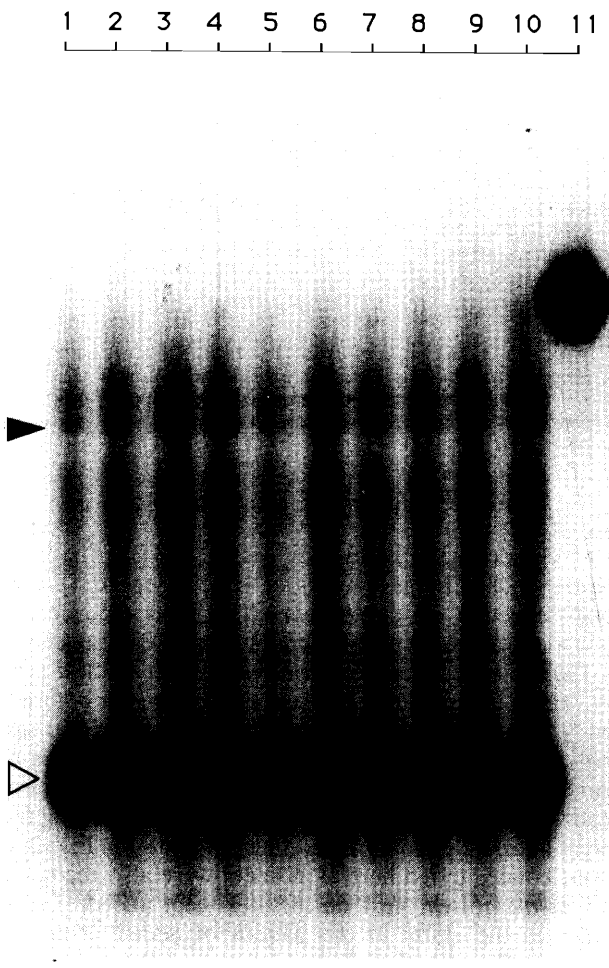

FIG. 1. Analysis of the effect of concentration on conformer formation of the oligomer $d(T_2G_4)_4$. Each lane of the nondenaturing 12% polyacrylamide gel contained 0.125 ng of oligodeoxyribonucleotide, ^{32}P-labeled at the 5′ end. Unlabeled $d(T_2G_4)_4$ was added to give final concentrations (in ng per 4 μL) of 0.125 (lane 1), 0.5 (lane 2), 1 (lane 3), 5 (lane 4), 10 (lane 5), 50 (lane 6), 100 (lane 7), 250 (lane 8), 500 (lane 9), and 1000 (lane 10). Lane 11 contained radiolabeled $d(T_2G_4)_4$ hybridized to the unlabeled, complementary, 24-base oligomer $d(C_4A_2)_4$, as a 24 base pair duplex marker. The gel was run at 7.5 V/cm at 5°C as described by Henderson *et al.* (1987). The bromphenol blue dye is marked with a solid arrowhead and the hairpin form of $d(T_2G_4)_4$ (Henderson *et al.* 1987), with an open arrowhead.

in the different lanes. The pattern of conformers, including the major, rapidly migrating, foldback form (open arrowhead; see Henderson *et al.*, 1987), is unchanged over an 8000-fold DNA concentration range.

To explore further the recognition of telomeric sequences by telomerase, we have tested several other DNA oligonucleo-

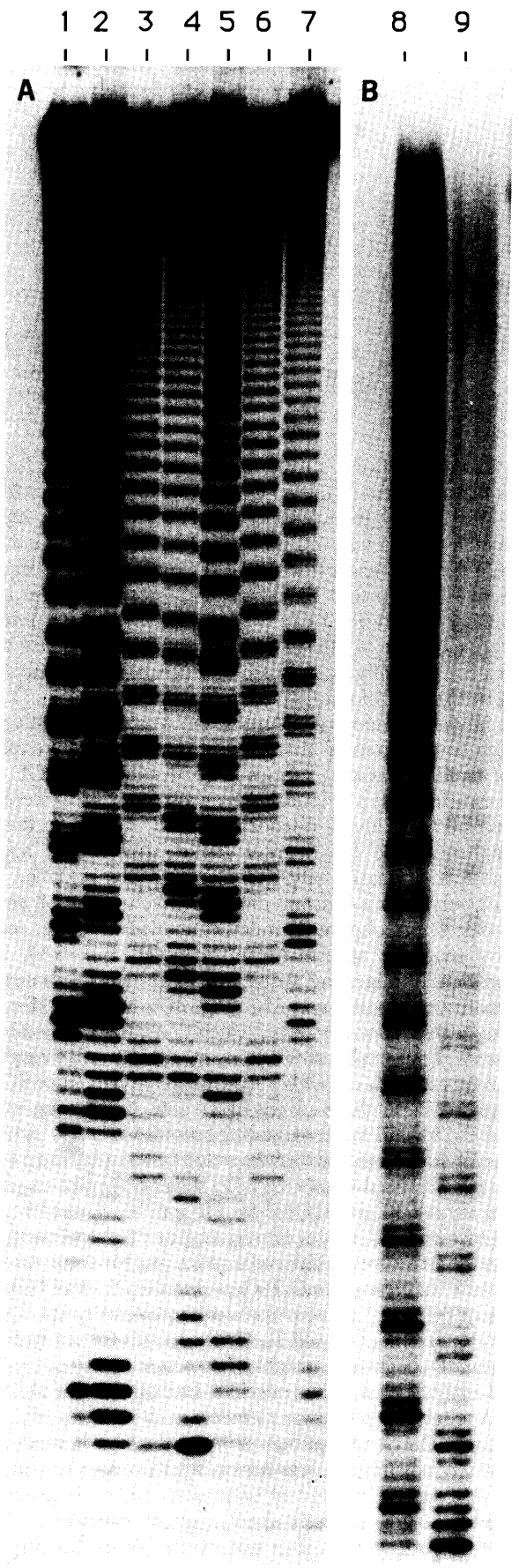

FIG. 2. Priming and 3′ end recognition of different DNA oligomers. Each oligomer primer was incubated with *Tetrahymena* telomerase as described previously (Greider and Blackburn 1987) and in Materials and methods. The reaction products were analyzed by electrophoresis on a denaturing 8 (Fig. 2A) or 6% (Fig. 2B) polyacrylamide DNA sequencing gel. Primers in Fig. 2A: lane 1, $(T_2G_4)_3$; lane 2, $G(T_2G_4)_2G_3$; lane 3, $(G_4T_2)_3$; lane 4, $(TG)_9$; lane 5, $(G_2T_2)_4G_2$; lane 6, $(T_3G_3)_3$; and lane 7, $T_3G_5T_5G_5$. Fig. 2B: lane 8, $(T_2G_4)_4$; and lane 9, $G_4T_2G_4$.

TABLE 1. Priming and 3′ end recognition by G-rich oligomers

Primer	Sequence and added nucleotides	Shift	Lane
$(TTGGGG)_3$	TTGGGGTTGGGGTTGGGGttggggttggggtt...	0	1
$G(TTGGGG)_2TTGGG$	GTTGGGGTTGGGGTTGGGgttggggttggggtt...	+1	2
$(GGGGTT)_3$	GGGGTTGGGGTTGGGGTTggggttggggtt...	+4	3
$(TG)_9$	TGTGTGTGTGTGTGTGTGgggttggggttggg...	+3	4
$(GGTT)_4$	GGTTGGTTGGTTGGTTGGggttggggttgggg...	+2	5
$(GGGTTT)_3$	GGGTTTGGGTTTGGGTTTggggttggggttgg...	+4	6
$T_3G_5T_5G_5$	TTTGGGGGTTTTTGGGGGttggggttggggtt...	0	7
$(TTGGGG)_4$	TTGGGGTTGGGGTTGGGGTTGGGGttggggtt...	0	8
$G_4T_2G_4$	GGGGTTGGGGttggggttggggttggggtt...	+4	9

NOTE: DNA sequences are shown 5′ to 3′, reading from left to right. The primer sequence is in capitals and the sequence of the first nucleotides inferred to be added to each primer by the *Tetrahymena* telomerase is shown in lower case letters. Lanes are those shown in Fig. 2. Shifts are those observed in the 6-base periodic patterns of bands in Fig. 2, relative to the $(T_2G_4)_3$ primer in Fig. 2A for lanes 2–7, or relative to the $(T_2G_4)_4$ primer for lane 9 in Fig. 2B.

tides for their ability to prime telomerase efficiently. Table 1 shows the sequences of these DNA oligonucleotides. Of the 18 nucleotide long oligonucleotides, short tracts of T_3G_3 repeats (oligonucleotide in lane 6, Fig. 2; Table 1) are found randomly intermingled with short tracts of G_4T_2 repeats in *Paramecium* telomeres (Forney and Blackburn 1988); none of the other 18-mer sequences in Table 1 are known to be telomeric sequences of any organism. As shown in Fig. 2, all these sequences efficiently primed the addition of T_2G_4 repeats by the *Tetrahymena* telomerase.

The ability of these nonduplex DNA oligonucleotides to undergo conformational change was assessed by their formation of rapidly migrating species in nondenaturing PAGE. The nondenaturing polyacrylamide gels shown in Fig. 3 were run as described previously (Henderson *et al.* 1987), so that the bromophenol blue dye marker (solid arrowhead), and an oligonucleotide unaffected by temperature, the hexamer T_2G_4 (arrow), were run a constant distance. Each of the 18 and 10 nucleotide DNA oligonucleotides contained a single, major, primary sequence species (denaturing gel, Fig. 3A). Each formed rapidly migrating species upon gel electrophoresis in nondenaturing conditions at 5°C (Fig. 3C). Multiple, discrete, fast-migrating species were clearly visible for all the oligomers. Each had a single, major, rapidly migrating species, except for $(TG)_9$, $(GGTT)_4GG$, and $(T_3G_3)_3$ (Fig. 3C). In the case of these three oligomers, some broadening of the major band toward faster-migrating species was seen, although with only minor amounts of discrete, faster-migrating forms (lanes 1–3). The decamer $G_4T_2G_4$ formed rapidly migrating species at both 30 and 5°C (Figs. 3B and 3C).Therefore, like the 18-mer primers, this decamer, despite its short length, can assume the structure we believe is necessary for recognition by telomerase. In contrast to $G_4T_2G_4$ and to the 18-mer $(TG)_9$, the decamer $(TG)_5$ was not active as a primer. As described previously (Henderson *et al.* 1987), $(TG)_5$ did not form any detectable, rapidly migrating, foldback structure.

These results extend our previous finding that the ability of a DNA oligomer to prime telomerase correlates well with formation of fast-migrating conformations, which become particularly apparent at low temperature.

Recognition of the 3′ end sequence of DNA primers

As described previously, telomerase from *Tetrahymena* recognizes the 3′ end sequence of a telomere sequence primer and adds the appropriate next nucleotide to complete a G_4T_2 hexamer repeat. This was shown with DNA oligomers consisting of repeats of T_2G_4, T_2AG_3, and the yeast telomeric repeat (Greider and Blackburn 1985, 1987). The initial nucleotides added in these reactions varied, depending on the 3′ end sequence of the primer. This was evident from examination of the elongation products on DNA sequencing gels. The products form a ladder of bands with a 6-base periodicity, which is caused by preferential pausing at or before the added T residues. Thus, for two input primers of the same length, a relative upward shift in the pattern of bands in one primer compared with another with a different 3′ end sequence indicates a shift in the register of added repeats, resulting from different nucleotides being added first. As shown in Fig. 2, the oligonucleotides tested here as primers were also recognized such that the correct next nucleotides were added. Table 1 summarizes the shifts in the pattern of added nucleotides observed in Fig. 1 and the interpretation of these shifts.

Length of the telomere repeat addition products synthesized by telomerase

As seen in Fig. 2, a significant fraction of the products of telomerase activity accumulate at the top of the 6 or 8% polyacryamide DNA sequencing gels we typically use for analysis of the products of the activity. To determine the size of these larger products, and to assess the time course of the telomerase elongation reaction, we analyzed aliquots taken at different time points from a reaction, and displayed them using alkaline agarose gel electrophoresis in order to resolve large (>400 nucleotide) products. Fig. 4 shows a typical analysis. Up to 8000 nucleotides of T_2G_4 repeats were added to the 24-nucleotide input primer in this reaction. Both the maximum length of products and the range of product sizes continued to increase during the 120-min reaction; in addition, the mean size of the products increased as a function of time of reaction for at least 30 min. The kinetics and processivity of the telomerase reaction are currently being investigated further.

Elongation of yeast telomeres in vivo

We have previously described the length microheterogeneity of telomeres in yeast (Shampay and Blackburn 1988). The mean lengths of individual chromosomal telomeres were shown to vary by up to a few hundred base pairs in different clones of a given haploid strain of yeast (Shampay and Blackburn 1988). This variation in mean telomere size results from length heterogeneity which is generated in the replicative descendents of a single telomere molecule, and can be detected in less than 30 cell generations. An example of this variation is shown in Fig. 5 for two different strains, A364A and a

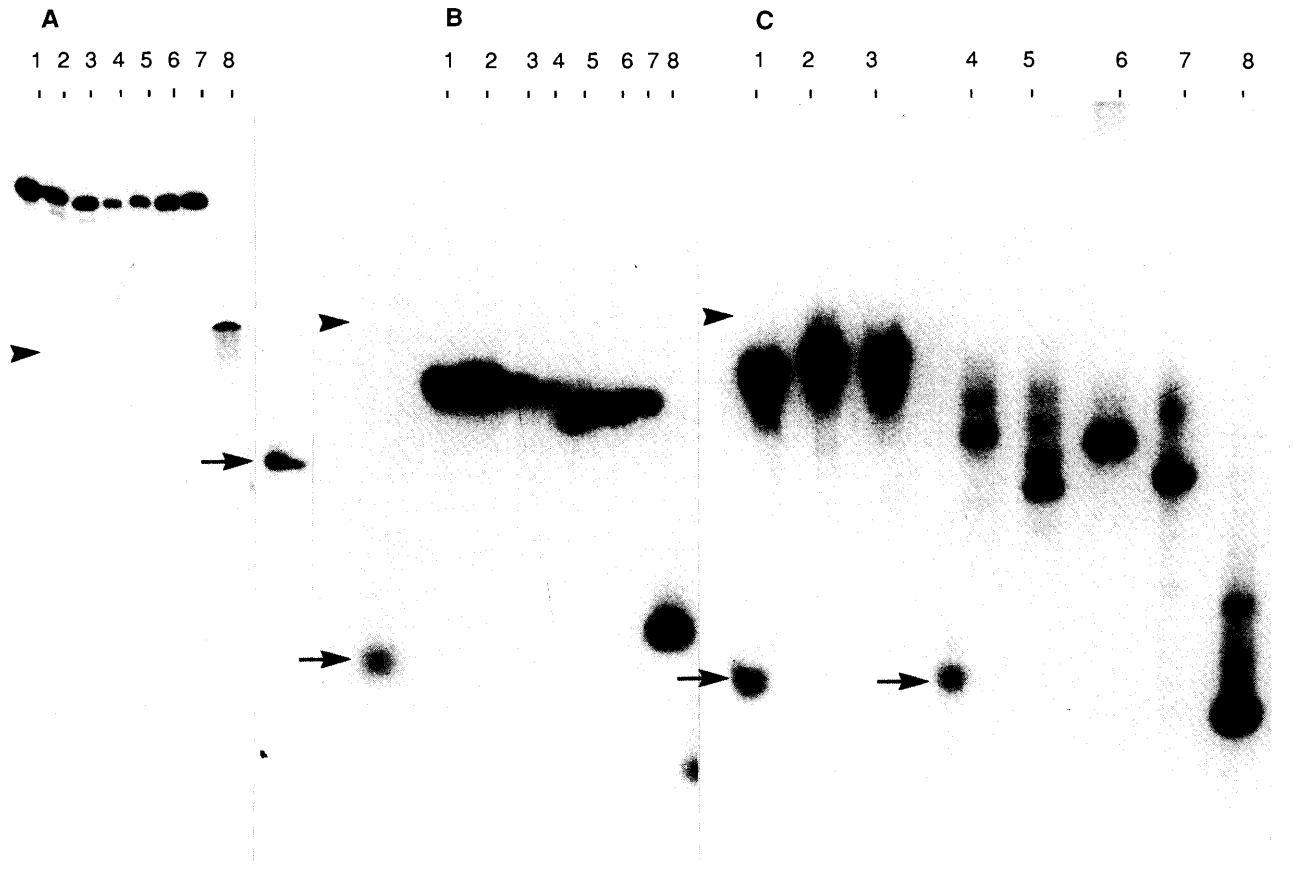

FIG. 3. Analysis of conformer formation by DNA oligomers with repeated telomeric and telomere-like sequences. Fig. 3A shows a 12% polyacrylamide DNA sequencing gel, containing 8 M urea and run under denaturing conditions. Figs. 3B and 3C show nondenaturing, 12% polyacrylamide gels run at 7.5 V/cm at either 30 (B) or 5°C (C). The arrowhead shows the position of the bromphenol blue dye marker, which was run to a constant distance (24 cm) for each gel. Arrows indicate the hexanucleotide marker T_2G_4, whose electrophoretic mobility is unaffected by temperature under nondenaturing conditions (Henderson *et al.* 1987). Lane 1, $(T_2G_4)_3$; lane 2, $G(T_2G_4)_2G_3$; lane 3, $(G_4T_2)_3$; lane 4, $(TG)_9$; lane 5, $(G_2T_2)_4G_2$; lane 6, $(T_3G_3)_3$; lane 7, $T_3G_5T_5G_5$; and lane 8, $G_4T_2G_4$.

temperature sensitive mutant, *cdc*17-1. The A364A and the *cdc*17-1 strains were each grown at a temperature semipermissive for *cdc*17-1 growth. Carson and Hartwell (1985) have shown previously that under these growth conditions, *cdc*17 strains exhibit slow elongation of their telomeres in the course of prolonged cell divisions. As shown in Fig. 5, telomere length heterogeneity and clonal variability seen after ~30 cell divisions were as pronounced in the *cdc*17-1 cells dividing at the semipermissive temperature (30°C) as in those dividing at 25°C. The increased length of telomeric fragments observed in *cdc*17-1 at higher temperatures would not be significant in the time frame of this experiment. The degree of heterogeneity was also indistinguishable from that in strain A364A. We confirmed that, as expected (Carson and Hartwell 1985), upon prolonged cell growth (~50 cell generations) at 30°C, the average length of telomeres in the *cdc*17-1 strain used in these experiments was greater than that seen after prolonged cell growth at 25°C (data not shown). We conclude that any change in the *cdc*17-1 gene activity at the semipermissive temperature has no discernible effect on the generation of telomere length heterogeneity under these conditions.

Discussion

Telomere recognition and elongation in vitro

We have extended the known range of DNA sequences that are recognized *in vitro* by the telomerase of *Tetrahymena*. Several repeated, telomere-like sequences, not currently identified in telomeres in nature, were active as primers for telomerase, and formed rapidly migrating conformations stabilized by low temperatures (Table 1; Figs. 2 and 3). These sequences include the alternating sequence $(TG)_9$ and the decamer $G_4T_2G_4$. However we showed previously that another decamer, $(TG)_5$, was not active as a primer for telomerase (Greider and Blackburn 1987). On nondenaturing PAGE $(TG)_5$ did not form any fast-migrating species, suggesting that it cannot form a G · G base-paired foldback structure like that found in active primers (Henderson *et al.* 1987). In contrast, as shown in Fig. 3C, a rapidly migrating species was observed at low temperature as the major component of the DNA oligomer $G_4T_2G_4$, and as a minor component of $(TG)_9$. These findings are consistent with the notion that the failure of $(TG)_5$ to prime telomerase is not because telomerase cannot recognize such a short (10 nucleotide) substrate, but rather because $(TG)_5$ cannot assume a sufficiently stable G · G base-paired structure. These findings extend our previous conclusion that the ability to prime telomerase correlates with the formation of G · G base-paired secondary structures.

Recognition of short G-rich telomeric sequences by telomerase is relevant to the situation *in vivo*. The natural telomeres of hypotrichous ciliates have 16- or 14-base 3′ overhangs of the G-rich strand (Klobutcher *et al.* 1981; Pluta *et al.*

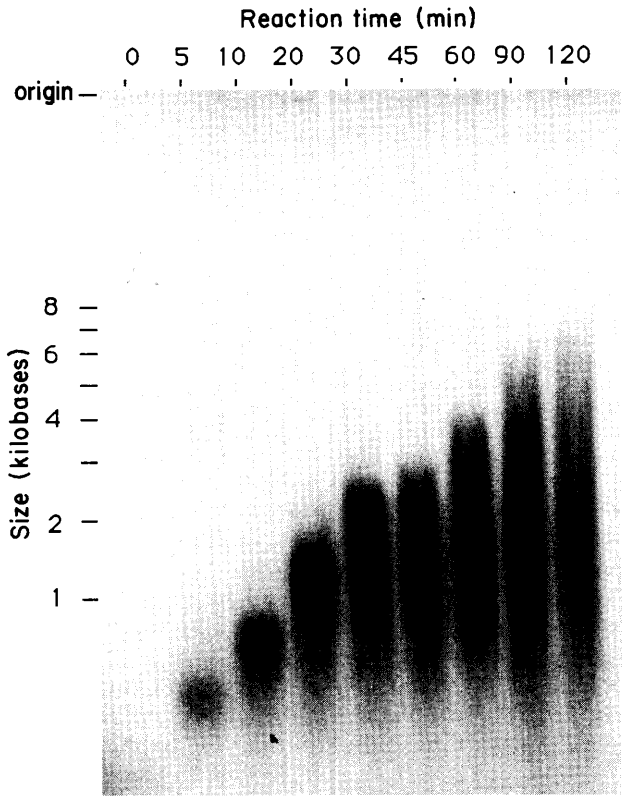

FIG. 4. Time course of telomere sequence addition by *Tetrahymena* telomerase. Radioactive addition products of a telomerase reaction primed by $(T_2G_4)_4$ were fractionated by alkaline agarose gel electrophoresis. Aliquots of a standard telomerase reaction (see Materials and methods) were taken at times 0, 5, 10, 20, 30, 45, 60, 90, and 120 min. Bars indicate single-stranded DNA marker fragments.

1982). Similarly, those of *Tetrahymena* and the slime mold *Didimium* have 12–16 base 3′ overhangs of the G-rich strand (Henderson and Blackburn 1989). Our work with synthetic, G-rich telomere sequence oligonucleotides as short or shorter than these overhangs provides good evidence that such overhangs are capable of forming G · G paired structures, and thus could be substrates for telomerase *in vivo*.

Specificity of sequence addition by telomerase

The ability of the *Tetrahymena* telomerase to add GGGGTT repeats to primers of differing sequences shows that the telomerase itself defines the sequence added, rather than that information being provided by the primer. Nevertheless, the 3′ end sequence of the primer dictates the next nucleotides added by telomerase, in a manner that always leads to completion of the first G_4T_2 repeat.

An active site of the *Tetrahymena* telomerase can be envisaged as having four G binding sites and two T binding sites. The arrangement of these sites could in theory be determined by an array of sites on a protein component, or as a complementary sequence on the RNA component of telomerase. Tests with various primers in the current and previous work allow us to conclude that the 3′ end recognition sequence must include at least the four nucleotides at the 3′ end of the primer, as indicated in Table 1. An 18-mer or 24-mer with a TGGG 3′ end is distinguished by telomerase from an 18-mer with a ·· G4 or ·· G5 (which appears to be equivalent to G4) sequence at the 3′ end, and also from an 18-mer with a · GGTTGG 3′ end. Taken together, our past results and this paper suggest a model for telomerase, as shown in Fig. 6. We propose that the *Tetrahymena* telomerase has a primer binding site with two components: one for secondary structure recognition, which allowes telomere or telomere-like sequences to be recognized, and a binding site for at least the four nucleotides at the 3′ end of the primer. The sequence 5′CAACCCCAA3′, complementary to one and a half GGGGTT repeats, has been found in the RNA component of the *Tetrahymena* telomerase (Greider and Blackburn 1989). One attractive possibility is that all, or part of this sequence, base-pairs with the 3′ end of the primer and then templates the added repeats. For example, if the CCCC sequence pairs with a GGGG sequence at the 3′ end of a primer, then the 5′CAA sequence in this RNA sequence could be used to template the first added nucleotides. Binding of appropriate triphosphates might then be dictated by this complementary RNA sequence in a manner analogous to DNA polymerases. Alternatively, the dGTP and dTTP binding sites might be dictated by the geometry of the protein component of the telomerase RNP in a manner analogous to the binding of rCTP and rATP to tRNA nucleotidyl transferase (Deutscher 1983).

Our finding that $G_4T_2G_4$ is active as a primer is interesting in this regard since, if the four G residues at the 3′ end of this primer are occupied in 3′ end sequence recognition, then only six nucleotides remain for secondary structure recognition. A single TTGGGG hexamer alone is apparently too short to assume an intramolecular structure like that seen for the decamer and other primers, as it neither forms rapidly migrating species nor is active as a primer for telomerase (Henderson *et al.* 1987; Greider and Blackburn 1987). Thus, the question is whether, in the $G_4T_2G_4$ oligomer primer, the 5′ G_4T_2 sequence alone is sufficient for structure formation, or whether the G residues at the 3′ end also contribute to the secondary structure, in addition to being involved in 3′ sequence recognition. In the latter case, if the telomerase RNA component has a templating role in directing the synthesis of GGGGTT repeats, then recognition of the 3′ end of the primer by base pairing with the telomerase RNA could, in the case of $G_4T_2G_4$, mean that the G residues at the 3′ end are involved in a 3-stranded structure, as they would interact both with the complementary sequence in the telomerase RNA and with the other G residues in the DNA primer. Alternatively, the $G_4T_2G_4$ oligomer could bind first to telomerase in a foldback conformation, which then unfolds, still associated with telomerase, making the four G residues at the 3′ end available for 3′ end recognition. Further work on the mechanism of telomerase recognition and on the role of the telomerase RNA will be necessary to answer these questions.

Tetrahymena *and yeast telomere elongation* in vivo

The results in Fig. 3 show that the telomerase of *Tetrahymena* can add thousands of nucleotides to a telomere primer. This suggests that the action of telomerase must be regulated *in vivo*. Two general types of regulation can be envisaged: first, telomerase activity itself could be regulated, or, second, regulatory activities, such as specific or nonspecific nucleases, could shorten telomeres after they have been lengthened by the addition of very long tracts of telomeric repeats similar to that observed *in vitro*.

Some insight into this question comes from examination of the properties of telomeres *in vivo*. In *Tetrahymena*, telomere length is relatively closely controlled, although during cell

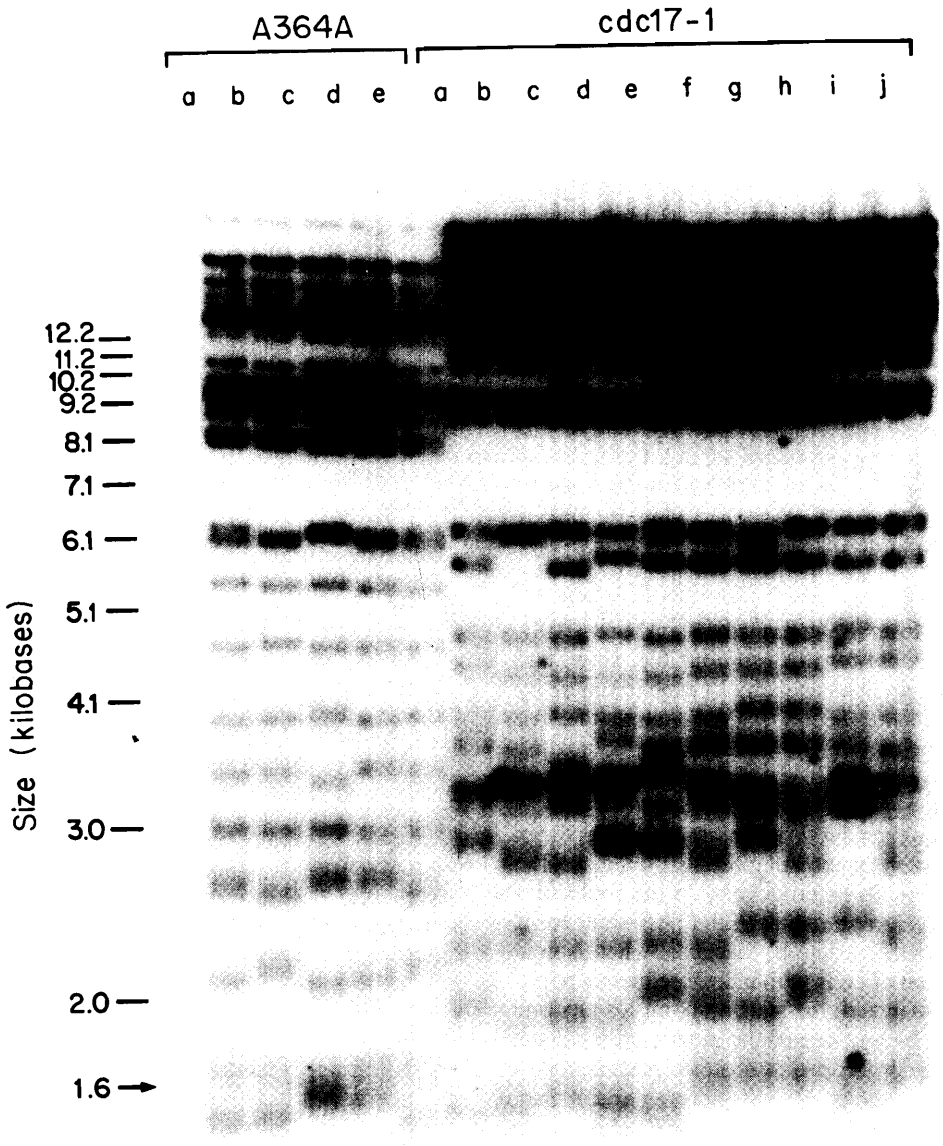

FIG. 5. Telomere lengths of individual yeast chromosomes vary among clonal populations in *cdc*17-1 and A364A strains. Each lane contains DNA prepared from a separate overnight culture, each derived from a separate single colony of the same strain. DNA was digested with *Pvu*II, displayed on a 0.8% agarose gel, blotted onto a nylon filter, and hybridized to a fragment containing yeast telomeric $C_{1-3}A$ repeats. The ten fuzzy bands at the bottom are the individual chromosomal telomeric restriction fragments. Cultures *a*–*e* of *cdc*17-1 were grown at 23°C; cultures *f*–*j* of *cdc*17-1 and *a*–*e* of A364A were grown at 30°C. The increased length of telomeric fragments observed in *cdc*17-1 at higher temperatures would not be significant in the time frame of this experiment. Bars indicate molecular weight markers.

divisions under exponential growth conditions the average length of all the telomeres in the somatic nucleus (the macronucleus) slowly increases. Measurement of the breadth of telomeric restriction fragments shows that under most conditions 95% of the telomeres vary by only $\pm \sim 100$ base pairs (Larson *et al.* 1987; Henderson *et al.* 1988). This is consistent with the notion that telomerase activity itself is controlled since telomeres do not show the wide variations in length that might be expected from regulation by nucleases, given the ability of telomerase to add thousands of bases of G_4T_2 repeats *in vitro*.

During prolonged cell divisions at the semipermissive temperature, the average telomere length of temperature sensitive *cdc*17 cells increases (Carson and Hartwell 1985, and data not shown). The *cdc*17 gene has been shown to encode the catalytic subunit of DNA polymerase I of yeast (M. Carson and L. Hartwell, personal communication). Interestingly, as shown in Fig. 5, the generation of heterogeneity and the consequent individual telomere length variability among clones is unaffected in the *cdc*17-1 cells at the semipermissive temperature.

The behavior of yeast telomeres *in vivo* is consistent with the notion that a telomerase activity is present in this species, and is regulated *in vivo*. We found previously that the molecular descendents of a single telomere undergo a gradual increase in length heterogeneity, starting from the single original length of the telomere being examined and producing both longer and shorter progeny within 30 generations, at an initial rate of ~ 7

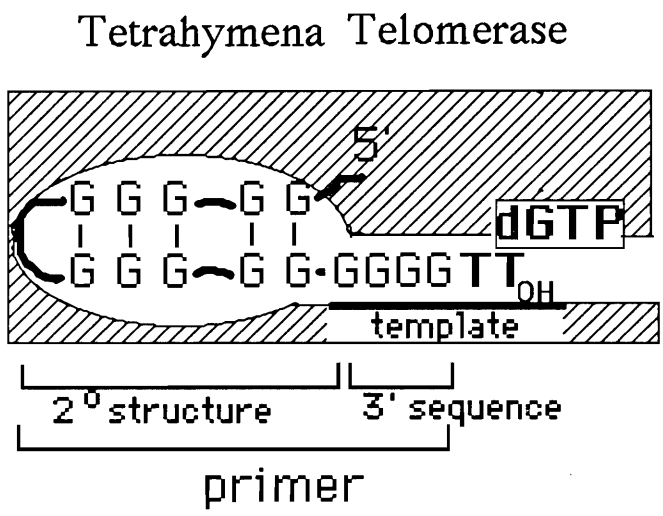

FIG. 6. Model for telomerase of *Tetrahymena* and its recognition of DNA primers. See text for details.

base pairs per cell generation (Shampay and Blackburn 1988). First, it is difficult to account for this reproducible, slow, and steady increase in the length heterogeneity of each telomere by a recombination mechanism. Second, it is notable that we never observe wide fluctuations in yeast telomere lengths, even in the short term. These observations are more easily explained by the action of a telomerase activity that is regulated *in vivo*, rather than large additions or losses from telomeres with only subsequent length adjustments. The generation of telomere length heterogeneity in the molecular descendents of a single telomere leads to the phenomenon of individual telomere length variation, because when single clones from the population of cells with a heterogeneous population of telomeres are analyzed, the starting point for any one telomere in a clone will be any one of the new lengths generated in the course of the preceding replication rounds.

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