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Telomere terminal transferase activity in the hypotrichous ciliate *Oxytricha nova* and a model for replication of the ends of linear DNA molecules

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ABSTRACT

We have found abundant telomere-specific terminal transferase activity in crude macronuclear extracts from vegetatively growing cells of the hypotrichous ciliate Oxytricha nova. This activity adds two to seven tandem repeats of the sequence GGGGTTTT (the Oxytricha telomeric repeat) to the 3' end of oligonucleotide primers ending in repeats of G_4T_4 and always adds the repeats in the proper phase. The activity requires the presence of micromolar amounts of dGTP and dTTP as well as single-stranded oligomer primers ending 3' with repeats of the Oxytricha telomeric sequence. A nuclease activity is present in the extracts which is closely balanced with telomere terminal transferase activity. We propose a simple model for replication of the ends of linear DNA molecules based on the telomere terminal transferase.

INTRODUCTION

Telomeres provide for the stability and replication of chromosome ends (1). Telomeres behave differently in cells than do ends of broken chromosomes. Muller (2) found in Drosophila that chromosomes broken by X-irradiation were highly unstable and fused together to form new arrangements, but normal ends did not fuse. Thus one function of telomeres is to forbid recombination of the ends of chromosomes. Telomeres must also be involved in replication of the ends of linear DNA molecules. DNA polymerase makes DNA chains in the 5'-to-3' direction from a 3'-to-5' template DNA strand and requires a primer with a free 3' OH group. Therefore, if DNA polymerase were the only enzyme involved in replication of the terminal bases of a linear DNA molecule, linear chromosomes would presumably shorten with each round of replication. Since they do not, other mechanisms must maintain telomere length (discussed in 3,4).

Telomeres from Tetrahymena and Oxytricha are recognized as functional telomeres in yeast (5,6). Pluta et al. (6) and Shampay et al. (7) both further showed that yeast added approximately 300 base pairs of yeast telomeric sequence repeats onto the Oxytricha and Tetrahymena telomeric repeats at the ends of linear transforming vectors. These experiments implied two things; that there is a functional relationship between the telomeric sequences of different lower eukaryotes, and that telomeric sequences can be added onto the ends of a molecule de novo.

Greider and Blackburn (8) have identified a telomere-specific terminal transferase activity in crude extracts of vegetative and developing macronuclei from Tetrahymena. This activity adds as many as 30 tandem repeats of the single-stranded sequence TTGGGG to the 3' end of a primer consisting of the sequence $(TTGGGG)_4$, which is the Tetrahymena telomeric repeat. In addition, oligomers containing the G-rich telomeric repeats of four other lower eukaryotes primed this TTGGGG addition activity (9). Efficient synthesis of TTGGGG repeats was dependent on the presence of micromolar amounts of dGTP and dTTP, and was sensitive to heat and proteinase K. Synthesis was also sensitive to treatment with RNase A, and it is believed that the telomere terminal transferase enzyme in Tetrahymena is a multicomponent enzyme complex with RNA as a subunit (9).

Telomere length in Tetrahymena changes according to the conditions of the culture (10). This is different from the telomeres of the gene-sized macronuclear DNA molecules of hypotrichous ciliates. The macronucleus of the hypotrich Oxytricha nova contains 24,000 different DNA molecules with an average copy number of 1000 for each. These macronuclear gene-sized molecules range in size from 400 to 20,000 base pairs with an average length of 2200 base pairs (for a review see 11). Each end of each gene-sized macronuclear DNA molecule has the same telomeric structure. It is:

$$\begin{array}{r} 5'C_4A_4C_4A_4C_4- \\ 3'G_4T_4G_4T_4G_4T_4G_4T_4G_4- \end{array} \text{-Gene-} \begin{array}{l} -G_4T_4G_4T_4G_4T_4G_4T_4G_43' \\ -C_4A_4C_4A_4C_45' \end{array}$$

(12,13,14). There is no apparent template for synthesis of the 3', 16 base terminal, single-stranded tail.

In this paper we describe assays with crude extracts of Oxytricha macronuclei that demonstrate a telomere terminal transferase activity that can add G_4T_4 repeats to a G_4T_4-containing single-stranded oligonucleotide. The enzyme activity described here was found in abundance in vegetative macronuclei. In contrast, telomere terminal transferase activity studies with Tetrahymena were done with developing macronuclei in exconjugant cells (8,9). Only weak activity was detected in vegetative Tetrahymena cells. We present a simple, testable hypothesis for how the ends of the linear DNA molecules in hypotrichs are replicated.

MATERIALS AND METHODS

Cell Cultures

Oxytricha nova strain H0 was grown under non-sterile conditions using Chlorogonium (15) as a food source. Macronuclei were prepared typically from 16 L of log phase cell culture. These preparations had between 18 and 28% of the macronuclei containing replication bands (replication bands reviewed in 11).

Macronuclei Preparation

Cells were cleaned by filtering through cotton and then concentrated to 50 ml with a 10 um Nitex filter. The cells were centrifuged at 250 x g in a 50 ml conical centrifuge tube for 5 minutes. The cell pellet was resuspended in 5 ml of ice cold lysis buffer (0.01 M Tris-Cl pH 8.0, 0.5% Triton X-100, 0.05% spermidine-PO_4). The lysate was pelleted through 5% sucrose lysis buffer at 500 x g for 10 minutes at 4°C. The pellet was resuspended in 2.5 ml of lysis buffer and pelleted again through 5% sucrose lysis buffer at 500 x g for 10 minutes. This pellet was resuspended in 0.65 ml of lysis buffer and 0.35 ml of a 60% Nycodenz/percoll (w/v) solution. This was layered onto a 60% Nycodenz/percoll (w/v) step gradient of 40 to 90% in 10% steps. This was centrifuged at 16,000 x g for 10 minutes. The macronuclei band approximately 1/3 of the way down this gradient. The band was removed and pelleted through 5% sucrose lysis buffer at 500 x g for 10 minutes.

The final pellet was resuspended in 3 ml of TMS (0.01 M Tris-Cl pH 8.0, 0.01 M $MgCl_2$, 0.003 M $CaCl_2$, 0.24 M sucrose),

transfered in 110 ul aliquots to eppendorf tubes, and stored at -70°C. Concentrations of macronuclei in these preparations were in the range of 1 to 5 x 10^6 macronuclei/ml. Once it was realized that the telomere terminal transferase activity is sensitive to RNase, RNase inhibitor from human placenta (Sigma) was added to all buffers in the macronuclei preparation at a concentration of 5.6 units/ml. This resulted in more telomere terminal transferase activity in the preparations. Telomere terminal transferase activity was stable at least four months when stored this way. Most macronuclei are lysed upon thawing and incubation in the reactions at 15°C.

Synthetic Oligomers

Synthetic oligonucleotides were made on an Applied Biosystems model 380A DNA synthesizer by Jon Binkley. Oligonucleotides were gel purified on a 15% acrylamide 7 M urea preparative gel.

Reactions

Telomere terminal transferase assays were done using slight modifications of the protocol described by Greider and Blackburn (8). Reaction mixtures were made on ice and done in a total volume of 40 ul. Each reaction contained 20 ul of thawed macronuclear extract, and 10 ul of 4X reaction buffer (200 mM Tris-Cl pH 7.5, 40 mM $(NH_4)_2SO_4$, 8 mM $MgCl_2$, 0.4 mM spermidine, 4 mM spermine, 8 mM dithiothreitol). The final 10 ul contained primers, dNTPs, and sufficient dH_2O to add up to 10 ul. Cold primers were generally added as 1 ul of a 25 uM solution to give a final concentration of 0.625 uM. 5' end labeled primers were generally added so that sufficient counts were present. dNTPs were generally added as 1 ul of a 5 mM solution to give a final concentration of 125 uM. Alpha ^{32}P dTTP was added as 2 ul of an 800 Ci/mmol, 10mCi/ml solution (New England Nuclear) which gave a final concentration of 0.625 uM dTTP in the reactions.

Reactions mixtures were incubated at 15°C for 90 minutes. Reactions were stopped by addition of 95 ul of TE buffer and 5 ul of 0.5 M EDTA. Reactions were then phenol extracted once and $CHCl_3$ extracted. 2 ul of 10 mg/ml yeast RNA, 20 ul of 5 M NH_4-Acetate, and 0.5 ml of 100% ethanol were then added. These were incubated 1 hour at -20°C. Ethanol precipitates were collected

by centrifugation for 15 minutes at 4°C in an Eppendorf microcentrifuge and the pellets were 70% ethanol rinsed and dried in a Speed-Vac.

Gel Electrophoresis

Samples were resuspended in 5 ul of an 80% formamide sequencing loading buffer containing bromophenol blue and xylene cyanol as marker dyes. Samples were boiled 5 minutes and cooled on ice before loading gel. Electrophoresis was done on 16% acrylamide (19:1) 7 M urea 40 cm sequencing gels in 1X TBE buffer. Approximately 2 ul of sample were run in each lane. Electrophoresis was typically carried out at 2000 volts for 3.5 hours which put the bromophenol blue band 5 cm from the bottom of the gel. After electrophoresis the gel plates were separated and the plate containing the gel was wrapped in plastic wrap. Autoradiography was carried out at -70°C with a Kodak X-Omatic intensifying screen.

RESULTS

We used the basic protocol described by Greider and Blackburn (8) to test for the presence of a telomere terminal transferase activity in O. nova macronuclear extracts. Figure 1 shows the result of our initial assays for this activity. In this experiment, a synthetic oligonucleotide, the 16mer 5'GGGGTTTTGGGGTTTT3', which is two repeats of the Oxytricha telomeric sequence G_4T_4, was incubated in assay buffer with 125uM dNTPs, 0.625uM alpha ^{32}P dTTP, and macronuclear extracts for 90 minutes at 15°C (lanes 1-5). The primer was extended only if both dGTP and dTTP were present. It was extended in an eight-base repeat pattern of four dark bands separated by four very light bands. In this exposure, two eight-base repeats are seen. In most experiments, two to seven repeats were detected.

In figure 1, four bands are present at and below the size of the input oligomer. This is in part due to the activity of a nuclease whose presence is shown in lane 6 of figure 1. Lane 6 shows a 5' end labeled $(G_4T_4)_2$ primer incubated under the same conditions as described above except that all four dNTPs were present at 125uM concentration but no ^{32}P dNTPs were present. This primer is degraded by a nuclease activity. Extension of

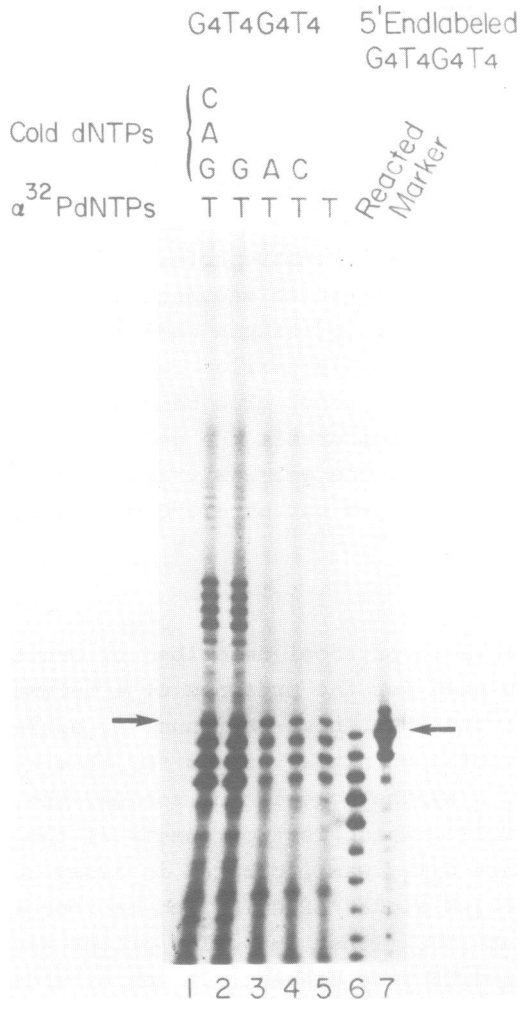

Figure 1. Initial Telomere Terminal Transferase Assays. In lanes 1 thru 5 the primer 5'$G_4T_4G_4T_4$3' was added to the reaction system with alpha ^{32}P dTTP (0.625 uM) and 125 uM of the indicated dNTPs. Lane 1) dGTP, dATP, and dCTP. Lane 2) dGTP. Lane 3) dATP. Lane 4) dCTP. Lane 5) No other dNTPs added. Lane 6 is a 5' end-labeled $G_4T_4G_4T_4$ primer incubated in the presence of all 4 dNTPs at 125 uM each (no ^{32}P added). Lane 7 is a 5' end-labeled $G_4T_4G_4T_4$ included as a control. Arrows indicate size of input oligomers.

this primer does occur and can be detected when more counts are loaded on the gel. Note that the 5' labeled 16mer runs slightly faster (halfway between a 15mer and a 16mer without a 5'

phosphate) than does the 16mer without a phosphate group at its 5' end. In figure 1, the four bands at or below the size of the 16mer input primer in lanes 1 thru 5, representing 13, 14, 15, and 16mers, correspond to the four terminal T residues of the primer. We propose that a telomere terminal transferase activity has replaced the 3' terminal T residues that have been removed by the nuclease. Strong evidence for this is in lane 5, where alpha ^{32}P dTTP was the only dNTP present, and the four terminal T residues are labeled without any extension beyond the length of the input oligonucleotide. In the presence of both dGTP and dTTP, four heavy bands occur that represent addition of 5 to 8 bases to the end of the primer (lanes 1 and 2). This indicates that in the presence of both dGTP and dTTP, GGGGTTTT is added to the 3' end of the primer GGGGTTTTGGGGTTTT. The observed pattern must mean that extended primers rarely end in G and that almost all end in T.

The apparent tendency to pause after addition of a T residue was tested by doing side-by-side terminal transferase reactions using the primers 5'GGGGTTTTGGGGTTTT3' and 5'TTTTGGGGTTTTGGGG3'. If telomeric repeats are added to the primers in the proper phase and if this addition activity has a preference for pausing after addition of a T residue, then the two primers should produce alternate patterns of T stops. Figure 2 shows the predicted pattern of stops with stops occurring most frequently where T residues are expected.

Figure 3 shows an experiment that relates telomere terminal transferase activity to dNTP concentration. In this experiment a 5' end labeled $G_4T_4G_4T_4$ oligonucleotide was added to the reaction system with varying concentrations of different dNTPs. In the lanes where the dTTP concentration was 125uM (lanes 1, 2, and 4), hesitation was pronounced only after addition of the fourth T residue. In lane 3, where the dTTP concentration was 100-fold less (1.25uM), a pattern of bands is seen corresponding to molecules ending in T residues. This is a similar pattern to that seen in experiments where cold primer is incubated with alpha ^{32}P dTTP, where the dTTP concentration is 0.625 uM. Low availability of dTTP in those experiments must cause the enzyme to add T slowly thus resulting in a pattern of bands corresponding to molecules ending in T. Reducing the dGTP

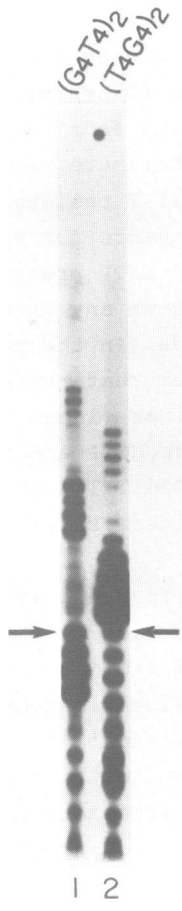

Figure 2. Comparison of $G_4T_4G_4T_4$ and $T_4G_4T_4G_4$ Primers in Telomere Terminal Transferase Assays. The primers 5'$G_4T_4G_4T_4$3' and 5'$T_4G_4T_4G_4$3' were added to the reaction system under identical conditions and run side-by-side. The concentration of each primer is 0.625 uM and the dNTPs are alpha ^{32}P dTTP (0.625 uM) and 125 uM dGTP. $(G_4T_4)_2$ is in lane 1 and $(T_4G_4)_2$ is in lane 2. Arrows indicate the size of input oligomers.

concentration (lane 4), had no effect on the pattern in comparison to lane 2, where the dGTP concentration is 100-fold higher. This suggests that the telomere terminal transferase catalyzes the addition of G residues much more efficiently than the addition of T residues. In each lane that contains dGTP and dTTP, six repeats of eight bases are present, consistent with alternate addition of four bases of G with four bases of T.

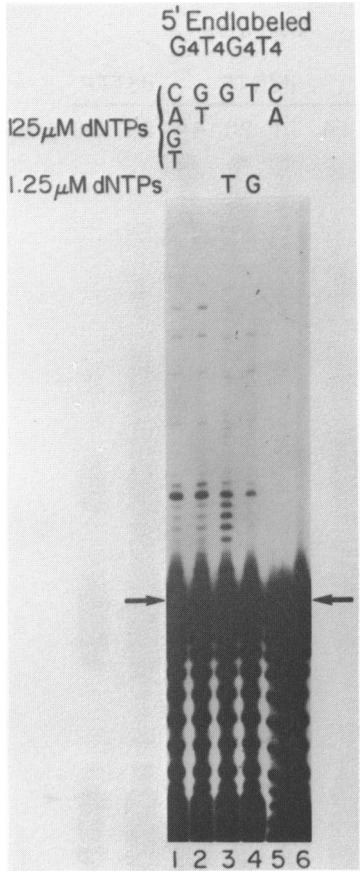

Figure 3. The Effects of dNTP Concentration on Patterns of Extension. The primer 5'$G_4T_4G_4T_4$3' labeled at its 5' end was added to the reaction system in the presence of different concentrations of dNTPs. Lane 1) 125 uM of each of the 4 dNTPs. Lane 2) 125 uM dGTP and 125 uM dTTP. Lane 3) 125 uM dGTP and 1.25 uM dTTP. Lane 4) 1.25 uM dGTP and 125 uM dTTP. Lane 5) 125 uM dCTP and 125 uM dATP. Lane 6) No dNTPs added. Arrows indicate size of input oligomer.

In an attempt to force the pattern of deoxynucleotide addition to the primer to stop at G residues, dideoxy GTP was added to the standard reaction mixture in varying ratios of ddGTP to dGTP so that ddGTP and dGTP together yielded the usual 125uM concentration in these reactions. Addition of ddGTP to a chain should cause termination because of a lack of a free 3' OH group needed for addition of the next residue. The results of

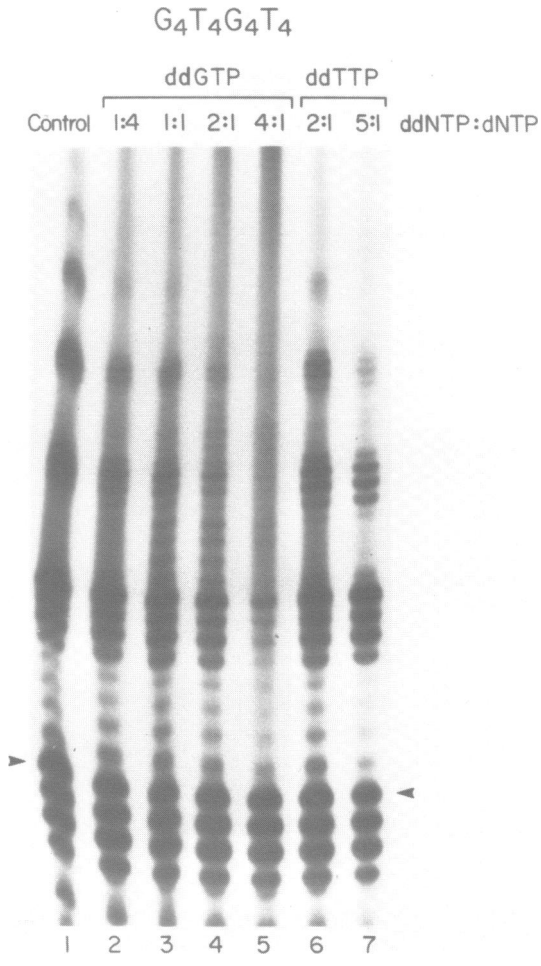

Figure 4. Telomere Terminal Transferase Assays in the Presence of Dideoxy NTPs. Lane 1) The primer 5'$G_4T_4G_4T_4$3' was added to the reaction system with 0.625 uM alpha ^{32}P dTTP and 125 uM dGTP. Lanes 2-5) Same as lane 1 with dideoxy GTP (ddGTP) added to each reaction in different ratios to dGTP so that the ddGTP and dGTP concentrations added up to 125 uM. Lane 2) 1:4 ddGTP:dGTP. Lane 3) 1:1 ddGTP:dGTP. Lane 4) 2:1 ddGTP:dGTP. Lane 5) 5:1 ddGTP:dGTP. Lane 6) Same as lane 1 but ddTTP has been added to a final concentration of 1.25 uM. 2:1 ddTTP:dTTP. Lane 7) ddTTP has been added to a final concentration of 3.125 uM. 5:1 ddTTP:dTTP. Arrows indicate size of input oligomer.

this experiment are shown in figure 4. As increasing amounts of ddGTP were added to the reactions (lanes 2-5), the amount of extension diminished and the banding patterns in the lanes

changed. Bands appear with more intensity at molecules ending in G residues, while the pattern of stops after addition of each T residue remained. It is important to note that the banding pattern for labeled molecules ending in G representing 17, 18, 19, and 20mers did not change as a result of adding ddGTP. This is because alpha ^{32}P dTTP was the radioactive label used, and since the 16mer primer had four T residues at its 3' end, 17, 18, 19, and 20mers ending in dideoxy G would not have incorporated any label. However, some of the 16mer primers were partially degraded by a nuclease and filled in with alpha ^{32}P dTTP. We assume that primers filled back to 16mers would be heavily competed against by the added, unlabeled 16mer primers, accounting for the low level of labeling at positions 17, 18, 19, and 20.

In lanes 6 and 7, ddTTP was added to the reaction in a ratio of 2:1 and 5:1 to the standard 0.625 uM concentration of alpha ^{32}P dTTP. In these lanes, the amount of extension diminished, but the pattern of stops after addition of T residues did not change. These dideoxynucleotide addition results provide additional evidence that repeats of the sequence GGGGTTTT are being added to the 3' end of the oligonucleotide.

We have characterized certain aspects of telomere terminal transferase in the crude extracts. The temperature optimum for this reaction is near 15°C, a temperature at which O. nova commonly lives in freshwater ponds. The maximum amount of extension is generally achieved by 60 minutes at this temperature; we do the standard reactions for 90 minutes to be certain of completion of the reaction. EDTA at 50mM, more than enough to bind to all the divalent cations in the reaction mixture, will stop the extension reaction as well as the the general nuclease reaction that degrades any oligonucleotide added to the reaction mixture (data not shown). EGTA at 25mM, a concentration high enough to chelate all the Ca^{++} ions in the reaction slightly enhanced the reaction. We have found that a 4x reaction buffer containing 200mM Tris-Cl pH 7.5 and 8mM $MgCl_2$ gave as much activity as the 4x reaction buffer described in the methods section that was used in all the experiments in this paper. We isolated macronuclei from a different hypotrich, a freshwater Euplotes sp., by a slight modification of the

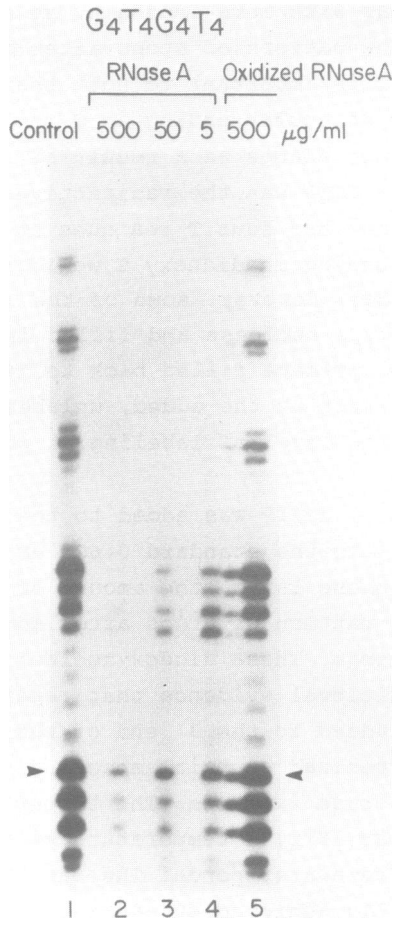

Figure 5. Effect of RNase A on the Telomere Terminal Transferase Activity. 20 ul of macronuclei and 10 ul of 4x reaction buffer were added to each tube. RNase A was added to give the following final concentrations. Lane 1) No RNase. Lane 2) 500 ug/ml RNase A. Lane 3) 50 ug/ml RNase A. Lane 4) 5 ug/ml RNase A. Lane 5) 500 ug/ml inactivated oxidized RNase A. Mixtures were incubated at 15°C for 15 minutes. 0.625 uM alpha ^{32}P dTTP, 125 uM dGTP, and 0.625 uM $G_4T_4G_4T_4$ were added to each tube. Mixtures were then incubated for 90 minutes at 15°C and stopped as described in the Materials and Methods section. Arrows indicate size of input oligomer.

protocol for Oxytricha nova macronuclear isolation. An activity, very similar to the telomere terminal transferase activity from Oxytricha nova was also present in this genus (data not shown).

Greider and Blackburn (9) report that the telomere terminal transferase in exconjugant cells of Tetrahymena is a ribonucleoprotein complex with an essential RNA component. To determine if the same is true for the telomere terminal transferase of Oxytricha nova we incubated the macronuclear extracts with assay buffer with varying concentrations of RNase A for 15 minutes at 15°C. The dNTPs and the oligonucleotide primer $G_4T_4G_4T_4$ were then added and the reactions were allowed to proceed at 15°C for 90 minutes. As can be seen in figure 5, RNase A had an inhibitory effect on the reaction. As a control we incubated the reactions with inactivated oxidized RNase A (Sigma) and no negative effect on the reaction was observed. Both RNase A and oxidized RNase A were tested for DNase activity, and none was detected (data not shown). These results imply that an RNA species is a necessary constituent of the telomere terminal transferase activity in Oxytricha nova.

Other oligomers besides the two already discussed were tested for priming ability. They are:

A) 5'CCCCAAAACCCCAAAACCCCTGCAG3'

B) 5'AATTCTGCAGGGGTTTTGGGGTTTTGGGG3'

C) 5'AATTCTGCAGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG3'

D) 5'GGACGCGTCCATATTGC3'

E) 5'GACGACTCCGGAACC3'

Results of telomere terminal transferase reactions with these oligomers are shown in figure 6. The C_4A_4-containing oligomer (A) tested in the presence of alpha ^{32}P dTTP and cold dGTP did show some type of extension (lane 1). In the absence of dGTP (lane 2), no incorporation of dTTP and therefore no extension of oligomer (A) was detected. Oligomer (A) can form a duplex structure with itself that may be a substrate for DNA polymerase:

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5' CCCCAAAACCCCAAAACCCCTGCAG 3'
                    3' GACGTCCCCAAAACCCCAAAACCCC 5'
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Any extension of this structure by DNA polymerase would require dGTP. The fact that a regular pattern of extension is not obtained with oligomer (A), along with the fact that its extension activity has different substrate requirements than the activity that extends G_4T_4-containing primers, leads us to believe that this incorporation of nucleotides is due to an

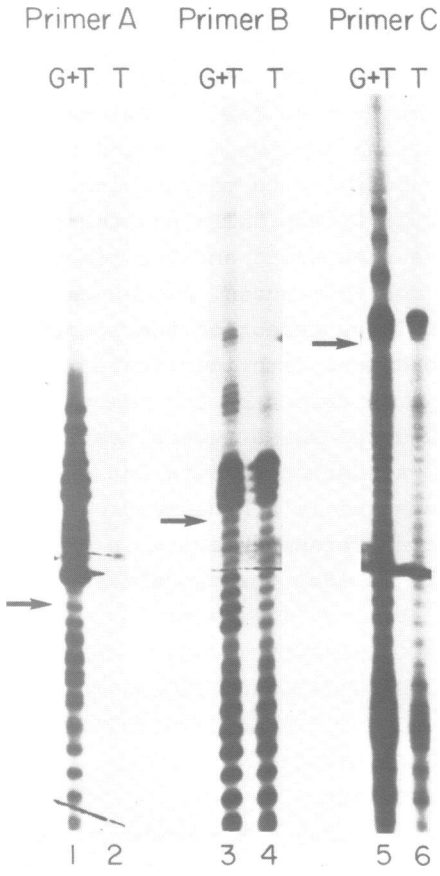

Figure 6. Additional Oligomers Tested in the Telomere Terminal Transferase Reaction System. Oligomers (A), (B), and (C) (see text for sequences) were tested in the reaction system. Lane 1) Oligomer (A) with alpha ^{32}P dTTP (0.625 uM) and 125 uM dGTP. Lane 2) Oligomer (A) with alpha ^{32}P dTTP (0.625 uM). Lane 3) Oligomer (B) with alpha ^{32}P dTTP (0.625 uM) and 125 uM dGTP. Lane 4) Oligomer (B) with alpha ^{32}P dTTP (0.625 uM). Lane 5) Oligomer (C) with alpha ^{32}P dTTP (0.625 uM) and 125 uM dGTP. Lane 6) Oligomer (C) with alpha ^{32}P dTTP (0.625 uM). Arrows indicate sizes of input oligomers.

enzyme activity other than the telomere terminal transferase. Both oligomers (B) and (C) promoted the addition of three eight-base repeats of the expected T-stop pattern (lanes 3 and 5), with oligomer (C), which contains 16 more bases of the Oxytricha telomeric repeat, being a slightly stronger primer. This

indicates that the enzyme does not count how many G_4T_4 repeats are present in the primer before it adds more repeats. This implies that some other activity is necessary to set the final telomere length of the highly defined hypotrich telomere. In lanes 4 and 6, where only dTTP was present in the reaction mixture, only four bands appear in each lane, as expected. Both primers end in GGGG; therefore when dTTP is the only dNTP present, only four Ts are added, and the bands seen in lanes 4 and 6 correspond to these Ts. Random oligomers (D) and (E) did not have any detectable extension activity.

The above oligomers were constructed so that when oligomer (A) was added to oligomer (B), a duplex would be formed containing a blunt-end telomere on one end and an EcoR1 overhang on the other end. When oligomer (A) is hybridized to oligomer (C), the resulting duplex contains an Oxytricha telomere complete with a 16 base 3' terminal extension on one end, and an EcoR1 overhang on the other end. Telomere terminal transferase assays with these double-stranded oligomers indicate that these duplexes do not act as substrates for telomere terminal transferase (data not shown).

DISCUSSION

Unlike the enzyme in Tetrahymena (8,9), telomere terminal transferase occurs abundantly in vegetative cells of Oxytricha and Euplotes. The enzyme has been studied previously in exconjugant cells of Tetrahymena; vegetative Tetrahymena show only a trace of enzyme activity (8). The earlier results in Tetrahymena are consistent with the building of telomeres on the fragments of DNA created by the breakup of chromosomes in exconjugant cells. Telomere addition is also characteristic for the fragments of DNA created by the breakup of chromosomes in exconjugant hypotrich cells (16). However, the strong enzyme activity reported here suggests that new telomeric sequences are synthesized as a regular part of DNA replication during the cell cycle of vegetatively reproducing cells.

The much greater abundance of telomere terminal transferase activity in vegetative macronuclei of hypotrichs compared to Tetrahymena was predictable. A single macronucleus of Oxytricha

nova contains 57 pg of DNA, representing 2.4 X 10^7 molecules with an average size of 2200 bp and 4.8 X 10^7 telomeres on these molecules (11). A macronucleus of Tetrahymena contains 12.2 pg of DNA, representing 2.5 X 10^4 molecules (of much larger size than in Oxytricha) and only 5 X 10^4 telomeres (270 macronuclear DNA molecules with an average copy number of 45 plus 10,000 copies of rDNA molecules (17,18)). Thus, on a per weight basis of DNA, Oxytricha must synthesize 200 fold more telomeres than Tetrahymena during each cell cycle. Thus, vegetative hypotrichs are a convenient, rich source of telomere terminal transferase.

The telomere terminal transferase activity in hypotrichs has, as expected, a different spelling specificity than the enzyme in Tetrahymena; the hypotrich enzyme specifies G_4T_4 repeats and the Tetrahymena enzyme specifies G_4T_2 repeats. The Tetrahymena enzyme in vitro can add as many as 30 repeats of G_4T_2 to a primer (8). The enzyme in Oxytricha adds two to seven repeats of G_4T_4 to a primer. The explanation of the difference might be trivial; it could reflect the way in which the extracts are made. It might also reflect the fact that the Tetrahymena enzyme has been assayed in developing macronuclei and the Oxytricha enzyme has been assayed in vegetative macronuclei. Different length telomeres may be made in the two cell states (exconjugant vs. vegetative cells). For example, in exconjugants, the telomeres on initial DNA fragments are several fold longer than those on the mature macronuclear DNA (16). Or finally the difference in the lengths produced in vitro by Tetrahymena vs. Oxytricha enzymes may simply reflect the fact that telomeres are indeed longer in DNA in vegetative macronuclei in Tetrahymena than in Oxytricha.

The telomere terminal transferase activity of Oxytricha nova has a tendency to pause after addition of the fourth T, regardless of deoxynucleotide concentration (figure 3). This indicates that the enzyme might dissociate from the oligonucleotide after addition of the fourth T and must rebind to the oligonucleotide before the next repeat can be added. This implies that the enzyme adds one repeat of GGGGTTTT, then dissociates from the primer and rebinds to add the next GGGGTTTT. This would result in a consistent pattern of stops after addition of a fourth T, which is in fact observed.

The telomere terminal transferase of Oxytricha extends primers with as many as seven repeats of G_4T_4 or T_4G_4. Yet, all telomeres in macronuclear DNA are exactly 36 nucleotides long (20 bases of G_4T_4 paired with 20 bases of C_4A_4, plus the 16 base 3' single-stranded tail). One possibility is that telomeres may be synthesized in oversized length in vegetative macronuclei and then cut back to a uniform size of 36 bases. Some observations suggest this. The macronuclear telomeres of Oxytricha form a very stable DNA-protein complex with two proteins that remain bound to the DNA even after extraction with 2 M NaCl (19). Gottschling and Zakian have isolated these two proteins and have shown that the 3' terminal extension is required for proper protein binding to the telomere (19). Price and Cech (20) have shown that the 3' terminal single-stranded extension and the adjacent duplex region form a specific complex with these proteins, which are 55 and 43 kDa in size, giving a distinctive pattern of protection of the telomere sequence from methylation by dimethyl sulfate (reviewed in 21). We have consistently observed the presence of a nuclease activity in our macronuclear crude extracts that removes deoxynucleotides from primers. We assume that this activity also removes deoxynucleotides from single-stranded 3' extensions at telomeres. In vivo, once a telomere has reached its full length, it is protected by telomere binding proteins. Telomeres may be overbuilt during DNA replication but cessation of transferase activity would allow the nuclease to reduce all 3' tails to the proper length - the 16 deoxynucleotides protected by the telomere binding proteins. The function of the telomere binding proteins during telomere replication is not known, however it seems likely that they must dissociate from the telomeres during replication.

We have shown that single-stranded oligonucleotides of 16 bases or more that end with 5'$G_4T_4$3' or 5'$T_4G_4$3' repeats can act as primers for telomere terminal transferase. Double-stranded oligonucleotides representing blunt-end telomeres or telomeres with a proper 3' terminal single-stranded extension do not act as primers for telomere terminal transferase. In vivo, the double-stranded portion of a telomere, composed of 5'$T_4G_4$3' presumably acts as a primer for addition of the 16-base single-stranded portion of the telomere by telomere terminal

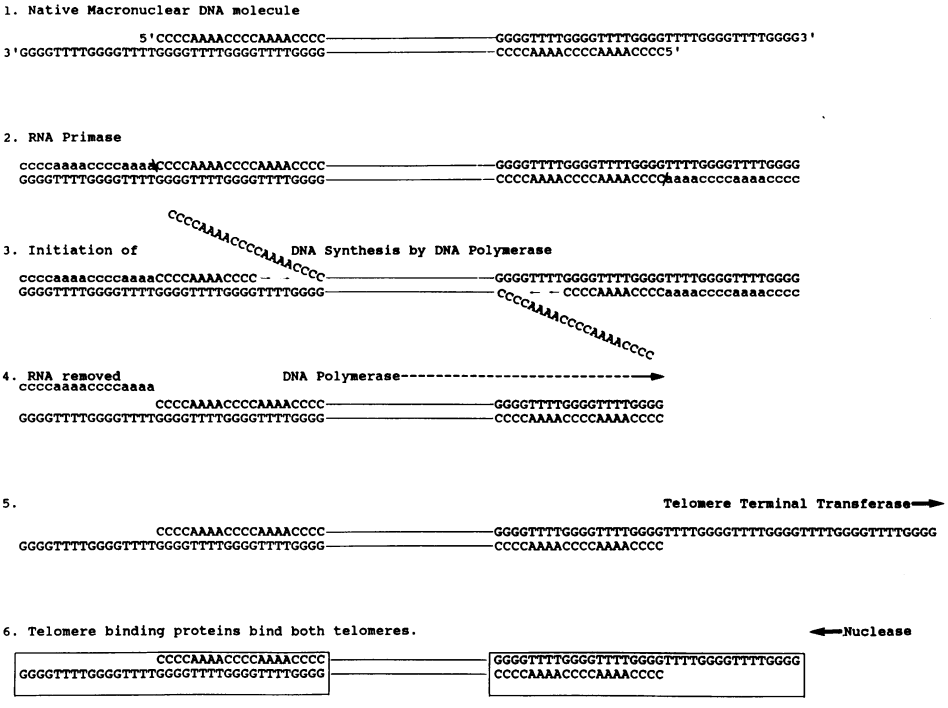

Figure 7. Model for Telomere Replication in the Macronucleus of Hypotrichous Ciliates.
1. Native macronuclear DNA molecule with telomere binding proteins removed prior to DNA replication.
2. RNA primer (lower case letters) is made on 3' terminal extension (5' gap) by an RNA primase activity.
3. DNA synthesis by DNA polymerase is initiated by RNA primer.
4. DNA polymerase replicates entire macronuclear gene but leaves a blunt end on the right end of the molecule because there is no template to create a new 3' terminal extension. RNA primer is removed.
5. Telomere terminal transferase makes a new 3' terminal extension whose length is overly long.
6. Telomere binding proteins bind to telomere with overly long single-stranded 3' terminal extension and protects first 16 bases of single-stranded region. Nuclease activity removes unprotected single-stranded region. Replication is complete.

transferase. We can think of two explanations why a similar double-stranded molecule does not work as a primer for telomere terminal transferase in vitro. First, a helicase or similar enzyme may be required to open up the double-stranded end of a newly replicated macronuclear DNA molecule to allow a single-stranded G_4T_4 strand to be recognized and thus extended by the

telomere terminal transferase. Second, the synthetic double-stranded primers may lack some property possessed by newly synthesized, blunt-ended macronuclear DNA.

We propose that replication of the ends of linear DNA molecules occurs as shown in figure 7. Replication of the 5' end is initiated by synthesis of an RNA primer, using all or part of the single-stranded telomere tail as a template. DNA polymerase replicates the entire molecule, producing a blunt-ended double-stranded telomere sequence of 20 bases at the far end of the molecule. The RNA primer is removed from the initiating end of the molecule by an RNase activity. The blunt end is extended by telomere terminal transferase with several repeats of single-stranded T_4G_4. Once this extension surpasses 16 bases it is stabilized by the binding of telomere binding proteins. Any excess over 16 bases in this single-stranded region is then removed by the nuclease activity associated with telomere terminal transferase in extracts.

Figure 7 provides a simple model for replication of the ends of a linear DNA molecule, the elements of which are testable. All enzyme activities involved in this model have been shown to exist with the exception of an RNA primase activity specific for the 3' terminal extension. In this model, the role of the 3' terminal extension made by telomere terminal transferase is to serve as a 5' gap; a region at the end of the molecule that provides a place for an RNA to be synthesized that will prime replication of the 5' end of the molecule. Synthesis of the 3' terminal extension by telomere terminal transferase as one of the last steps in replication of the telomere insures that a proper 5' end will be synthesized during the next round of replication.

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