November 1997

Coordinate Regulation of G- and C Strand Length during New Telomere Synthesis

Xinqing Fan
University of Nebraska - Lincoln

Carolyn Mary Price
University of Nebraska - Lincoln

Molecular Biology of the Cell
Vol. 8, 2145–2155, November 1997

Coordinate Regulation of G- and C Strand Length during New Telomere Synthesis

Xinqing Fan and Carolyn Mary Price*

Departments of Chemistry and Biochemistry, University of Nebraska, Lincoln, Nebraska 68588

Submitted June 23, 1997; Accepted August 25, 1997
Monitoring Editor: Joseph Gall

We have used the ciliate *Euplotes* to study the role of DNA polymerase in telomeric C strand synthesis. *Euplotes* provides a unique opportunity to study C strand synthesis without the complication of simultaneous DNA replication because millions of new telomeres are made at a stage in the life cycle when no general DNA replication takes place. Previously we showed that the C-strands of newly synthesized telomeres have a precisely controlled length while the G-strands are more heterogeneous. This finding suggested that, although synthesis of the G-strand (by telomerase) is the first step in telomere addition, a major regulatory step occurs during subsequent C strand synthesis. We have now examined whether G- and C strand synthesis might be regulated coordinately rather than by two independent mechanisms. We accomplished this by determining what happens to G- and C strand length if C strand synthesis is partially inhibited by aphidicolin. Aphidicolin treatment caused a general lengthening of the G-strands and a large increase in C strand heterogeneity. This concomitant change in both the G- and C strand length indicates that synthesis of the two strands is coordinated. Since aphidicolin is a very specific inhibitor of DNA polα and polδ, our results suggest that this coordinate length regulation is mediated by DNA polymerase.

INTRODUCTION

In most organisms telomere length is determined by a balance between activities that elongate and shorten the telomeric DNA (Greider, 1996). Telomere shortening is caused by incomplete replication of the 5′ end of the chromosome or by nuclease action, whereas elongation is caused either by telomerase, the specialized terminal transferase that extends the G-rich strand of the telomere, or by recombination. The balance between telomere lengthening and shortening is extremely important for long-term cell viability as continuous telomere lengthening or shortening results in increased cell death (Lundblad and Szostak, 1989; McEachern and Blackburn, 1995; Krauskopf and Blackburn, 1996; Wright *et al.*, 1996). For example, in human somatic cells the absence of telomerase leads to progressive loss of telomeric DNA at each population doubling until the cells eventually become senescent and die (Greider, 1996; Wright *et al.*, 1996). A similar effect is observed in yeast where mutations in telomerase components or telomere proteins lead to telomere shortening and senescence (Singer and Gottschling, 1994; Nugent *et al.*, 1996; Virta-Pearlman *et al.*, 1996).

Genetic experiments in yeast have shown that telomere length regulation is a complex process that involves many different molecules. For example, telomere length is affected by mutations not only in telomerase, but also in telomere-binding proteins, the DNA replication machinery, helicases, and check point-related proteins (Carson and Hartwell, 1985; Schulz and Zakian, 1994; Singer and Gottschling, 1994; Morrow *et al.*, 1995; Zakian, 1995; Adams and Holm, 1996; Nugent *et al.*, 1996; Virta-Pearlman, 1996; Cooper *et al.*, 1997). One general theme to emerge is that telomere proteins such as Rap1, Rif1, Rif2, Cdc13, and Est1 set up a complex chromatin structure that regulates telomerase access to the DNA terminus (Zakian, 1995; Krauskopf and Blackburn, 1996; Cooper *et al.*, 1997; Marcand *et al.*, 1997). This in turn determines whether or not telomerase can add additional telomeric DNA. However, it is still unclear what role(s)

* Corresponding author: Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588.

the checkpoint proteins, DNA polα, and replication factor C play in the length regulation process.

It has been difficult to address the role of DNA polymerase in telomere length regulation as mutations in replication proteins usually affect general chromosomal replication in addition to any telomeric functions. Thus, although mutations in yeast polα and replication factor C cause telomere lengthening (Carson and Hartwell, 1985; Adams and Holm, 1996), it is unclear whether these proteins are directly involved in telomere length regulation or whether the mutations cause a secondary effect on telomere length as a result of a general replication defect. It is widely assumed that DNA polα/primase is an active participant in telomere maintenance as the normal DNA replication machinery is thought to generate the telomeric C-strand once telomerase has extended the G-strand (Greider, 1996; Skopp *et al.*, 1996; Reveal *et al.*, 1997). However, there is little experimental evidence to support this contention.

The ciliate *Euplotes crassus* has proved particularly useful for studying telomere biochemistry because this organism has literally millions of telomeres that are generated by a multistep process during the sexual stage of the life cycle (Prescott, 1994). Since no general DNA replication occurs during the new telomere synthesis, *Euplotes* provides a unique opportunity to study the role of DNA polymerase in telomeric C strand synthesis without the complication of simultaneous DNA replication. We have made use of this aspect of *Euplotes* biology to examine the link between C strand synthesis and telomere length regulation.

Like other ciliates, *Euplotes* has two structurally and functionally distinct nuclei: the germline micronucleus and the transcriptionally active macronucleus. The micronucleus contains 50–100 large chromosomes whereas the macronucleus contains millions of linear gene-sized DNA molecules [average size 2 kilobases (kb)] that have telomeres on each end (Prescott, 1994). The macronucleus is formed from a copy of the micronucleus as a result of a complex genomic reorganization that takes place when *Euplotes* cells mate (see Figure 1 and Jahn, 1991). During this ~100-h process the DNA in the developing macronucleus (or anlage) first replicates to form polytene chromosomes (Figure 1), after which various noncoding DNA sequences [e.g., internal eliminated sequences (IESs) and the transposon-like TEC elements] are eliminated (Frels and Jahn, 1995). Subsequently, the individual genes are excised as free linear DNA molecules, and telomeres are added to each end (Roth and Prescott, 1985). These newly synthesized telomeres are longer and more heterogeneous in length than the telomeres on mature macronuclear molecules (Vermeesch and Price, 1994). They are later trimmed to the mature size (Roth and Prescott, 1985; Vermeesch *et al.*, 1993). *E. crassus* is ideal for analyzing the various steps in de novo telomere synthesis because this species will mate synchronously, thus allowing one to obtain cultures in which all the cells are at a particular stage of macronuclear development.

Telomere length is very precisely regulated in mature *Euplotes* macronuclei as the G-strands are exactly 42 nucleotides long while the C-strands are 28 nucleotides long. As a result, each telomere consists of 28 base pairs of $C_4A_4{\cdot}G_4T_4$ duplex DNA and a 14-nucleotide G strand overhang (Klobutcher *et al.*, 1981). In contrast, the G-strands of newly synthesized telomeres are heterogeneous in length and range from 33–106 nucleotides. The median length is 94–95 nucleotides (Vermeesch and Price, 1994). Surprisingly, the C-strands of newly synthesized telomeres are much less heterogeneous than the G-strands as the majority are exactly 84 nucleotides long. Thus, C strand length is much more tightly regulated than G strand length. This finding suggests that although synthesis of the G-strand (by telomerase) is the first step in new telomere addition, a major regulatory step occurs during subsequent C strand synthesis.

Our discovery, that C- rather than G strand length is tightly regulated during new telomere addition, led us to wonder whether G- and C strand synthesis might be regulated coordinately rather than by independent mechanisms. Since the telomeric C-strand is thought to be synthesized by DNA pol α/primase, we decided to test for coordinate G- and C strand regulation by determining what happens to G strand length when C strand synthesis is inhibited using a DNA polymerase inhibitor. We show here that if the drug aphidicolin is used to partially inhibit DNA polymerase, both G- and C strand length are altered. These results indicate that telomeric G- and C strand synthesis are indeed coordinately regulated. Our results also suggest that DNA polymerase is involved in this coordinate length regulation.

MATERIALS AND METHODS

Growth of E. crassus *and Isolation of Anlagen DNA*

Euplotes cells were grown and mated as previously described (Price 1990; Price *et al.*, 1994) except that the cultures were concentrated fivefold just before aphidicolin addition. The timing of telomere addition was determined by Southern hybridization and dimethylsulfate (DMS) cleavage (Vermeesch *et al.*, 1993). The concentration of aphidicolin required to inhibit DNA replication and hence cell division, was determined by growing *Euplotes* cells in 10–100 μg/ml of the drug and counting the number of cells on a daily basis. A concentration of 40 μg/ml was found to reversibly inhibit cell division without causing cell death. Anlagen were isolated using the guanidinium thiocyanate method as previously described (Vermeesch and Price, 1994). Mature macronuclei and anlagen and macronuclear DNA were isolated as described (Price *et al.*, 1994).

Southern Hybridization

Three micrograms of anlagen DNA or 1.5 μg macronuclear DNA were separated on 1.0% agarose gels and transferred to nylon mem-

brane (Magna Nylon, Micron Separations, Inc., West Borrough, MA) as previously described (Vermeesch and Price, 1993). To detect the long C-strands from newly synthesized telomeres but not the shorter mature macronuclear telomeres, the filters were hybridized overnight with a 5′ end-labeled 64-base oligonucleotide $(G_4T_4)_8$ at 46°C in 400 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA, 50% formamide, 0.1% SDS, and 5× Denhardt's solution. The filters were then washed three times for 20 min at 66°C with 0.1% SSC and 0.2% SDS and exposed to film. Hybridization at temperatures greater than 46°C resulted in a weak hybridization to the newly synthesized telomeres, while lowering the temperature by even 1–2°C resulted in hybridization to the shorter mature telomeres. Optimal hybridization to the short macronuclear telomeres was observed when the filters were hybridized at 40°C and then washed at 60°C.

Monitoring of DNA Replication.

Levels of DNA replication were determined by measuring [^{3}H]thymidine incorporation as described by Frels and Jahn (1995). To determine the timing of the rounds of DNA replication that immediately precede telomere addition, mated cells were concentrated four- to fivefold and aliquoted into a microtiter dish. [^{3}H]thymidine (10 μCi/ml) was added to the wells at 2-h intervals between 28 and 40 h of development. After a 2-h incubation, total cellular DNA was isolated from each sample, and the amount of ^{3}H incorporation was determined by scintillation counting. To determine the extent to which aphidicolin inhibited DNA replication, 10 μCi/ml [^{3}H]thymidine and 0 or 40 μg/ml aphidicolin were added to either vegetative cells or mated cells at 35 h of development. The DNA was isolated 5 h later. Three samples of DNA were isolated for each time point, and each experiment was repeated three times.

Guanine and Thymine-specific Cleavage

Guanine-specific modification and cleavage were performed as previously described (Vermeesch and Price, 1994). One microliter of a 1:100 dilution of DMS was added to 19 μl 3′ end-labeled DNA and allowed to stand for 10 min at 25°C. Twenty microliters of 2 M pyrrolidine were added to stop the reaction, and the DNA was cleaved at methylated guanines by heating to 90°C for 15 min. Cleavage at single-stranded thymines was achieved using $KMnO_4$ as described previously (Williamson *et al*., 1989). Ten microliters of 1 mM $KMnO_4$ were added to 10 μl of 3′ end-labeled DNA in Tris-EDTA and allowed to stand for 5 min at 25°C. The reaction was stopped by addition of 1 μl allyl alcohol. The DNA was then cleaved at modified thymidines by adding 20 μl pyrrolidine and heating to 90°C for 15 min.

Telomerase Assays

Telomerase-containing extracts were prepared from mated *Euplotes* cells as described by Bednenko *et al.* (1997). Telomerase assays were performed in 20 μl reactions containing 2 μl of telomerase extract, variable amounts of aphidicolin, 0.4 μM primer, 5 mM $MgCl_2$, 20 mM EGTA, 50 mM Tris, pH 8.0, 1 mM spermidine, 1 mM dithiothreitol, and 0.5 μM [^{32}P]deoxyguanosine triphosphate (dGTP) (800 Ci/mmol)(Bednenko *et al.*, 1997). Reactions were incubated at 30°C for 1 h. They were extracted with phenol/chloroform, and the products were separated on 8% sequencing gels. Each assay was performed in duplicate, and each experiment was repeated three times. The amount of reaction product was determined using a PhosporImager (Molecular Dynamics, Sunnyvale, CA) and analyzed for significant differences using a one-way analysis of variance. To compensate for uneven losses during sample preparation and gel loading, the total amount of reaction product measured for each lane was compared with an internal standard that was added after the telomerase reaction was complete but prior to DNA isolation. The processivity for each reaction was compared by measuring the amount of product in repeat 1 relative to repeat 10 for each lane.

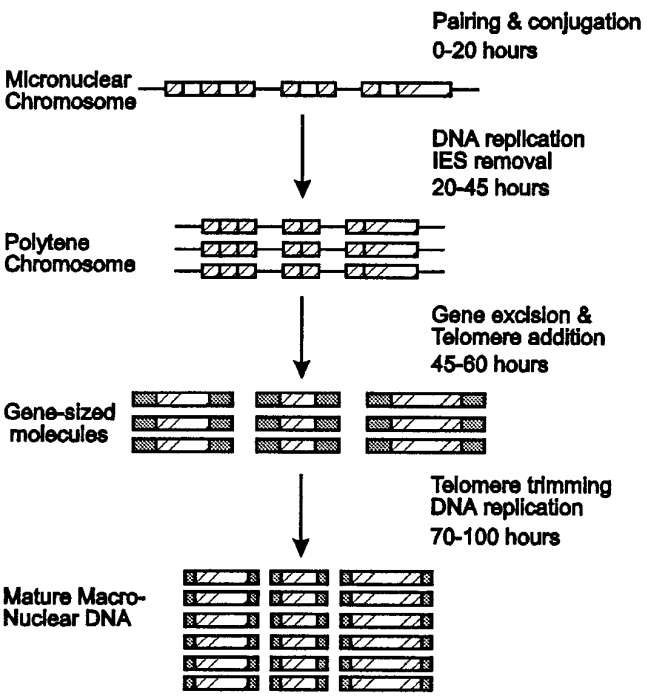

Figure 1. Stages in *Euplotes* macronuclear development. The hatched boxes represent coding sequences; the shaded boxes represent telomeric DNA; the open boxes represent IESs or internal eliminated sequences; and the thin line represents other micronuclear sequences.

Measuring G- and C Strand Length by Cloning and Sequencing

Cloning and sequencing were performed essentially as described (Vermeesch and Price, 1993). To make the C-tailed library, *Euplotes* DNA was treated with deoxycytidine triphosphate and terminal transferase for 5 min at 25°C while the linearized plasmid (pTZ19R) was treated with dGTP and terminal transferase for 15 min at 37°C. The G- and C-tailed DNAs were mixed, incubated at 65°C for 10 min and at 57°C for 90 min, and then used to transform *Escherichia coli* DH5α cells. The blunt-end libraries were made by treating *Euplotes* DNA with T4 DNA polymerase in the presence of dGTP and deoxythymidine triphosphate. The blunt-ended anlagen DNA was then ligated to vector DNA that had been cut with Ecl135II (New England Biolabs, Beverly, MA). Plasmids containing anlagen DNA inserts were obtained by picking white colonies and then sequenced using a sequenase kit (United States Biochemical, Cleveland, OH). It was difficult to sequence through more than 25 G_4T_4 repeats; thus the numbers given in Figure 6 are a minimum estimate. The distribution of terminal nucleotides obtained from the sequencing experiments was different from that observed in the DMS cleavage experiment (Figure 4) because in the DMS cleavage experiment the low [^{32}P]cordycepin concentration caused molecules ending in G to be preferentially labeled [see Vermeesch and Price (1994) for discussion].

RESULTS

Effect of Aphidicolin on Telomerase Activity

Since aphidicolin is a very specific inhibitor of DNA polymerase α and δ (Spadari *et al*., 1982; Byrnes, 1984)

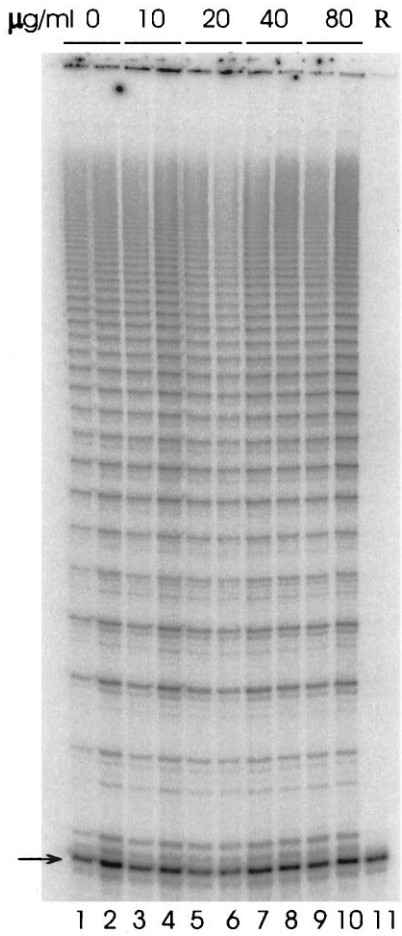

Figure 2. Effect of aphidicolin on telomerase activity. The amount of aphidicolin added to each reaction is shown above each lane. Each reaction was done in duplicate. In lane R, the telomerase extract was treated with RNase. The arrow marks the internal standard that was added to monitor sample loss.

and is known to inhibit DNA replication in *Euplotes* (Olins and Olins, 1994), it seemed likely that we could use this drug to selectively inhibit C strand synthesis during de novo telomere addition. Previous experiments had shown that aphidicolin has no effect on telomerase from *Tetrahymena* (Greider and Blackburn, 1985); however, no experiments had been done with the *Euplotes* enzyme. To ensure that *Euplotes* telomerase is also unaffected by the drug, we set up a series of in vitro telomerase reactions that contained increasing concentrations of aphidicolin. As shown in Figure 2, even 80 μg/ml aphidicolin had no apparent effect on the telomerase activity. The concentration 80 μg/ml was double that needed to completely inhibit growth of *Euplotes* cells (see MATERIALS AND METHODS). To monitor both the extent of the reaction and the processivity of the enzyme more closely, we used a PhosphorImager to measure either the total amount of reaction product in each lane or the amount of product in repeat 1 relative to repeat 10 (the ratio is a useful measure of processivity).When we analyzed the results using a one-way analysis of variance, we found no evidence that the drug treatment had altered either the amount of reaction product ($p > 0.96$; $df = 4, 20$) or the processivity ($p > 0.967$; $df = 4, 20$). Thus, we conclude that *Euplotes* telomerase is unaffected by aphidicolin.

Inhibition of DNA Replication by Aphidicolin

As growth of vegetative *Euplotes* cells was completely but reversibly inhibited by 40 μg/ml aphidicolin (see MATERIALS AND METHODS), we next investigated how efficiently this drug concentration inhibits DNA polymerase in living cells. This was done by measuring ^{3}H incorporation when either vegetative or mated cells were treated with aphidicolin and pulse labeled with [^{3}H]thymidine. To ensure that the ^{3}H was incorporated by DNA polymerase rather than telomerase, we performed the pulse labeling of the mated cells during the rounds of DNA replication that accompany polytene chromosome formation immediately preceding telomere addition (see Figure 1). This was necessary because dATP and dCTP, the only nucleotides used by DNA polymerase during telomere addition, can be converted to the telomerase substrates dGTP and dTTP by various biosynthetic pathways. To precisely identify the timing of the rounds of DNA replication, we performed preliminary experiments in which samples of mated cells were incubated with [^{3}H]thymidine for 2-h intervals during early macronuclear development (Frels and Jahn, 1995). When we isolated the DNA and measured the extent of ^{3}H incorporation, we found that replication peaked between 35 and 40 h of development (Fan and Price, unpublished results).

To compare the level of DNA replication in the presence or absence of aphidicolin, we added [^{3}H]thymidine plus 0 or 40 μg/ml aphidicolin to samples of vegetative cells or mated cells at 35 h of development. After a 5-h incubation, we isolated the DNA and measured the extent of ^{3}H incorporation. As shown in Figure 3, the aphidicolin caused a 65–70% decrease in ^{3}H incorporation by the vegetative cells but only a 30–35% decrease for the developing cells. Despite this relatively low level of inhibition for the mated cells, we chose to use a maximum of 40 μg/ml aphidicolin in subsequent experiments because higher concentrations were toxic to vegetative cells. The same relatively inefficient inhibition of DNA replication during polytene chromosome formation has been reported by other researchers (Frels and Jahn, 1995). It is likely to result, in part, from decreased drug uptake due to the changes to the cell pellicle that accompany mating. Also, the DNA degradation that occurs at this time

may yield high nucleotide pools that compete with aphidicolin for polymerase binding (Sheaff *et al.*, 1991).

Effect of Aphidicolin on New Telomere Synthesis

To maximize the effect of aphidicolin treatment on new telomere synthesis, we added the drug to mated *Euplotes* cells a number of hours before new telomere addition and then isolated the developing macronuclei (anlagen) several hours after telomere addition would normally be complete. Since preliminary experiments established that telomere addition starts ~48 h post mating (Fan and Price, unpublished results), we allowed mated *Euplotes* cells to proceed through macronuclear development for 44 h to ensure that DNA replication was complete. We then concentrated the cells fivefold (to reduce the total amount of drug needed) and added the aphidicolin. The anlagen were isolated 16 h later.

The effect of the aphidicolin treatment on G strand length was determined using the Maxam and Gilbert G-sequencing reaction. Anlagen DNA was 3′ end labeled, treated with DMS, cleaved at methylated G's, and separated on 8% sequencing gels. Figure 4A shows the G-cleavage pattern obtained with DNA from mature macronuclei and mated cells that were treated with 0, 10, 20, or 40 μg aphidicolin. Figure 4B shows the pattern obtained with mature macronuclei, with anlagen DNA from various control cultures (untreated cells, concentrated cells, and cells treated with dimethyl sulfoxide (DMSO), the solvent for aphidicolin), and anlagen DNA isolated from cells treated with 40 μg/ml aphidicolin. As expected, the DNA from mature macronuclei gave a characteristic G_4 cleavage pattern that extended 42 nucleotides up the gel (Figure 4, A and B, lane 1). Similarly, the anlagen DNA samples from untreated cells, the various control cultures, and cells treated with the lower concentrations of aphidicolin all gave a cleavage pattern that extended the expected ~98 nucleotides (Figure 4A, lanes 2–4; Figure 4B, lanes 2–4). However, the G-cleavage pattern from cells treated with 40 μg/ml aphidicolin extended considerably further up the gel and was clearly visible for about 150 nucleotides (Figure 4, A–C, lane 5). The cleavage pattern also showed a slight shift relative to the untreated samples, which probably reflected the effect of the aphidicolin on the identity of the 3′ terminal nucleotide (see Figure 6). The extension of the G-cleavage pattern beyond the normal ~98 nucleotides indicated that the aphidicolin had caused a disruption in G strand length regulation. This result suggested that DNA polymerase plays a role in regulating G strand synthesis.

As DNA polymerase was only partially inhibited when mated cells were treated with 40 μg/ml aphidicolin, we did not expect the drug treatment to prevent C strand synthesis. To confirm this suspicion, we used

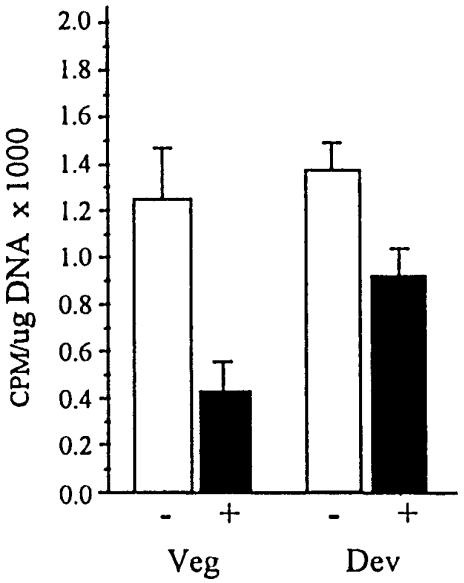

Figure 3. Inhibition of DNA replication by aphidicolin. The shaded columns show the extent of ^{3}H incorporation by aphidicolin-treated cells; the unshaded columns show ^{3}H incorporation by untreated cells. The columns marked VEG show the ^{3}H incorporation by vegetative cells; those marked DEV show the ^{3}H incorporation by cells at 35 h of development. The bars above each column represent the SD.

Southern hybridization to look for newly synthesized C-strands on the telomeres from aphidicolin-treated cells. Since preparations of anlagen DNA always contain some DNA from the old macronucleus, it was necessary to develop hybridization conditions that would detect the long stretches of C_4A_4 sequence characteristic of newly synthesized telomeres, but not the shorter stretches present on telomeres from mature and old macronuclei. This was achieved by using a 64-nucleotide T_4G_4 probe and carefully regulated hybridization conditions (see MATERIALS AND METHODS). When the probe was hybridized to DNA from mature macronuclei or anlagen DNA isolated at 44 h of development (i.e., before telomere addition), no hybridization signal was observed (Figure 5, lanes 1 and 6), whereas a strong hybridization signal was observed with anlagen DNA isolated from control cells at 60 h of development (lanes 2–4). As shown in lane 5, DNA isolated from cells treated with 40 μg/ml aphidicolin also gave rise to a strong hybridization signal. This demonstrated that 40 μg/ml aphidicolin does not prevent C strand synthesis even though it does affect G strand length.

Interestingly, the DNA from the aphidicolin-treated cells gave a much more intense hybridization signal near the top of the gel than the DNA from control cells. Since this apparent increase in DNA size was too large to be accounted for by the addition of longer telomeres, we initially suspected that it might be

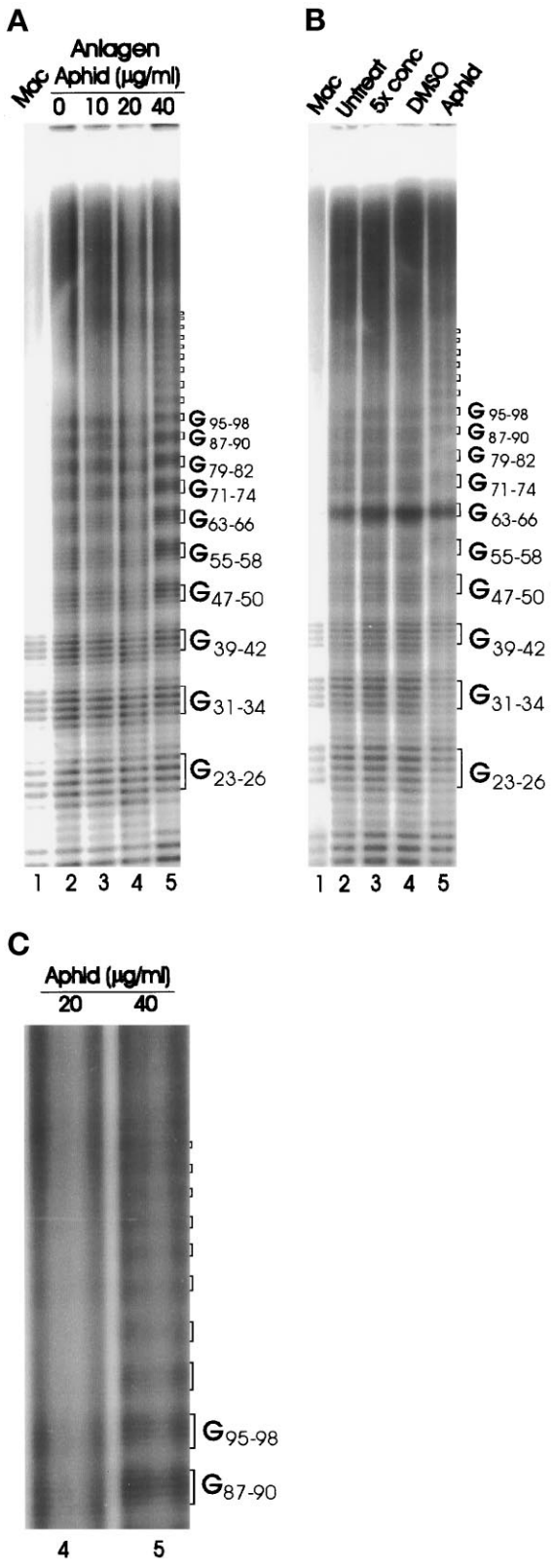

Figure 4.

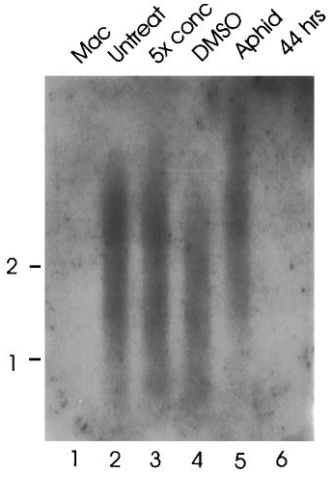

Figure 5. Detection of long C-strands by Southern hybridization $(T_4G_4)_8$. Lane 1, DNA from mature macronuclei; lanes 2–4, anlagen DNA isolated from control cells at 60 h of development; lane 2, DNA from untreated cells; lane 3, DNA from fivefold concentrated cells; lane 4, DNA from concentrated cells treated with 0.4% DMSO; lane 5, anlagen DNA isolated from aphidicolin-treated cells at 60 h of development; lane 6, anlagen DNA isolated at 44 h of development. The position of 1 kb and 2 kb markers are shown to the right.

caused by either a decrease in TEC or IES element removal, or an inhibition of chromosome fragmentation. However, when we performed quantitative polymerase chain reaction across known sites of TEC and IES removal or chromosome fragmentation (Frels and Jahn, 1995), we were unable to detect a decrease in any of these events (Fan and Price, unpublished results). Instead, we were able to show that the apparent change in size could be abolished by heating the DNA before it was loaded on the gel. Thus, it is likely that intermolecular interactions between regions of single-stranded G_4T_4 sequence (see later experimental results) caused the DNA to migrate abnormally. G_4T_4 sequences have a strong tendency to form G-quartets and other unusual structures (Williamson *et al.*, 1989).

G- and C Strand Length in Aphidicolin-treated Cells

While the DMS cleavage experiment shown in Figure 4 demonstrated that aphidicolin treatment causes an increase in the length of the newly synthesized G-strands, the experiment did not provide information about the length distribution of these overlong telomeres. Likewise, the Southern blot shown in Figure 5 merely demonstrated that the newly synthesized C-strands were longer than the C-strands from mature

Figure 4 (cont). Examination of G strand length by DMS cleavage. (A) lane 1, G-cleavage pattern of mature macronuclear DNA. Lanes 2-5, anlagen DNA isolated from cells treated with 0, 10, 20, or 40 μg/ml aphidicolin. (B) lane 1, G-cleavage pattern of mature macronuclear DNA; lane 2, anlagen DNA from untreated cells; lane 3, anlagen DNA from fivefold concentrated cells; lane 4, anlagen DNA from concentrated cells treated with 0.4% DMSO; lane 5, anlagen DNA from concentrated cells treated with 40 μg/ml aphidicolin. (C) Enlargement of lanes 4 and 5 from panel A showing the G-cleavage pattern extending beyond G_{95-98}. All anlagen DNA was isolated at 60 h of development. The positions of the guanines relative to the 3′ terminus are marked at the side of each gel.

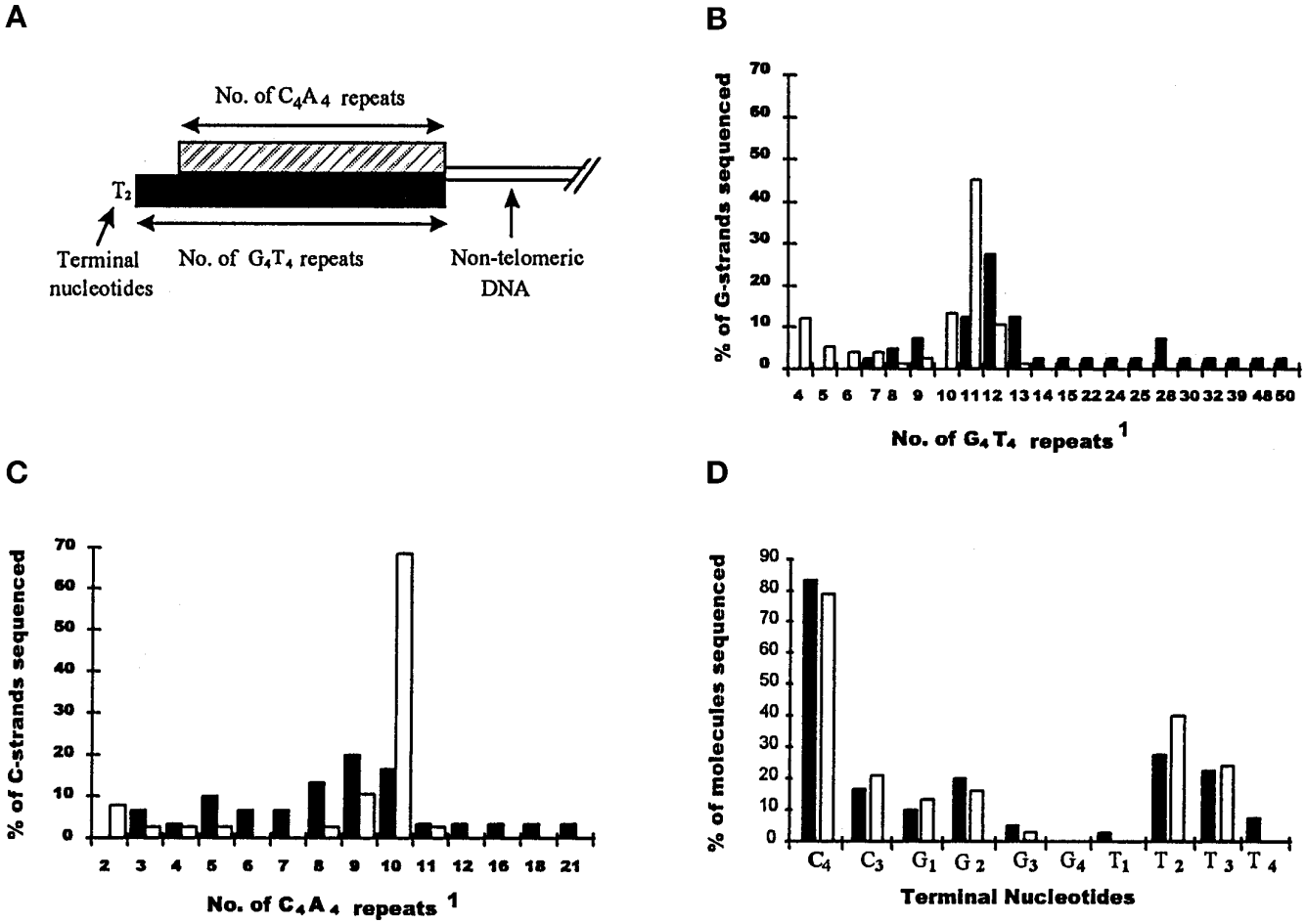

Figure 6. G- and C strand length distribution. (A) Features of a *Euplotes* telomere. (B) Comparison of the length of the telomeric G-strands from control cells (the clear bars) and aphidicolin- treated cells (black bars). For molecules with telomeres longer than 200 nucleotides (25 repeats), the number of G_4T_4 repeats represents the minimum number of repeats present. (C) Comparison of the length of the telomeric C-strands from control cells (clear bars) and aphidicolin-treated cells (black bars). The data for the control cells in panels B and C are a combination of the data mentioned in the results and in Vermeesch and Price (1994). [1], Note the scale on the x axis is not linear. (D) Identity of the terminal nucleotides on the G- and C-strands of telomeres from untreated cells (clear bars) or aphidicolin-treated cells (black bars).

telomeres (the probe only hybridized to longer telomeres), but gave no indication of their actual size. Thus, to obtain a more accurate picture of both G- and C strand length, we cloned individual anlagen DNA molecules and determined the length of either the G- or C-rich strands by direct sequencing.

Libraries that contained the entire telomeric G-strand were made by C-tailing anlagen DNA and G-tailing linearized vector DNA, allowing the tailed DNAs to anneal, and then transforming them into *E. coli* (Vermeesch and Price, 1993). Libraries that contained only the duplex region of the telomere were created by removing the G strand overhang with T4 polymerase (Vermeesch and Price, 1993). The resulting blunt-ended molecules were ligated to vector DNA that had been linearized with a blunt cutter, and transformed into *E. coli*. Clones containing insert DNA were isolated and the DNA was sequenced. To ensure that our results were representative, we made C-tailed libraries from three different cultures of aphidicolin-treated cells and blunt-end libraries from two different cultures. In each case, one library was prepared with DNA from untreated cells.

G Strand Length. When we analyzed the telomeres from the C-tailed library made with DNA from untreated cells, we observed the same G strand length distribution as was reported previously (Vermeesch and Price, 1994). While the G-strands were heterogeneous in length, the majority of the telomeres contained 11 G_4T_4 repeats and none contained more than 13 (see Figure 6A and B). A significant population of the molecules had one telomere that was much shorter and contained only four or five repeats. As previously observed, the identity of the terminal nucleotide var-

ied considerably. About 80% of the G-strands terminated in either T_2 or T_3, and the remainder ended in G_1, G_2, or G_3. None terminated in T_1 or T_4 (Figure 6D).

The G strand length distribution from aphidicolin-treated cells was quite different from that of the control cells (Figure 6B). The most striking difference was that about one-third of the G-strands contained more than 13 G_4T_4 repeats, and many were considerably longer. The longest telomere measured had 50 repeats (400 nucleotides) of G_4T_4 sequence instead of the normal 10–13 repeats (80–100 nucleotides). Furthermore, the population of molecules with one short telomere (four to six repeats) had disappeared, and while about half of the molecules still had telomeres that contained 10–13 G_4T_4 repeats, the median number of repeats was shifted from 11 to 12. The aphidicolin treatment also affected the identity of the terminal nucleotide as some of the G-strands now terminated in T_1 and T_4 (Figure 6D). This change in terminal nucleotide distribution was particularly obvious for the molecules with very long G-strands.

Although only one-third of the telomeres sequenced had unusually long G-strands, we suspect that molecules with very long G-strands were underrepresented in our libraries. First, the intensity of the DMS cleavage pattern (Figure 4) suggests that a large fraction of the telomeres had G-strands of more than 100 nucleotides. Second, the relative fraction of molecules with overlong G-strands varied considerably between the different libraries. As discussed in the next section, many of the telomeres from aphidicolin-treated cells have extended G strand overhangs. Since we did not include fill in and ligation steps when we made the C-tailed libraries, molecules with long overhangs were probably cloned with reduced frequency. The cloning efficiency was probably also reduced by the long overhangs being removed by nucleases or folding to form G-quartet structures.

C Strand Length. When we analyzed C strand length using the blunt-end library made with DNA from untreated cells, we found that both the length distribution and the identity of the terminal nucleotide were quite tightly regulated. As reported by Vermeesch and Price (1993), the majority of the C-strands were 84 nucleotides in length and 80% were between 72 and 84 nucleotides long (i.e., 9 or 10 C_4A_4 repeats, see Figure 6C). As previously observed, almost 90% of the molecules terminated in C_4 and the remainder ended with a C_3 (Figure 6D). When we analyzed the C-strands from aphidicolin-treated cells, we found that the drug treatment had altered the C strand length distribution, but the effect was different from that observed with the G-strands. Instead of becoming generally longer, the C strands' length distribution had become much more heterogeneous (Figure 6C). Only ~40% of the molecules had C-strands of 72 or 84 nucleotides while ~40% were 64 nucleotides or less and ~20% were 92 nucleotides or longer. The longest C-strand sequenced was 168 nucleotides long (21 repeats). The drug treatment had no effect on the terminal nucleotide distribution (Figure 6D).

Since aphidicolin treatment caused an alteration in both G- and C strand length, we conclude that G- and C strand synthesis must be coordinately regulated. Our findings also suggest that DNA polymerase plays a role in this coordinate length regulation.

Length of the G Strand Overhang

In normal *Euplotes* cells the G-strands of newly synthesized telomeres are slightly longer than the C-strands; therefore, most telomeres have a 9- to 14-nucleotide G strand overhang (Vermeesch and Price, 1994). Since the G-strands from aphidicolin-treated cells were on average considerably longer than the C-strands, it seemed likely that many of the telomeres would have abnormally long G strand overhangs. To assay for these long tracts of single-stranded DNA, we used T4 polymerase to remove any 3′ overhang (Vermeesch and Price, 1994) and then looked for a decrease in the length of the G_4T_4 repeat pattern when the DNA was 3′ end labeled and cleaved at G residues. The T4 polymerase digestion did appear to reduce the G-cleavage pattern by three to four repeats (Fan and Price, unpublished results), suggesting that long G strand overhangs were present on some molecules. However as the data were rather indistinct, we decided to use potassium permanganate footprinting to verify this result. Potassium permanganate reacts with non–base-paired thymidines.

To perform the footprinting, DNA isolated from mature macronuclei or the anlagen of control or aphidicolin-treated cells was 3′ end labeled with [^{32}P]cordycepin. Half of the DNA was then denatured by boiling while half was maintained in the native state. Both the denatured and native DNA samples were treated with potassium permanganate and cleaved at the modified T's by treatment with pyrrolidine, the DNA fragments were then separated on sequencing gels. Figure 7A, lanes 1–3, shows the T-cleavage pattern obtained with denatured DNA from aphidicolin-treated cells, control cells, and mature macronuclei. As expected, the T-cleavage pattern for the denatured DNA extended the full length of the telomere. This was 40 nucleotides for the macronuclear DNA (lane 3) and ~94 nucleotides for the control anlagen DNA (Figure 7A, lane 2; and Figure 7B, lanes 2 and 3).

Unlike the cleavage pattern obtained with the denatured DNA, the pattern obtained with the native DNA samples only extended the length of the G strand overhang (Figure 7A, lanes 4–6). For mature macronuclear DNA this was 13–14 nucleotides while for the anlagen DNA from untreated cells the pattern faded

out at 13–15 nucleotides. The cleavage pattern for the DNA from aphidicolin- treated cells was strongest for the first 10–15 nucleotides. However an additional three to four sets of T_4 repeats were also clearly visible. Thus, a significant proportion of the telomeres had an unusually long G strand overhang. PhosphorImager analysis of the relative amount of product in the various sets of T_4 repeats revealed that approximately 40% of telomeres had G strand overhangs of 38 nucleotides or more.

Testing of Other DNA Polymerase Inhibitors

The most obvious interpretation of our results is that the deregulation of G- and C strand length was caused by the partial inhibition of DNA polymerase by aphidicolin. However, it was possible that the observed alterations in G- and C strand length were caused by a secondary effect of the drug. We attempted to address this possibility by using other replication inhibitors to interfere with C strand synthesis. Unfortunately, experiments with araA and araC, the only relatively specific DNA polymerase inhibitors to be taken up by *Euplotes* cells, were unsuccessful. We could not achieve sufficiently high concentrations of araA to inhibit DNA replication, and the araC treatment caused extensive nicking of the chromosomal DNA; therefore, we were unable to use any of our standard techniques to analyze telomere length. Thus, we cannot formally exclude the possibility that the deregulation of G- and C strand length is caused by the aphidicolin affecting something other than DNA polymerase. However, since numerous studies have shown that aphidicolin is a very specific inhibitor of eukaryotic replicative polymerases and does not affect DNA methylation, transcription, translation, or nucleotide biosynthesis (Spadari *et al.*, 1982), this is unlikely to be the case.

DISCUSSION

Regulation of telomere length is a critical aspect of telomere function because this is how most eukaryotes solve the chromosome end replication problem (Lundblad and Szostak, 1989; Bacchetti, 1996; Nugent and Lundblad, 1996). It is now clear that G strand length is strongly influenced by various telomere-binding proteins that regulate access of telomerase to the DNA terminus (Krauskopf and Blackburn, 1996; Lin and Zakian, 1996; Nugent *et al.*, 1996; Virta-Pearlman *et al.*, 1996; Cooper *et al.*, 1997; van Steensel and de Lange, 1997). While several studies have suggested that C strand synthesis may be also be involved in regulating telomere length (Carson and Hartwell, 1985; Adams and Holm, 1996), very little is known about this aspect of telomere biology. We have now examined whether synthesis of the telomeric C-strand influences G strand length during de novo telomere synthesis in *Euplotes*. We did this by using the drug aphidicolin to interfere with C strand synthesis and then determining the effect of the drug treatment on both G- and C strand length. The aphidicolin treatment caused a concomitant change in G- and C strand length, indicating that synthesis of the two strands of the telomere is coordinately regulated. Since aphidicolin is a very specific inhibitor of DNA polα and polδ (Spadari *et al.*, 1982), our results suggest that this coordinate length regulation is mediated by DNA polymerase.

One relatively straightforward way to achieve coordinate regulation of G- and C strand synthesis would

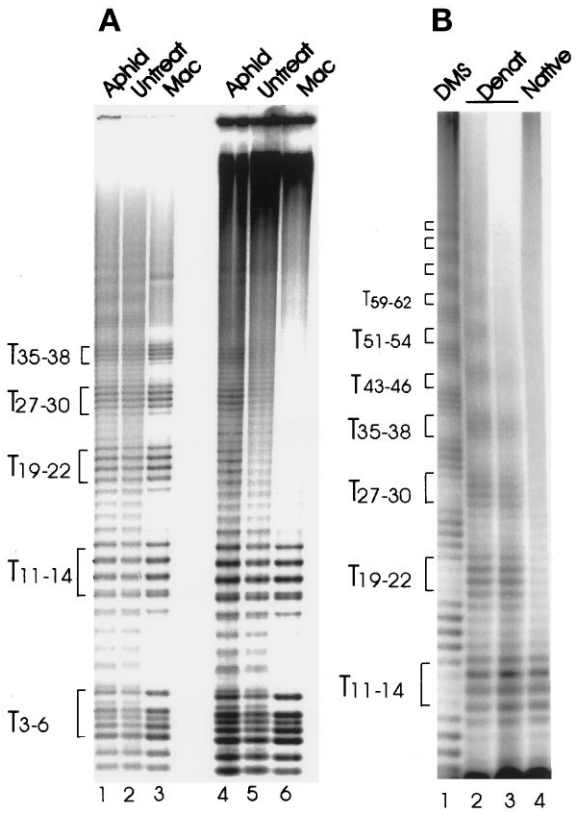

Figure 7. Examination of 3′ overhang length by $KMnO_4$ footprinting. The samples in panel A were run on a 15% sequencing gel while the samples in panel B were run on an 8% gel. (A) Lanes 1–3, denatured DNA from anlagen of aphidicolin-treated cells (lane 1), anlagen of untreated cells (lane 2), or mature macronuclei (lane 3). Lanes 4–6, nondenatured DNA from aphidicolin-treated cells (lane 4), untreated cells (lane 5), or mature macronuclei (lane 6). The heterogeneity in the lower bands of the T_4 repeat pattern was caused by end labeling of molecules ending in both G_2 and G_1 (the macronuclear DNA was slightly degraded). (B) Lane 1, marker lane showing the G-cleavage pattern obtained after DMS treatment of anlagen DNA from untreated cells. Lanes 2 and 3, T-cleavage pattern obtained after $KMnO_4$ footprinting of denatured anlagen DNA from untreated cells; lane 2, 1 mM $KMnO_4$; lane 3, 2 mM $KMnO_4$. Lane 4, T-cleavage pattern obtained with nondenatured anlagen DNA from untreated cells after footprinting with 1 mM $KMnO_4$.

be for the initiation of C strand synthesis to prevent further addition of T_4G_4 repeats to the G-strand. This might be achieved by polα/primase displacing or inhibiting telomerase. Such a scenario could explain the normal heterogeneity in the G-strands of newly synthesized telomeres because the precise length of the G-strand would be determined by the speed with which polα/primase initiates C strand synthesis after the first 84 nucleotides of G-strand have been generated. If primer synthesis normally occurs very promptly, G strand length would be kept under 100 nucleotides. The increase in G strand length in response to aphidicolin treatment could be explained by the drug causing a delay in primer synthesis. This delay would give telomerase more time to add on G_4T_4 repeats, enabling longer G-strands to be generated. The delay in initiation could result from the aphidicolin slowing down elongation of previously initiated C-strands by competing for the deoxynucleoside triphosphate binding site on polα. Another possibility is that chromosome fragmentation and telomere addition are achieved by a multiprotein complex that includes not only the cleavage factors and telomerase (Fan and Yao, 1996), but also polα/primase. The association between telomerase and polα could be critical for ensuring timely initiation of C strand synthesis. This association might be disrupted by the aphidicolin treatment.

At present the process that determines C strand length is unknown; however it must involve a length-measuring activity that directs polα/primase to initiate primer synthesis at a set distance along the G-strand. The broadening of the C strand length distribution in response to aphidicolin treatment indicates that the drug disrupts this precise positioning of primer synthesis. There are several obvious mechanisms by which this might occur. First, an association between telomerase and polα could be important for regulating not only the timing but also the position at which C strand synthesis is initiated. Disruption of this association by aphidicolin might lead to primer synthesis at random sites along the C-strand. An alternative, but not mutually exclusive, explanation for the effect of aphidicolin is that the long G-strands titrate out a single-strand binding protein that normally binds along the G-strand and directs polα/primase to initiate C strand synthesis at a set distance from the junction with the nontelomeric DNA. This protein could measure G strand length in a manner analogous to the measurement of poly A tail length by poly A binding protein (Keller, 1995).

Although the extensive new telomere synthesis that takes place in *Euplotes* is unique, the coordinate regulation of G- and C strand synthesis that we have observed in this ciliate is likely to be a common feature in all organisms in which significant C strand synthesis takes place (Price, 1997). Indeed, the yeast cdc17 (polα) and cdc44 (replication factor C) mutations provide evidence for coordinate regulation of G- and C strand length in yeast (Carson and Hartwell, 1985; Adams and Holm, 1996). These mutants both exhibit telomerase- dependant telomere elongation as well as increased telomere length heterogeneity. These changes in telomere length are likely to result from impaired C strand synthesis. In yeast, long G strand overhangs are generated during late S phase; these then disappear in G2 (Wellinger *et al.*, 1993, 1996). Since yeast telomeres do not suddenly shorten in G2, the G strand overhang is thought to be filled in by DNA polymerase rather than being removed. In the case of the polα and replication factor C mutations, disruption of this C strand fill-in would give telomerase more time to add repeats to the telomeric G-strand and hence lead to a net telomere lengthening in a manner analogous to what we have observed in *Euplotes*.

ACKNOWLEDGMENTS

We thank Paul Carlson for help with the telomerase assays and Carolyn Jahn for help with the quantitative polymerase chain reaction to analyze IES and Tec excision. We are most grateful to the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program at the National Cancer Institute for supplying us with aphidicolin. This work was supported by grant GM-41803 from the National Institutes of Health.

REFERENCES

Adams, A.K., and Holm, C. (1996). Specific DNA replication mutations affect telomere length in *Saccharomyces cerevisiae*. Mol. Cell. Biol., *16*, 4614–4620.

Bacchetti, S. (1996). Telomere dynamics and telomerase activity in cell senescence and cancer. Semin. Cell. Dev. Biol, *7*, 31–39.

Bednenko, Melek, J.M., Greene, E.C., and Shippen, D.E. (1997). Developmentally regulated initiation of DNA synthesis by telomerase: evidence for factor-assisted de novo telomere formation. EMBO J, *16*, 2507–2518.

Byrnes, J. (1984). Structural and functional properties of DNA polymerase delta from rabbit bone marrow. Mol. Cell. Biochem., *62*, 13–24.

Carson, M.J., and Hartwell, L. (1985). CDC17: an essential gene that prevents telomere elongation in yeast. Cell, *42*, 249–257.

Cooper, J.P., Nimmo, E.R., Allshire, R.C., and Cech, T.R. (1997). Regulation of telomere length and function by a Myb-domain protein in fission yeast. Nature, *385*, 744–747.

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

Frels, J.S., and Jahn, C.J. (1995). DNA rearrangements in *Euplotes crassus* coincides with discrete periods of DNA replication during the polytene chromosome stage of macronuclear development. Mol. Cell. Biol., *16*, 6488–6495.

Greider, C.W. (1996) Telomere-length regulation. Annu. Rev. Biochem., *65*, 337–65.

Greider, C.W., and Blackburn, E.H. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell, *43*, 405–413.

Jahn, C. (1991). The nuclear genomes of hypotrichous ciliates: maintaining the maximum and the minimum of information. J. Protozool., *38*, 252–258.

Keller, W. (1995). No end yet to messenger RNA 3′ processing. Cell, *81*, 829–832.

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

Krauskopf, A., and Blackburn, E.H. (1996). Control of telomere growth by interactions of *RAP1* with the most distal telomeric repeats. Nature, *383*, 354–357.

Lin, J.J., and Zakian, V.A. (1996). The *Saccharomyces* CDC13 protein is a single-strand $TG_{1–3}$ telomeric DNA-binding protein in vitro that affects telomere behavior in vivo. Proc. Natl. Acad. Sci. U.S.A., *93*, 13760–13765.

Lundblad, V., and Szostak, J.W. (1989). A mutant with a defect in telomere elongation leads to senescence in yeast. Cell, *57*, 633–643.

Marcand, S., Gibson, E., and Shore, D. (1997). A protein-counting mechanism for telomere length regulation in yeast. Science, *275*, 986–990.

McEachern, M.J., and Blackburn, E.H. (1995) Runaway telomere elongation caused by telomerase RNA gene mutations. Nature, *376*, 403–409.

Morrow, D.M., Tagle, D.A., Shiloh, Y., Collins, F.S., and Hieter, P. (1995). TEL1, an *S. cerevisiae* homolog of the human gene mutated in Ataxia telangiectasia, is functionally related to the yeast checkpoint gene *MEC1.* Cell, *82*, 831–840.

Nugent, C.I., Hughes, T.R., Lue, N., and Lundblad, V. (1996). CDC13 is a single-stranded telomere binding protein with a dual role in yeast telomere maintenance. Science, *274*, 249–252.

Olins, D.E., and Olins, A.L. (1994). The replication band of ciliated protozoa. Int. Rev. Cytol., *153*, 137–170.

Prescott, D.M. (1994) The DNA of ciliated protozoa. Microbiol. Rev., *58*, 233–267.

Price, C.M. (1990). Telomere structure in *Euplotes crassus*: characterization of DNA-protein interactions and isolation of a telomere-binding protein. Mol. Cell. Biol., *10*, 3421–3431.

Price, C.M. (1997). Synthesis of the telomeric C-strand. Biochemistry, In press.

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

Reveal, P.M., Henkels, K.M., and Turchi, J.J. (1997). Synthesis of the mammalian telomere lagging strand in vitro. J. Biol. Chem. *272*, 11678–11681.

Roth, M., and Prescott, D.M. (1985). DNA intermediates and telomere addition during genome reorganization in *Euplotes crassus*. Cell, *41*, 411–417.

Schulz, V.P., and Zakian, V.A. (1994). The *Saccharomyces PIF1* DNA helicase inhibits telomere elongation and de novo telomere formation. Cell, *76*, 145–156.

Sheaff, R., Ilsley, D., and Kuchta, R. (1991). Mechanism of DNA polymerase α inhibition by aphidicolin. Biochemistry, *30*, 8590–8597.

Singer, M.S., and Gottschling D.E. (1994). *TLC1*: template RNA component of *Saccharomyces cerevisiae* telomerase. Science, *266*, 404–409.

Skopp, R., Wang, W., and Price, C.M. (1996). rTP: a candidate telomere protein that is associated with DNA replication. Chromosoma, *105*, 82–91.

Spadari, S., Sala, F., and Pedrali-Noy, G. (1982). Aphidicolin: a specific inhibitor of nuclear DNA replication in eukaryotes. Trends Biochem. Sci., *7*, 29–32.

van Steensel, B., and de Lange, T. (1997). Control of telomere length by the human telomeric protein TRF1. Nature, *385*, 740–743.

Vermeesch, J.R., and Price, C.M. (1994). Telomeric DNA sequence and structure following de novo telomere synthesis in *Euplotes crassus*. Mol. Cell. Biol., *14*, 554–566.

Vermeesch, J.R., Williams, D., and Price, C.M. (1993). Telomere processing in *Euplotes*. Nucleic Acids Res., *21*, 5366–5371.

Virta-Pearlman, V., Morris, D.K., and Lundblad, V. (1996). EST1 has the properties of a single-strand telomere end-binding protein. Genes Dev., *10*, 3094–3104.

Wellinger, R.J., Ethier, K., Labrecque, P., and Zakian, Z.A. (1996). Evidence for a new step in telomere maintenance. Cell, *85*, 423–433.

Wellinger, R.J., Wolf, A.J., and Zakian, Z.A. (1993). Origin activation and formation of single-strand $TG_{1–3}$ tails occur sequentially in late S phase on a yeast linear plasmid. Mol. Cell. Biol., *13*, 4057–4065.

Williamson, J.R., Raghuraman, M.K., and Cech, T.R. (1989). Monovalent cation-induced structure of telomeric DNA: the G-quartet model. Cell, *59*, 871–880.

Wright, W.W., Brasiskyte, K., Piatyszek, M.A., and Shay, J.W. (1996). Experimental elongation of telomeres extends the lifespan of immortal x normal cell hybrids. EMBO J., *15*, 1734–1741.

Zakian, V.A. (1995). Telomeres: function, structure and replication. In *Telomeres*, ed. E.H. Blackburn and C.W. Greider, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 107–138.