3698–3704 *Nucleic Acids Research, 1997, Vol. 25, No. 18*

dGTP-dependent processivity and possible template switching of *Euplotes* telomerase

Philip W. Hammond[+] and Thomas R. Cech*

Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, University of Colorado, Boulder, CO 80309-0215, USA

Received May 2, 1997; Revised and Accepted August 1, 1997

ABSTRACT

We have measured the processivity of telomeric DNA extension by *Euplotes aediculatus* telomerase at various concentrations of the nucleotide substrates dGTP and dTTP. The maximum processivity (~3 repeats) was observed at ~100 μM of each dNTP. Processivity decreased as the dNTP concentrations were reduced and, surprisingly, as the concentration of dGTP was increased. Also, the characteristic banding pattern generated by telomerase extension of DNA primers shifted in response to changes in dGTP concentration. One pattern with 8 nt periodicity was predominant at dGTP concentrations ≤16 μM, while at ≥250 μM an 8 nt repeat pattern out-of-phase with the first was observed; at intermediate concentrations the two patterns co-existed. We propose that two different segments of the RNA subunit can serve as the template for repeat synthesis; nt 42–49 at low dGTP concentrations and nt 36–43 at high dGTP concentrations. An alternative model for the low dGTP pattern involves an internal pause site but no pause at the end of the template and is, therefore, considered less likely. Because the effects of dGTP on processivity and banding pattern appear to be distinct from nucleotide binding in the polymerase active site, we propose a second dGTP binding site involved in template selection and processivity.

INTRODUCTION

In most eukaryotes, the DNA sequence at the chromosome termini consists of an array of simple tandem repeats. This repetitive DNA and associated proteins make up a terminal structure called the telomere (for reviews see 1,2). The telomere provides a protective cap to prevent degradation or terminal fusion of the chromosomes (3,4). The DNA repeat sequence varies between organisms, but is often 6–8 nt long and rich in G and T nucleotides in the strand running 5′ to 3′ towards the end of the chromosome (5). This holds true for organisms as diverse as ciliated protozoa (T_4G_4 or T_2G_4), vertebrates (T_2AG_3) and plants [$T_2(T/A)G_3$ or T_3AG_3], although exceptions have been found (6). These repetitive sequences are maintained during DNA replication by the enzyme telomerase, which can also synthesize new telomeres after chromosome breakage (7,8) or during programmed development of the macronucleus of the hypotrichous ciliates (for reviews see refs 9,10).

Telomerase is a ribonucleoprotein enzyme that contains a single RNA subunit and associated protein components. Two protein components of telomerase have been reported for the holotrichous ciliate *Tetrahymena thermophila* (11), one of which has homologs in mammals (12,13). Two unrelated protein components have been found in the hypotrichous ciliate *Euplotes aediculatus* (14), and one of these (p123) is a reverse-transcriptase related protein proposed to serve as the catalytic subunit (15). The yeast homolog of p123, Est2p (Ever Shorter Telomeres) (16), is essential for telomerase activity *in vivo* and *in vitro* (15).

The sequence of the RNA moiety has been determined for many organisms, and each contains a region complementary to the telomeric repeat (17). Modifications of the telomere-complementary sequence produce correspondingly altered telomeric repeats, demonstrating that this RNA provides the template for telomere synthesis (18–20). The region of telomere complementarity is ~1.5–2 repeats in length. However, only a segment coding for one repeat appears to serve as the actual template. The adjacent sequence has been shown to align the 3′-end of DNA substrates by base-pairing, so that correct repeats are synthesized (21).

The telomerase RNA secondary structure was determined for the ciliates by phylogenetic analysis (22,23), and while the primary sequence of the RNA varies greatly between species, the overall architecture is very similar. The telomerase RNA from *E.aediculatus*, studied herein, is shown schematically (Fig. 1) with a primer positioned on the template (shown in bold). Also shown is the UGUCA motif that is conserved among the ciliates and is located 2 nt 5′ of the template (23).

In vitro, ciliate telomerases are processive (24,25). This requires that after each repeat is added, the newly extended 3′-end be repositioned (or translocated) without dissociation of the primer from the enzyme. Translocation is possible because the 5′-region of the DNA substrate binds at a second site on the enzyme, designated the anchor site (26). This additional binding site may be especially important for telomerase's role in *de novo* telomere synthesis. Both 'chromosome healing' events and ciliate macronuclear development require extension of substrates that have anchor site binding regions, but non-telomeric 3′-ends (7,8). The anchor site of the *Euplotes* telomerase resides on a protein subunit of ~130 kDa (25) (believed to be the same 123 kDa protein that has been isolated and sequenced; 14,15), and can also include a region of the telomerase RNA (25).

*To whom correspondence should be addressed. Tel: +1 303 492 8606; Fax +1 303 492 6194; Email: thomas.cech@colorado.edu

[+]Present address: Mosaic Technologies, Inc., 1106 Commonwealth Avenue, Boston, MA 02215, USA

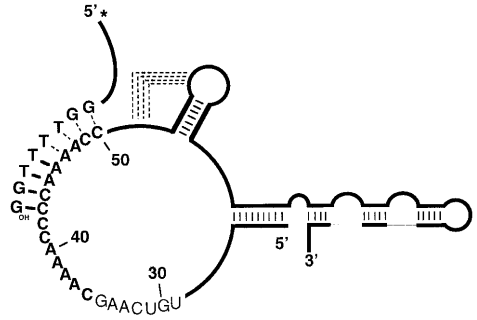

Figure 1. Schematic of the telomerase RNA from *E.aediculatus*. The RNA secondary structure (23) is shown in line form. Nucleotides of the template and alignment region (bold face type) and conserved upstream UCUGA motif are shown explicitly; nucleotides are numbered from the 5′-end. A 5′-labeled (*) telomeric DNA primer is shown bound in the register described by Lingner *et al.* (23). A minimum of 4 bp (thick lines) is required to give a unique register, and additional pairing (dashed lines) is possible.

We previously measured the processivity of the *Euplotes* telomerase using a bind-and-chase assay for primer extension (25). However, little is known about what factors influence translocation efficiency and the resulting enzyme processivity. The study reported herein was initiated to examine the effects of dNTP concentration. Unexpectedly, it was found that enzyme processivity decreases at high dGTP concentration, and also that the segment of the RNA used as the template may change in response to the dGTP concentration.

MATERIALS AND METHODS

Growth of *Euplotes* and preparation of nuclear extract

Euplotes aediculatus was grown as described (27) under non-sterile conditions in aerated 15 gallon reactors, with *Chlorogonium* as the food source. Cells were collected on a 15 µm Nytex filter and lysed in the presence of Nonidet P-40 non-ionic detergent. Nuclei were isolated by sucrose cushion centrifugation and a nuclear extract was prepared by Dounce homogenization as previously described (14).

Partial purification of telomerase

Nuclear extracts were partially purified as previously described (25). Extracts were fractionated by centrifugation in a 15–40% glycerol gradient. Fractions containing telomerase were identified by a gel-shift assay for the presence of telomerase RNA, using a radiolabeled telomeric primer. Peak fractions were pooled and dialyzed against telomerase reaction buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 50 mM sodium glutamate, 1 mM dithiothreitol, 10% glycerol) using a Spectra/Por CE membrane with a 100 kDa molecular weight cutoff.

Primer synthesis, purification and 5′-end labeling

Primers were prepared by standard phosphoramidite synthesis and purified by polyacrylamide gel electrophoresis as previously described (25). The non-telomeric competitor primer had the sequence 5′-ATTGAATGACTACGAGATGAA. Telomeric primers all contained 5-iododeoxyuridine (^{I}U) substitutions for thymidine at positions 1 and 3, as they were previously used for cross-linking studies; the ^{I}U substitution is isosteric with thymidine and does not disrupt normal base-pairing. All of the telomeric primers are composed of full and partial T_4G_4 repeats. The sequence of the 21 nt reference primer was 5′-^{I}UT^{I}UTGGGG-TTTTGGGGTTTTG. Shorter and longer primers were all altered at the 3′-end such that the 5′-end remained constant. Purified primers were 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol; NEN), then purified using Beckman G-25 TE spin columns.

Primer extension assay of telomerase activity

Partially purified telomerase was pre-incubated with the non-telomeric primer (6.5 µM), then 5′-end-labeled telomeric primer was added (0.015 µM) and allowed to bind for 10 min at 25°C. Aliquots of the reaction (10 µl) were mixed with an equal volume of telomerase reaction buffer containing dGTP and dTTP (or ddTTP) and specific competitor primer (5 µM), then incubated at 25°C. The concentrations of the added nucleotides and the incubation times were varied in individual experiments (see below). Control reactions with no dNTP's were included where indicated. The specific competitor primer for each reaction had the same sequence as the labeled primer used in that reaction. Extension reactions were stopped by addition of 100 µl of proteinase K buffer (20 mM Tris–HCl pH 7.9, 10 mM EDTA, 1% sodium dodecyl sulfate) and incubated for 3 min at 85°C. The nucleic acid component was isolated by digestion with proteinase K (80 µg/ml) for 45 min at 45°C, followed by ethanol precipitation. In reactions where the dGTP and dTTP concentrations were varied independently, the proteinase K was pre-mixed with the proteinase K buffer and no 85°C denaturation was performed. Primer extension products were separated by denaturing polyacrylamide gel electrophoresis. Reaction products were visualized and quantitated using a Molecular Dynamics phosphorimager.

To verify the effectiveness of the specific competitor, pre-chased reactions were performed in which the chase and ^{32}P-labeled primers were mixed before incubation with telomerase. To ensure that all endogenous nucleotides were removed during the telomerase purification, a control with no addition of nucleotides was included. In neither of these reactions did significant extension of labeled primer occur. The correct alignment of primers on the telomerase template was verified by substituting ddTTP for dTTP in the reaction. For each of the primers tested, extension was terminated at the first adenosine in the template, except where noted in the Results.

RESULTS

Nucleotide concentration dependence of telomerase processivity

For *in vitro* telomerase assays, a characteristic pattern of product bands is seen on polyacrylamide gels corresponding to additions of complete repeats to a substrate primer. These bands are believed to arise from primer pausing and/or dissociating upon extension to the end of the template (17). Processive extension of DNA primers by telomerase is observed as the addition of multiple repeats without intervening dissociation of the bound primer. We have previously developed a bind-and-chase assay to measure processivity by a quantitative analysis of the telomerase banding pattern (25). This assay is used herein to test the effects of nucleotide concentration on processivity.

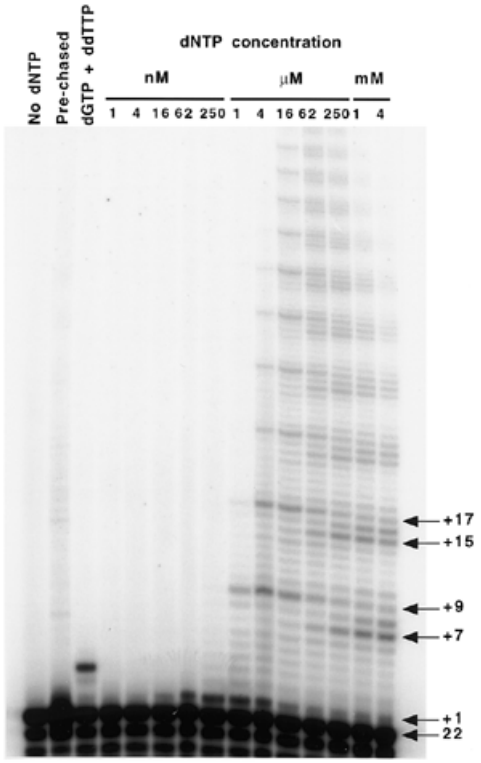

Figure 2. The effect of dNTP concentration on telomerase activity. Phosphorimager printout of a polyacrylamide gel showing primer extension reactions at various dNTP concentrations. The 22 nt primer 5′-[^{32}P]^{l}UT^{l}UTGGGGTTTT-GGGGTTTTGG was used in a bind-and-chase assay at concentrations of dGTP ranging from 1 nM to 4 mM; the concentration of dTTP always equaled that of dGTP. A sample without nucleotide addition was included in the first lane. In the pre-chased control, ^{32}P-labeled primer and chase primer were mixed before incubation with telomerase and 250 μM dNTPs. To verify correct alignment of the primer on the template, a reaction was included that contained 250 μM dGTP and 250 μM ddTTP.

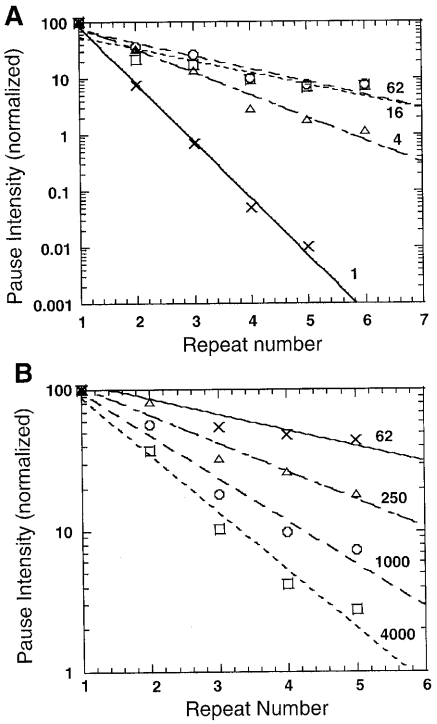

Figure 3. Telomerase processivity at various dNTP concentrations (μM). The intensity of each major band from the processivity assay (Fig. 2) was quantitated by phosphorimager analysis, and the first band in each lane was used to normalize the intensities of subsequent bands. Normalized intensities were then plotted versus the repeat number. (**A**) Processivity of the banding pattern observed at low dNTP concentrations (bands at +1, +9, +17, etc.). (**B**) Processivity of the banding pattern observed at high dNTP concentrations (+7, +15, +23, etc.).

A 22 nt primer (^{l}UT^{l}UTGGGGTTTTGGGGTTTTGG) was 5′-end-labeled and incubated with telomerase, followed by addition of the nucleotides dGTP and dTTP (from 1 nM to 4 mM) to allow primer extension and unlabeled specific competitor primer (>300-fold excess over labeled primer) to prevent re-binding of any labeled primer that dissociated. Primer extension products were then separated on a denaturing polyacrylamide gel (Fig. 2). At low concentrations of nucleotides (from 1 to 250 nM), the extension reaction was inefficient and only the addition of 1 nt was observed (+1 product). At higher nucleotide concentrations (from 1 to 16 μM), telomerase became processive and yielded longer products corresponding to multiple repeat additions (+9, +17, etc.). The +1 product observed at low nucleotide concentrations corresponds to the first step in this 8 nt periodicity and may accumulate due to low processivity at low nucleotide concentrations. At the highest concentrations tested (from 62.5 μM to 4 mM), a new pattern emerged that also showed an 8 nt periodicity (extension products of +7, +15, +23, etc.). As the new pattern appeared at high nucleotide concentration, the pattern observed at low nucleotide concentration simultaneously disappeared. This indicated that a shift was occurring from one pattern to the other, rather than simply the emergence of a new pattern.

At each dNTP concentration, the intensity of each major repeat band was measured, normalized to the intensity of the first band and then plotted versus the repeat number (Fig. 3). The data were fit to a single exponential; the processivity is inversely related to the steepness of the slope. For the low dNTP pattern (Fig. 3A), the processivity increased with increasing dNTP concentration until it reached a maximum at ~16 μM. The high dNTP banding pattern (Fig. 3B) had its maximal processivity at ~62 μM, which then decreased as the dNTP concentration was increased.

One concern in interpretation of such measurements is that the concentration of available Mg^{2+} can be influenced by complexation with nucleotides. In order to keep the available Mg^{2+} constant, the highest concentration of dNTP (4 mM each of dGTP and dTTP) was prepared with 8 mM $MgCl_2$ and the lower concentrations obtained by serial dilution of this stock solution with H_2O. In this way, it was ensured that the observed effects were due to the dNTP concentration and not the available Mg^{2+} concentration. This was also directly tested by observing the low and high dNTP banding patterns while varying the $MgCl_2$ concentration from 6 to 14 mM. No changes in either banding pattern occurred over this range of $MgCl_2$ concentrations (data not shown).

Cause of the alternative banding pattern

The banding pattern observed for telomerase primer extension changed as the dNTP concentration was varied. To determine if the change was due specifically to either dGTP or dTTP, one of

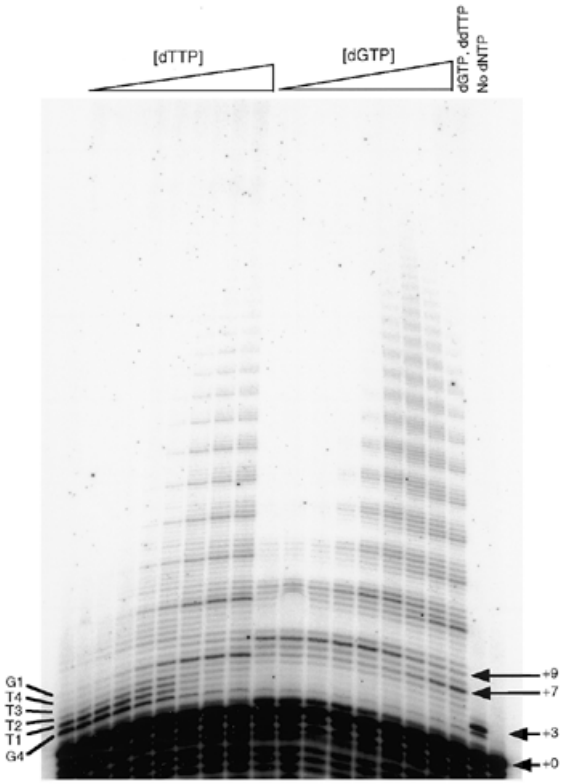

Figure 4. The effect of individual nucleotides (dTTP and dGTP) on processivity and banding pattern. Polyacrylamide gel of telomerase primer extension assays under bind-and-chase conditions. For each set of lanes, the concentration of the indicated nucleotide was varied from 10 nM to 656 μM in 4-fold increments while the other nucleotide was held constant at 656 μM. Controls were included with ddTTP substituted for dTTP and with no dNTP addition.

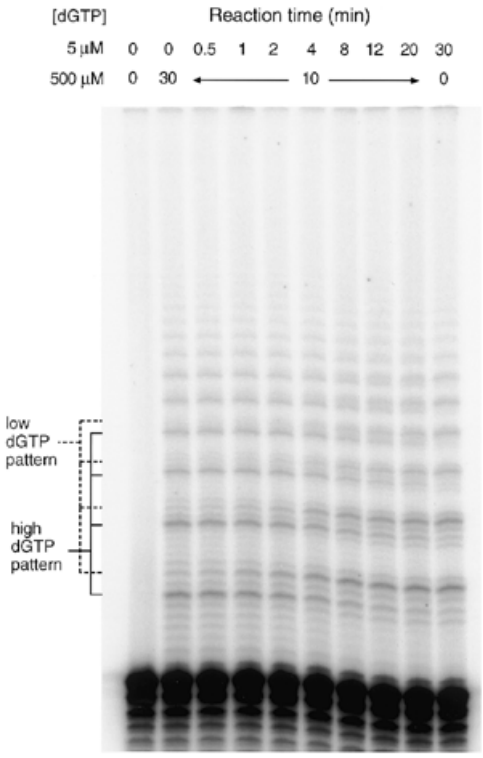

Figure 5. The same enzyme–primer complex can produce either banding pattern. The 5′-^{32}P-labeled 22 nt primer was bound to telomerase, then chased with unlabeled primer, 5 μM dGTP and 125 μM dTTP. After the indicated time at 5 μM dGTP, these reactions were chased for an additional 10 min with 500 μM dGTP. Single-chase controls were included with either 5 μM (lane 30, 0) or 500 μM dGTP (lane 0, 30) in the initial chase (along with unlabeled primer and 125 μM dTTP) and also with no nucleotides in either chase (lane 0, 0).

the nucleotides was held constant at 656 μM while the other was varied from 10 nM to 656 μM (Fig. 4). Comparison of the banding pattern in the dTTP lanes to that in the dGTP lanes showed that the banding pattern was shifting in response to the dGTP concentration. The same concentration dependence seen when the nucleotides were varied together was observed in the dGTP reactions. With ≤10 μM dGTP, the major pauses occurred at +1, +9, +17, etc., whereas with >164 μM dGTP the pattern shifted to +7, +15, etc; intermediate concentrations gave a mix of the two patterns.

The specificity of this shift was tested by primer extension using 5 μM dGTP (where the low dGTP banding pattern was expected) supplemented with concentrations of its ribo-analog (GTP) up to 1 mM (data not shown). All of these reactions gave the low dGTP banding pattern, indicating a high level of specificity for dGTP.

The effect of each dNTP on processivity was also different. Low dTTP concentrations limited the processivity primarily because partial extension products accumulated, as one would expect; these products are labeled G4, T1, T2, T3, T4 and G1 on the left of Figure 4. Assuming correct G_4T_4 repeats were added (see below), G4 corresponds to the fourth G in the GGGGTTTT repeat, T1 to the first T, etc. Once the build-up of these intermediate products was minimized at concentrations >41 μM dTTP, the processivity did not increase further, nor did it decrease. The dGTP concentration had a different impact on the processivity; as the concentration increased, so did the processivity, but at the two highest concentrations it began to decrease again. Decreased processivity is apparent by visual inspection of Figure 4, where it can be seen that the intensity of the banding pattern at higher molecular weights falls off more rapidly at the two highest dGTP concentrations, 164 and 656 μM. This was confirmed by quantitation as in Figure 3. An identical effect was observed when the concentrations of dGTP and dTTP were varied together.

Either banding pattern can be generated by the same primer–telomerase complex

The presence of two different banding patterns could conceivably be due to the presence of two species of telomerase that were active at low and high dGTP concentrations, respectively. To test this possibility we used a double chase assay in which primer was bound to telomerase, then first chased with unlabeled primer, dTTP (125 μM) and low concentration dGTP (5 μM); after incubation for various times at low dGTP, high dGTP (500 μM) was added for a second chase period (Fig. 5). Controls were included in which the first chase had high dGTP, the second chase had no dGTP or no nucleotides were present in either chase. All of the reactions were incubated for the same length of time (10 min)

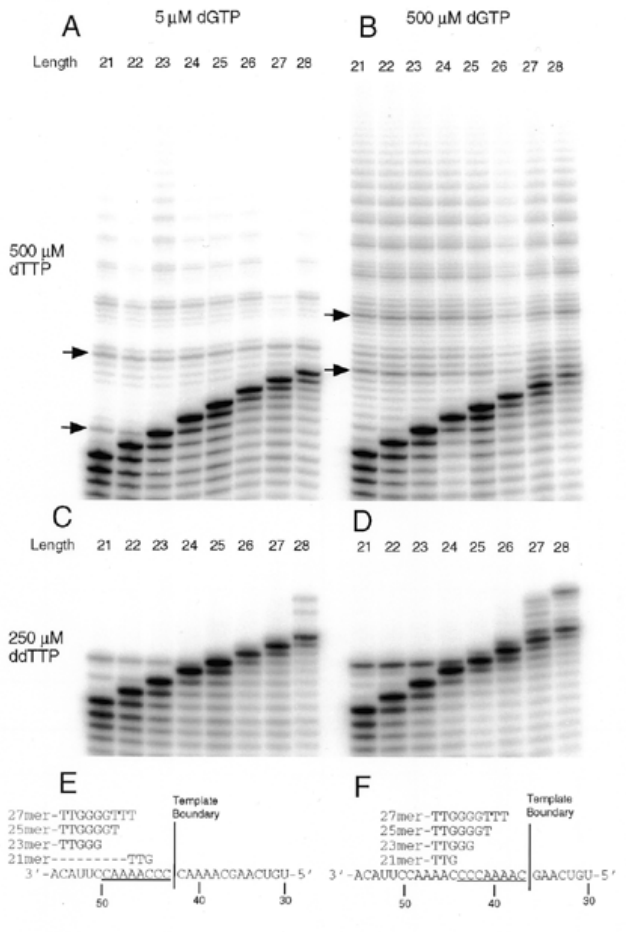

Figure 6. Mapping the template at low and high dGTP concentrations. Primers ranging from 21 to 28 nt in length, differing only at the 3′-end, were used in primer extension assays. To map the templates, each primer was extended at 500 µM dTTP and either (**A**) 5 µM dGTP or (**B**) 500 µM dGTP. To verify the addition of correct G_4T_4 repeats, reactions were also performed with 250 µM ddTTP and either (**C**) 5 µM dGTP or (**D**) 500 µM dGTP. Models of proposed primer–template alignments under conditions of (**E**) low dGTP and (**F**) high dGTP.

after the addition of high dGTP, so that if the high dGTP banding pattern was due to a separate species of enzyme, the intensity of the high dGTP pattern would be constant. The opposite was actually observed: the intensity of the high dGTP pattern was inversely proportional to the amount of time spent at low dGTP. Reactions that were incubated for 0.5–2 min at low dGTP gave mostly the high dGTP banding pattern while those incubated 12–20 min at low dGTP gave virtually no high dGTP pattern. In addition, the total amount of extended product was similar for all of the reactions, consistent with a single species of enzyme–primer complex giving rise to either banding pattern depending on the reaction conditions.

This experiment also demonstrates that the banding pattern at high dGTP was not due to a nuclease activity in the extract trimming back the extended product in response to stimulation by high dGTP. If such a nuclease were involved, the pattern in all the samples that had been incubated for 10 min at high dGTP should have been equivalent since the exposure to high dGTP was the same.

Both banding patterns represent addition of the correct repeat sequence

One possibility for the origin of the alternative banding pattern was the misalignment of the primer on the RNA template. This was tested by comparing the extension products for primers that differed at their 3′-ends (Fig. 6).

These primers were first used in a processive extension assay with 500 µM dTTP and either 5 µM dGTP (Fig. 6A) or 500 µM dGTP (Fig. 6B). The expected low-dGTP and high-dGTP banding patterns were produced for each of the primers (arrows in Fig. 6A and B, respectively). This result indicated that each of the different primer 3′-ends was properly aligned so that the end of the template occurred at the same location for each. This is the expected result if the banding pattern arises from either pausing or dissociation after primers are extended to the end of the template.

A second test for addition of correct repeats was to substitute ddTTP for dTTP, again under low-dGTP (Fig. 6C) and high-dGTP (Fig. 6D) reaction conditions. Incorporation of ddTTP causes chain termination, and will occur the first time that adenosine is encountered in the RNA template. The same pattern of dideoxynucleotide termination was observed at low dGTP as at high dGTP, revealing that the sequence being added to the primer was the same for each. The only significant deviation occurred with the 27mer in the high-dGTP reaction. The 27mer ends with the sequence -GTTT and apparently some misalignment (pairing of the T's with the template positions 45, 46 and 47) allowed the addition of -GGGGddT as well as the expected single nucleotide addition of ddT. This may be due in part to a relatively low efficiency of incorporation of ddTTP as was reported for the *Tetrahymena* enzyme (28).

These results can be explained if telomerase from *Euplotes* uses different regions of its RNA subunit as the template at low and high dGTP concentrations (Fig. 6E and F). The region of the RNA used as the template under each condition can be determined from the site of the first major pause when primers of different lengths are extended (Fig. 6A and B) and from the observation that an 8 nt repeat is added at either concentration. At low dGTP concentrations (Fig. 6E), translocation occurs after addition of the third G in the G_4T_4 repeat, indicating that the 8 nt template extends from C49 to C42. At high dGTP concentrations (Fig. 6F), translocation occurs after addition of the first G in the G_4T_4 repeat, indicating that the region from C43 to C36 serves as the template.

An alternative explanation for the low dGTP pattern would require pausing or dissociation prior to reaching the end of the C43–C36 template. For this model to explain the data, two conditions would have to be met. First, the addition of the fourth G, templated by C41, must be much less efficient than that of the first three Gs and second, translocation at C36 would have to be very efficient such that it did not result in the accumulation of significant product (Fig. 6A, 2 nt below the upper arrow). Correspondingly, as the dGTP concentration is increased, the resulting increase in efficiency of G addition at position 41 would have to be accompanied by an almost exactly compensating decrease in the efficiency of translocation. (The relative efficiency of each translocation is evidenced by the total amount of primer extended beyond the corresponding pause.) The first condition is conceivable; the K_m for dGTP could for some reason be especially high at the C41 template position, or the rate constant for addition of the fourth G could be lower than that for the first

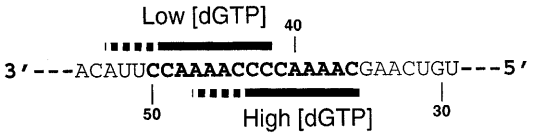

Figure 7. Two different templates used by *Euplotes* telomerase. Nucleotides 29–55 of the telomerase RNA are shown. The templates proposed for use at low and high dGTP concentrations are indicated by the solid lines and the corresponding alignment regions by dashed lines. The primer alignment region for the low-dGTP template includes nucleotides that are not phylogenetically conserved among hypotrichs. Perhaps the protein component of telomerase ameliorates the need for base-pairing in this region.

three. The second condition seems unlikely, however, because the processivity of *Euplotes* telomerase is modest (only ~2 repeats; 25), and a vast excess of unlabeled primer in the reaction prevents rebinding after dissociation; thus, some accumulation of product corresponding to polymerization to the end of the template is expected. Therefore, while we cannot rule out this 'internal pause site' model for the alternative banding pattern, the template switching model seems simpler because it involves fewer *ad hoc* assumptions.

DISCUSSION

Telomerase can use two different regions of the RNA as a template

Telomerase adds repeats to DNA primers using its RNA subunit as the template. After extension to the end of the template, the primer can translocate without dissociation so that additional repeats can be added. A characteristic banding pattern with a periodicity equal to the telomeric repeat length is observed by gel electrophoresis of DNA primers extended by telomerase. Such a pattern may in principle arise from either pausing or dissociation at the end of the template prior to or during translocation, or at an internal site due to a slow polymerization step or the absence of the next cognate nucleotide.

In primer extension assays with *Euplotes* telomerase at different dGTP concentrations, two different banding patterns were observed (Figs 2 and 4). For both patterns, correct 8 nt repeats are synthesized as judged by the position of stops in the presence of ddTTP (Fig. 6C and D). The two patterns arise from the same initial population of telomerase-bound primer (Fig. 5), indicating that both patterns can be generated by the same enzyme. The change in banding pattern occurs at dGTP concentrations between 40 and 160 μM. This is well above the concentration required to minimize stalling within the repeat which is observed at lower nucleotide concentrations (Figs 2 and 4, and data not shown). We therefore propose that these two banding patterns are the result of dGTP-dependent template switching, such that nucleotides C49–C42 or nucleotides C43–C36 of the RNA subunit provide the template at low and high dGTP concentrations, respectively (Fig. 7).

In another hypotrichous ciliate, *Oxytricha nova*, two groups have reported different regions of the RNA being used as the template (23,29). These different results can now be reconciled by comparing the assay conditions used for the two studies. The high-dGTP pausing pattern was observed in the study performed with ^{32}P-labeled primer and high dNTP concentrations (23), and the low-dGTP pausing pattern was observed when telomerase was assayed with unlabeled primers and low concentrations of [α-^{32}P]dGTP (29). Thus, we propose that dGTP-dependent template switching occurs for the telomerase of *Oxytricha* as well as *Euplotes*.

Telomerase can count

In ciliates, the telomerase template is preceded by a conserved UCUGA sequence motif (Fig. 1) (23). Based on site-directed mutagenesis of the *Tetrahymena* enzyme, it has been proposed that this motif serves to define the 5′-end of the template (30). Correspondingly, insertion or deletion of nucleotides within the *Tetrahymena* telomerase RNA template results in repeats that are longer or shorter, respectively (30,33). However, in *Euplotes* a different mechanism may contribute to definition of the template boundaries. Since the two templates are shifted by several nucleotides within the RNA sequence, it does not appear that sequences outside the template region can define both templates. Instead, *Euplotes* telomerase appears to have the ability to count nucleotides, as the use of either template results in the addition of a complete 8 nt repeat prior to translocation. We therefore propose that telomerase proteins help to define and maintain a fixed template size of 8 nt.

Why have two different template regions?

Telomerase has two distinct roles in the life cycle of the hypotrichous ciliates. One role is the maintenance of the chromosomal 3′-overhang during DNA replication by synthesis of telomeric sequences (31,32), and the second is *de novo* telomere synthesis during the development of the macronucleus (33; reviewed in 10). It has been suggested that telomerase may use different template regions for each of these processes (30). If primers are aligned exclusively by base-pairing interactions, for *Euplotes* telomerase there must be at least four primer-template base-pairs formed during primer alignment in order to confer a single unique alignment for each 3′-end. Comparison of the segments of telomerase RNA used for primer alignments under the high-dGTP and low-dGTP conditions reveals that they consist of the telomeric sequence, 5′-CAAAACC-3′, and the distinctly non-telomeric sequence 5′-CUUACA-3′, respectively. Only the former is phylogenetically conserved among hypotrichous ciliates.

This raises the possibility that the high-G and low-G templates are used for telomere maintenance and macronuclear development, respectively, due to the different base-pairing regions for primer alignment. During DNA replication, the substrate for telomerase presumably has a normal telomeric repeat sequence and may be easily aligned on the high-G template. In contrast, a comparison of the sequences of the micronuclear and macronuclear genomes revealed that there is no substantial telomere homology at the sites of telomere addition (34,35). More flexibility in the sequences that can be aligned on the low-G template may be allowed by the ability of U to form stable base-pairs with either G or A. Alternatively, telomerase protein–DNA interactions may supplant base-pairing for primer alignment on the low-G template.

Studies in the related organism *Euplotes crassus* have shown that extension of non-telomeric primers *in vitro* always begins with four dG residues, leading to the postulation of a default binding register (36). Extension from the default register may in fact be occurring not within the canonical template, but rather on the alternative template that is proposed herein to be used at low dGTP conditions. *Trans*-acting factor(s) that were specifically

present in extracts from cells undergoing macronuclear development enhance the use of DNA substrates lacking 3′ telomeric sequences *in vitro* (39). Perhaps one of the functions of this factor(s) is to direct telomerase to use the template observed herein at low dGTP concentrations.

The effect of dGTP concentration on telomerase processivity

The concentration of dGTP not only affected the template usage, but also had a marked effect on the processivity of *Euplotes* telomerase. The processivity appears to be affected by the nucleotide concentration in two distinct ways. The first is the efficiency of nucleotide incorporation within each repeat, which is expected to be related to the affinity of the active site for nucleotide binding. Indeed, in this study and others (37,38), increasing nucleotide concentrations improve repeat synthesis and longer products are observed. This is expected for any simple model in which primer extension competes with primer dissociation, with the rate of primer extension being dependent on the nucleotide concentration.

A second and novel effect was observed for dGTP in the experiments presented herein. At nucleotide concentrations above those required to prevent stalling within a repeat, dGTP appears to modulate the number of repeats added. That is, increasing the dGTP concentration first causes an increase in processivity and then at concentrations >100 μM causes a decrease in processivity.

A second binding site for dGTP

As a polymerase, telomerase clearly must bind dGTP in the active site as a substrate for primer extension, and this could influence which template is used. However, primers for which the next cognate nucleotide is T also use two different templates in a dGTP-dependent manner (Fig. 6A and B). For these primers the active site must bind dTTP, which would prevent simultaneous binding of dGTP at the same site. These results indicate the presence of at least one additional dGTP binding site.

The mechanism by which dGTP binding at such a secondary site stimulates translocation is unknown. One possibility is that dGTP hydrolysis may be required by a telomerase associated helicase-like activity that opens primer–template base-pairs and allows translocation. Alternatively, a simple bind-and-release of dGTP could activate translocation. Distinguishing between these and other possibilities is complicated by the fact that dGTP is required for telomeric repeat addition. Future testing of non-hydrolyzable dGTP analogs may provide some support for one of these models.

It is not clear whether the effects of dGTP on the processivity and the template switching phenomena are coupled. However, translocation and template switching both require a movement of the RNA relative to the polymerase active site. Identification and further characterization of a second dGTP binding site would provide additional insight into the mechanism of primer translocation by telomerase.

ACKNOWLEDGEMENTS

We thank Loren Denton and Brent Dickinson for growing *E.aediculatus* cells; Cheryl Grosshans and Elaine Podell for oligonucleotide synthesis; Lara Weinstein and Stacie Froelich-Ammon for critical reading of the manuscript; Evelyn Jabri and Lara Weinstein for helpful discussions. This work was supported by NIH grant GM28039. T.R.C. is an investigator of the Howard Hughes Medical Institute and an American Cancer Society Professor.

REFERENCES

1 Blackburn, E. H. (1991) *Nature*, **350**, 569–573.
2 Zakian, V. A. (1995) *Science*, **270**, 1601–1607.
3 Gottschling, D. E. and Zakian, V. A. (1986) *Cell*, **47**, 195–205.
4 Sandell, L. L. and Zakian, V. A. (1993) *Cell*, **75**, 729–739.
5 Henderson, E. (1995) In Blackburn, E. H. and Greider, C. W. (eds) *Telomeres*. Cold Spring Harbor Laboratory Press, NY. pp. 11–34.
6 McEachern, M. J. and Blackburn, E. H. (1995) *Nature*, **376**, 403–409.
7 Morin, G. B. (1991) *Nature*, **353**, 454–456.
8 Harrington, L. A. and Greider, C. W. (1991) *Nature*, **353**, 451–454.
9 Blackburn, E. H. and Karrer, K. M. (1986) *Annu. Rev. Genet.*, **20**, 501–521.
10 Blackburn, E. H. (1995) In Blackburn, E. H. and Greider, C. W. (eds) *Telomeres*. Cold Spring Harbor Laboratory Press, NY. pp. 11–34.
11 Collins, K., Kobayashi, R. and Greider, C. W. (1995) *Cell*, **81**, 677–686.
12 Harrington, L., McPhail, T., Mar, V., Zhou, W., Oulton, R., Program, A. E., Bass, M. B., Arruda, I. and Robinson, M. O. (1997) *Science*, **275**, 973–977.
13 Nakayama, J., Saito, M., Nakamura, H., Matsuura, A. and Ishikawa, F. (1997) *Cell*, **88**, 875–884.
14 Lingner, J. and Cech, T. R. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 10712–10717.
15 Lingner, J., Hughes, T. R., Shevenko, A., Mann, M., Lundblad, V. and Cech, T. R. (1997) *Science*, **276**, 561–567.
16 Lendvay, T. S., Morris, D. K., Sah, J., Balasubramanian, B. and Lundblad, V. (1996) *Genetics*, **144**, 1399–1412.
17 Greider, C. W. and Blackburn, E. H. (1989) *Nature*, **337**, 331–337.
18 Yu, G. L., Bradley, J. D., Attardi, L. D. and Blackburn, E. H. (1990) *Nature*, **344**, 126–132.
19 Autexier, C. and Greider, C. W. (1994) *Genes Dev.*, **8**, 563–575.
20 Singer, M. S. and Gottschling, D. E. (1994) *Science*, **266**, 404–409.
21 Greider, C. W. and Blackburn, E. H. (1987) *Cell*, **51**, 887–898.
22 Romero, D. P. and Blackburn, E. H. (1991) *Cell*, **67**, 343–353.
23 Lingner, J., Hendrick, L. L. and Cech, T. R. (1994) *Genes Dev.*, **8**, 1984–1998.
24 Greider, C. W. (1991) *Mol. Cell Biol.*, **11**, 4572–4580.
25 Hammond, P. W., Lively, T. N. and Cech, T. R. (1997) *Mol. Cell Biol.*, **17**, 296–308.
26 Collins, K. and Greider, C. W. (1993) *Genes Dev.*, **7**, 1364–1376.
27 Swanton, M. T., Heumann, J. M. and Prescott, D. M. (1980) *Chromosoma*, **77**, 217–227.
28 Strahl, C. and Blackburn, E. H. (1994) *Nucleic Acids Res.*, **22**, 893–900.
29 Melek, M., Davis, B. T. and Shippen, D. E. (1994) *Mol. Cell Biol.*, **14**, 7827–7838.
30 Autexier, C. and Greider, C. W. (1995) *Genes Dev.*, **9**, 2227–2239.
31 Zahler, A. M. and Prescott, D. M. (1988) *Nucleic Acids Res.*, **16**, 6953–6972.
32 Lingner, J., Cooper, J. P. and Cech, T. R. (1995) *Science*, **269**, 1533–1534.
33 Yu, G. L. and Blackburn, E. H. (1991) *Cell*, **67**, 823–832.
34 Baird, S. E. and Klobutcher, L. A. (1989) *Genes Dev.*, **3**, 585–597.
35 Klobutcher, L. A., Jahn, C. L. and Prescott, D. M. (1984) *Cell*, **36**, 1045–1055.
36 Melek, M., Greene, E. C., Shippen, D. E. (1996) *Mol. Cell Biol.*, **16**, 3437–3445.
37 Greider, C. W. (1995) In Blackburn, E. H. and Greider, C. W. (eds) *Telomeres*. Cold Spring Harbor Laboratory Press, NY. pp. 35–68.
38 Lee, M. S. and Blackburn, E. H. (1993) *Mol. Cell Biol.*, **13**, 6586–6599.
39 Bednenko, J., Melek, M., Greene, E. C. and Shippen, D. E. (1997) *EMBO J.*, **16**, 2507–2518.