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Identification of a Specific Telomere Terminal Transferase Activity in Tetrahymena Extracts

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Summary

We have found a novel activity in Tetrahymena cell free extracts that adds tandem TTGGGG repeats onto synthetic telomere primers. The single-stranded DNA oligonucleotides $(TTGGGG)_4$ and TGTGTGGGTGTGTGGGTGTGTGGG, consisting of the Tetrahymena and yeast telomeric sequences respectively, each functioned as primers for elongation, while $(CCCCAA)_4$ and two nontelomeric sequence DNA oligomers did not. Efficient synthesis of the TTGGGG repeats depended only on addition of micromolar concentrations of oligomer primer, dGTP, and dTTP to the extract. The activity was sensitive to heat and proteinase K treatment. The repeat addition was independent of both endogenous Tetrahymena DNA and the endogenous α-type DNA polymerase; and a greater elongation activity was present during macronuclear development, when a large number of telomeres are formed and replicated, than during vegetative cell growth. We propose that the novel telomere terminal transferase is involved in the addition of telomeric repeats necessary for the replication of chromosome ends in eukaryotes.

Introduction

Telomeres, the ends of eukaryotic chromosomes, are essential elements that stabilize chromosome ends (Muller, 1938; McClintock, 1941) and allow the complete replication of linear DNA molecules (reviewed in Blackburn and Szostak, 1984). A common feature of telomeres is a terminal DNA region consisting entirely of tandemly repeated units of simple, G+C-rich sequences. All of the known repeat units conform to the general formula $C_n(A/T)_m \cdot (T/A)_mG_n$ where n = 1–8 and m = 1–4 (reviewed in Blackburn, 1984). The orientation of these repeats with respect to the chromosome end is always the same; the C-rich strand runs 5′ to 3′ from the end of the chromosome toward the interior. Structural and functional studies of chromosomes and linear plasmids in yeast have shown that the only DNA elements essential for telomere function are the simple G+C-rich telomeric sequence repeats, in the correct orientation (Szostak and Blackburn, 1982; Murray and Szostak, 1983; Shampay et al., 1984; J. W. Szostak, personal communication).

Despite the conserved nature of telomeric sequences, the number of tandem sequence repeats on a given telomere is not fixed. Telomeric restriction fragments are commonly variable in length, forming diffuse bands upon gel electrophoresis (Blackburn and Gall, 1978; Johnson, 1980; Emery and Weiner, 1981; Shampay et al., 1984). The length variability of these fragments lies entirely within the region of telomeric repeats (Blackburn and Gall, 1978; Emery and Weiner, 1981; Blackburn et al., 1983). In addition to this variability, a net increase in telomere length occurs during long term logarithmic phase growth of trypanosomes and the ciliate Tetrahymena. In both Trypanosoma brucei and Tetrahymena thermophila the telomeres lengthen steadily, by 4–10 base pairs per cell generation, over the course of 200–300 cell generations (Bernards et al., 1983; D. Larson and E. Spangler, unpublished results). The length increase of the total population of macronuclear telomeres in Tetrahymena is entirely attributable to an increase in the number of telomeric sequence repeats on the telomeres (D. Larson and E. Spangler, unpublished results).

Together, these findings show that telomeres are dynamic structures capable of a net increase in length. However, DNA polymerases function in the 5′ to 3′ direction and require a template and primer for DNA synthesis, which means that the ends of chromosomal DNA should become progressively shortened over the course of many rounds of DNA replication (Cavalier-Smith, 1974). The fact that chromosome length is maintained, and even increased, strongly suggests that the replication of telomeric ends is not accomplished solely by the action of conventional DNA replication enzymes.

The telomeric sequences of the ciliates Tetrahymena and Oxytricha stabilize the ends of linear DNA molecules in yeast, allowing them to be maintained and replicated in this organism (Szostak and Blackburn, 1982; Pluta et al., 1984). Yeast repeats are added onto the ends of Tetrahymena telomeres after maintenance and replication of a linear plasmid in yeast; this is evident because the $C_{1\text{-}3}A \cdot TG_{1\text{-}3}$ telomeric repeat units of yeast are distinct from the telomeric CCCCAA·TTGGGG repeats of Tetrahymena (Shampay et al., 1984). Recombination between the Tetrahymena repeats and resident yeast telomeres is not likely to account for these findings because of the lack of sequence homology of the repeats and the lack of a requirement for the *RAD52* recombination function in yeast (Dunn et al., 1984). Based on all these considerations, the proposal was made that telomere replication involves a terminal transferase-like activity which adds the host cell telomeric sequence repeats onto recognizable telomeric ends (Shampay et al., 1984). In this model, shown schematically in Figure 1, a telomeric sequence of G-rich DNA is added de novo onto the preexisting telomeric end. Once this protruding G-rich strand is formed, it can serve as the template for the synthesis of the complementary C-rich strand by conventional primase and DNA polymerase activities. Removal of RNA primer and incomplete synthesis or ligation of the most recently added terminal repeats would account for the single-strand breaks found in the distal part of this strand in several organisms (Blackburn and Gall, 1978; Johnson, 1980; Katzen et al., 1981; Szostak and Blackburn, 1982; Blackburn and Challoner, 1984).

During macronuclear development in mated Tetra-

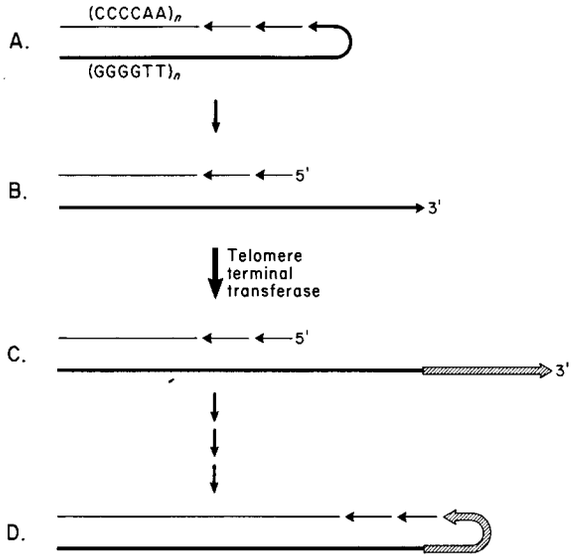

Figure 1. Model for Telomere Elongation

(A) The Tetrahymena telomere structure is shown schematically with a fold-back TTGGGG strand and small gaps on the CCCCAA strand (Blackburn and Gall, 1978; Blackburn et al., 1983; Blackburn, 1985). (B) The terminal region of the TTGGGG strand is made accessible for elongation at its 3′-OH end. (C) Telomere terminal transferase adds TTGGGG repeats to the TTGGGG strand. (D) DNA polymerase and associated primase activities copy the TTGGGG sequence, and incomplete ligation results in single-strand gaps on the CCCCAA strand. The newly synthesized single-stranded $(GGGGTT)_n$ self-associates into a fold-back secondary structure.

hymena cells, the five pairs of germ-line micronuclear chromosomes are fragmented into more than 200 pieces, and new telomeres are generated at the ends of these fragments. The DNA is then replicated to an average final ploidy level of 45C (reviewed in Gorovsky, 1980). We reasoned that the activity of telomere-replicating enzymes might therefore be greater during macronuclear development than during vegetative growth. Thus we made extracts both from synchronously mating Tetrahymena cultures and from nonmated cells in logarithmic phase growth.

We describe the discovery of an activity in Tetrahymena cell free extracts that synthesizes tandem TTGGGG repeats de novo, as predicted by the model described above. The DNA oligonucleotides that prime the elongation activity are telomeric sequences made up of the G-rich strand which is predicted to prime the addition of telomeric repeat units. From these findings, we propose that the novel terminal transferase-like activity in the Tetrahymena extracts is involved in the de novo elongation step of telomere replication.

Results

Cell Free Extracts Contain a Telomere Elongation Activity That Incorporates Only dGTP and dTTP

The proposed de novo addition of telomeric sequence repeats requires the free 3′-OH of the telomeric G+T strand as depicted in Figure 1. Therefore, the synthetic single-stranded DNA oligomer 5′-TTGGGGTTGGGGTTGGGGT-

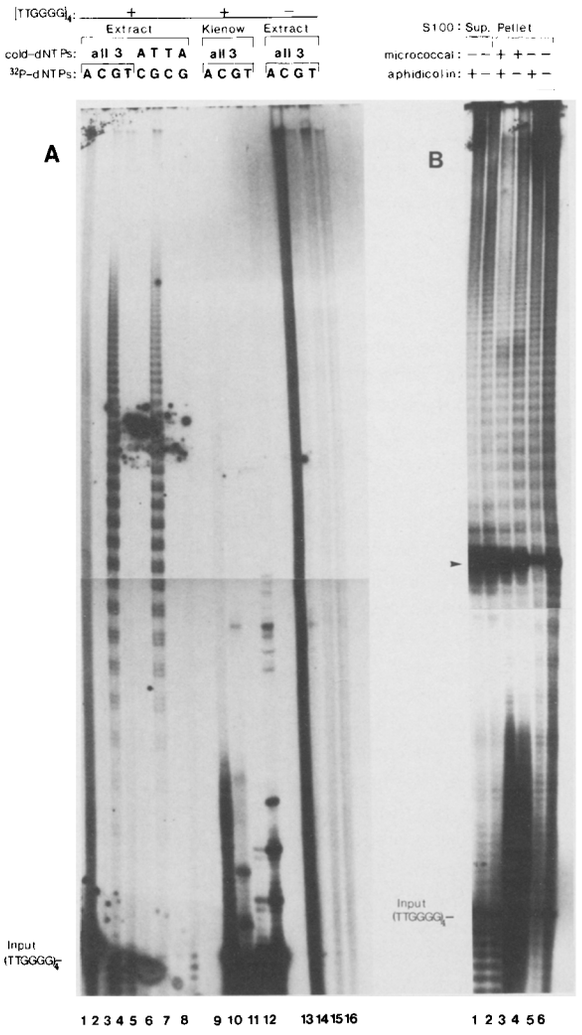

Figure 2. Nucleotide and Enzyme Requirements of the Elongation Reaction

(A) Each α-^{32}P-dNTP was added in turn to an in vitro reaction in the presence of the other three unlabeled dNTPs (lanes 1–4). In addition, four combinations of only two nucleotides were tested (lanes 5–8). Unlabeled oligomer was at 0.25 μM, the indicated α-^{32}P-dNTPs were present at 1.25 μM, and the unlabeled dNTPs were present at 150 μM. In the control lanes, 13–16, no oligomer was added. Lanes 9–12 show the repeat patterns synthesized when purified E. coli DNA polymerase I Klenow fragment (New England Biolabs) was substituted for the extract.

(B) The extracts were separated into S100 pellet and supernatant fractions and tested for activity in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of 20 μg/ml aphidicolin. The reactions contained 0.25 μM unlabeled $(TTGGGG)_4$, 1.25 μM α-^{32}P-dGTP, and 50 μM dTTP. In addition, the pellet fraction was pretreated with micrococcal nuclease to destroy the endogenous Tetrahymena DNA (lanes 3 and 4) or mock digested by incubation with BSA (New England Biolabs) for 10 min at 37°C (lanes 5 and 6). The dark band that appears in all lanes (arrowhead) is due to trapping of labeled material by carrier tRNA.

TGGGG-OH-3′ [$(TTGGGG)_4$] was used as a primer to assay for telomere elongation activity. The $(TTGGGG)_4$ primer was added to the Tetrahymena cell free extracts along with various combinations of α-^{32}P-labeled deoxynucleoside triphosphates (α-^{32}P-dNTPs) and unlabeled nucleoside triphosphates (dNTPs). After incubation for 90 min at 30°C, the reaction mixtures were phenol extracted,

ethanol precipitated, and analyzed by electrophoresis in a 6% polyacrylamide, 7 M urea sequencing gel.

The autoradiograms in Figure 2 show that an activity in extracts made from cells during macronuclear development elongated the unlabeled input $(TTGGGG)_4$ oligomer by the addition of DNA with a 6 base repeating pattern; up to 30 repeats were added. In Figure 2A, each of the four α-^{32}P-dNTPs was added in turn in the presence of the other three dNTPs, or different combinations of only two nucleotides were added at a time. The 6 base repeat pattern was seen only with α-^{32}P-dGTP and three unlabeled dNTPs (lane 3) or α-^{32}P-dGTP plus unlabeled dTTP (lane 6). α-^{32}P-dGTP was not incorporated into a 6 base repeat pattern in the absence of added dTTP (lane 8). In the absence of added oligomer, no repeats were synthesized (lanes 13–16). Poly(dG) is known to self-associate (Dugaiczyk et al., 1980; reviewed in Saenger, 1984), and dG can base pair with dT in a non-Watson-Crick manner (Brown et al., 1985; reviewed in Saenger, 1984). The possibility of repeat synthesis through self-priming and template slippage is eliminated by the absence of label incorporation when α-^{32}P-dCTP and dATP are added (Figure 2A, lanes 2 and 5).

The 6 base periodicity of the reaction products, and the dependence of α-^{32}P-dGTP incorporation on the presence of dTTP, suggested that repeats of the sequence TTGGGG, $[(TTGGGG)_n]$, were being synthesized. Although in Figure 2A, lane 4, α-^{32}P-dTTP is not incorporated into the elongation products when the three other dNTPs are present, as would be expected for the synthesis of TTGGGG, α-^{32}P-dTTP incorporation was seen in later experiments (see Figure 3). This lack of label incorporation will be discussed below. In a control experiment, when the single-stranded pBR322 sequence 5′-GGAGCCACTATCGACTTCGCGATCATGGCGACCA-OH-3′ was tested as a primer under conditions identical to those in Figure 2A, no repeat addition was seen with any combination of added nucleotides (data not shown).

Further evidence that self-priming and template slippage do not account for the observed elongation was obtained by substituting pure E. coli DNA polymerase I Klenow fragment for the Tetrahymena extract. Lanes 9–12 in Figure 2A show that this purified enzyme was capable of synthesizing a few repeats from $(TTGGGG)_4$. However, both the pattern and length of the products were strikingly different from those in the reactions catalyzed by the Tetrahymena extracts. With Klenow fragment, the input oligonucleotide was extended by only a few repeats, and each α-^{32}P-dNTP was incorporated when the other three cold dNTPs were present. Interestingly, these findings also showed that $(TTGGGG)_4$ must be able to self-associate, by either intermolecular or intramolecular interactions, into the antiparallel duplex structure required as template-primer for E. coli polymerase I (reviewed in Kornberg, 1980). Such higher order interactions of telomeric G+T strands may be important for both telomere structure and function. No addition products were seen when the single-stranded pBR322 oligomer was incubated with Klenow fragment and various combinations of dNTPs (data not shown).

In order to determine whether the repeats are added onto the input $(TTGGGG)_4$ oligomer, we added oligomer labeled with ^{32}P at the 5′ end to the extract along with all six possible combinations of two unlabeled dNTPs. The input end-labeled oligomer was elongated with the characteristic 6 base repeat pattern only when both dGTP and dTTP were added to the reaction. In addition, the action of nucleases in the extracts on the input 5′-end-labeled oligomer was examined. Endogenous nuclease activity created a single base ladder with bands of uniform intensity from 1 to 24 bases (data not shown). This result indicates that the characteristic 6 base repeat pattern seen in Figure 2 is not due to a sequence-specific nuclease activity.

Addition of Telomeric Sequence Repeats Is Template Independent

The crude extracts used in these experiments contain endogenous Tetrahymena DNA including long stretches of double-stranded telomeric sequence $(CCCCAA)_n \cdot (TTGGGG)_n$. To test whether the repeat addition was independent of this endogenous Tetrahymena DNA, the whole cell extracts were separated into S100 supernatant and pellet fractions. The pellet fraction, which contains endogenous cellular DNA, was pretreated with micrococcal nuclease. Under the nuclease conditions used, over 90% of a double-stranded $(CCCCAA)_{46} \cdot (TTGGGG)_{46}$ DNA restriction fragment, added to the S100 fraction as an internal control, was digested (data not shown; see Experimental Procedures). The DNA-containing S100 pellet fraction had the same amount of elongation activity with or without pretreatment with micrococcal nuclease (Figure 2B, lanes 3–6). In addition, the S100 supernatant fraction promoted the addition of many repeats (lanes 1 and 2), although the amount of endogenous DNA in this fraction was negligible. The low molecular weight material in lanes 3 and 4 results from an endogenous repair activity (see later) acting on the endogenous DNA that has been reduced to oligonucleotides of less than 40 bases in length by micrococcal nuclease treatment.

To test the possibility that hybridization of the input synthetic oligomer to endogenous $(CCCCAA)_n \cdot (TTGGGG)_n$ tracts allowed templated repeat synthesis via either strand displacement or nick translation, we tested the ability of a 5′-CCCCAACCCCAACCCCAACCCCAA-3′ $[(CCCCAA)_4]$ oligomer to prime repeat synthesis. No elongation of the $(CCCCAA)_4$ oligomer occurred with any combination of added nucleotides (data not shown). Thus the addition is specific to $(TTGGGG)_4$; neither $(CCCCAA)_4$ nor the pBR322 oligomer can prime repeat synthesis. As an additional control, we showed that the synthetic oligomer 5′-TAACCCCAACTCAATGGGGAACCCGTTGAG-OH-3′, which ends in a 3′ G residue, as does the $(TTGGGG)_4$ oligomer, was also unable to prime repeat addition (data not shown).

We tested for involvement of the endogenous Tetrahymena α-type DNA polymerase in the repeat synthesis by adding the drug aphidicolin to the reaction. Aphidicolin is a specific inhibitor of eukaryotic DNA polymerase α (reviewed in Huberman, 1981). Concentrations of 5 μg/ml

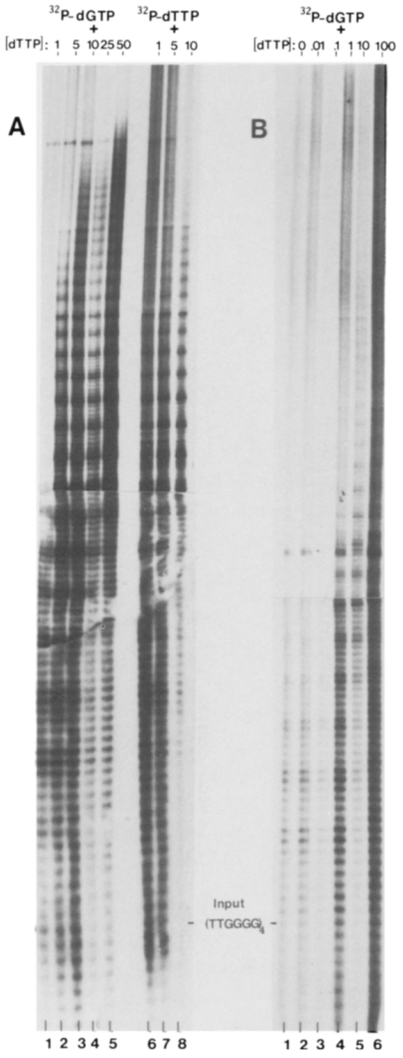

Figure 3. Dependence of the Reaction on dTTP Concentration

(A) Increasing concentrations of cold dTTP were added to in vitro reactions containing 0.25 μM unlabeled $(TTGGGG)_4$ and 1.25 μM α-^{32}P-dGTP (lanes 1–5). Alternatively, increasing concentrations of unlabeled dTTP were added to reactions containing 1.25 μM α-^{32}P-dTTP and 2 μM unlabeled dGTP (lanes 6–8). The number above each lane indicates the micromolar concentration of added dTTP. Note that in lanes 6–8 the specific activity of the label in the reaction is decreasing with added unlabeled dTTP.

(B) dTTP was added, in a wide range of concentrations (from 0.01 μM to 100 μM) to reactions containing 1.25 μM α-^{32}P-dGTP and 0.25 μM unlabeled $(TTGGGG)_4$ (lanes 2–6). In lane 1 no dTTP was added. The position of the original input primer is indicated.

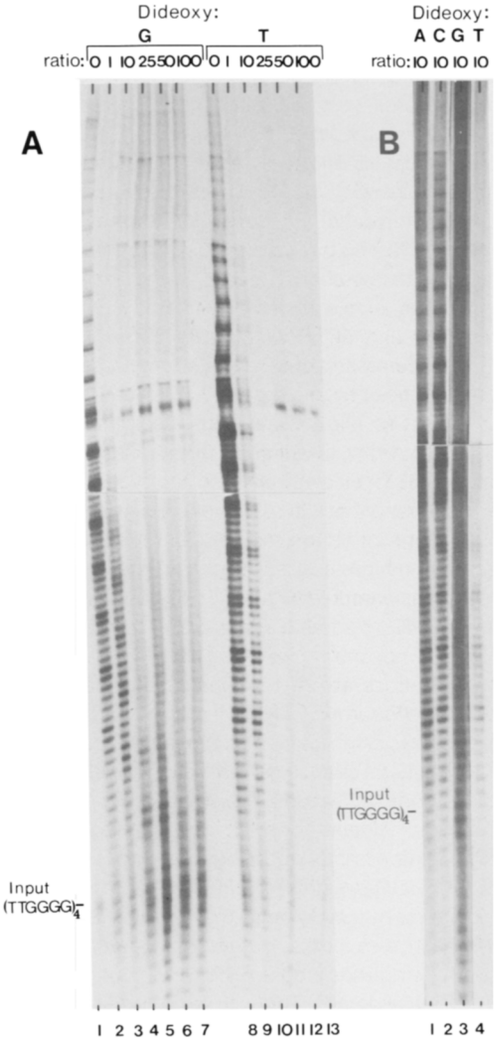

Figure 4. Effects of Dideoxynucleotide Triphosphates on the Elongation Reaction

(A) Dideoxy GTP or dideoxy TTP were added to reactions containing 1.25 μM α-^{32}P-dGTP, 10 μM dTTP, and 0.25 μM unlabeled $(TTGGGG)_4$. The molar ratios of ddGTP or dGTP (lanes 2–7) or ddTTP to dTTP (lanes 8–13) were varied from 0 to 100 as indicated. Lane 1 shows end-labeled oligomer to indicate the size of the input primer.

(B) Each ddNTP was added in turn to an in vitro reaction containing 1.25 μM α-^{32}P-dGTP, 10 μM each of unlabeled dATP, dCTP, and dTTP, and 0.25 μM unlabeled $(TTGGGG)_4$. In lanes 1 and 2, ddATP and ddCTP were added at ratios of 10:1 with the dNTPs. Ratios of 1:1 and 10:1 of ddGTP to dGTP (lanes 3 and 4) or ddTTP to dTTP (lanes 5 and 6) were added to the reactions.

aphidicolin inhibit Tetrahymena α-type DNA polymerase in vitro (Sakai and Watanabe, 1982), and concentrations of 10–20 μg/ml inhibit incorporation of ^{3}H-thymidine in Tetrahymena in vivo (B. Haller and E. Orias, personal communication). We have shown that 10–20 μg/ml of aphidicolin strongly inhibits α-type DNA polymerase activity in our crude Tetrahymena extracts, as assayed by the incorporation of α-^{32}P-dGTP with activated calf thymus DNA as template-primer (data not shown). The repeat synthesis was completely insensitive to 20 μg/ml aphidicolin (Figure 2B, lanes 1, 3, and 5). These experiments indicate that the addition activity is independent of both endogenous DNA and endogenous DNA polymerase α.

The Sequence $(TTGGGG)_n$ Is Added to the Synthetic Telomere Primer

The addition of DNA with a 6 base repeating pattern to the $(TTGGGG)_4$ oligomer, in the presence of only dGTP and dTTP, suggested that tandemly repeated TTGGGG was being synthesized. Several different experiments confirmed this hypothesis. In a preliminary characterization, the in vitro labeled material was subjected to depurination

(Burton, 1967) and pyrimidine tract analysis (Ling, 1972). When α-^{32}P-dGTP and three unlabeled dNTPs were added to the extracts, the labeled depurination products were pTpTp and inorganic phosphate (data not shown). This result indicated that DNA containing the sequences 5′-purine-pTpTpG-3′ and purine-pG, consistent with the sequence $(TTGGGG)_n$, were being synthesized.

Both the nucleotide concentration dependence of the reaction and the pattern of repeat addition are consistent with the synthesis of $(TTGGGG)_n$ in the extracts. Figure 3A shows the effects of varying the dTTP concentration. When dATP and dCTP were omitted from the reaction and the concentration of dGTP was kept low (2 μM), α-^{32}P-dTTP was efficiently incorporated into the in vitro elongation products (lanes 6–8), in contrast to the experiment shown in Figure 2A, in which no repeats were synthesized when α-^{32}P-dTTP was at 1 μM, and dATP, dCTP, and dGTP were each present at 150 μM. These findings confirm that only dGTP and dTTP are required for repeat addition and suggest that high concentrations of other nucleoside triphosphates compete with dTTP. The minimum concentration of dGTP needed to achieve elongation is less than 0.1 μM (data not shown), whereas dTTP must be present at concentrations above 1 μM before significant elongation is seen (Figure 3B).

The banding pattern of the elongation products was dependent upon the relative concentrations of dNTPs added to the reactions. In Figure 2A, where a high concentration (150 mM) of dTTP was added, a repeating pattern consisting of three dark bands and three light bands is apparent. When the concentration of dTTP relative to dGTP is dropped, a reproducible pattern of two dark and four light bands is observed for the synthesized material (Figure 3). The two dark bands begin 6 bases above the position of the input oligomer. Thus, if the telomeric TTGGGG repeats are accurately synthesized beginning at the 3′ end of the primer, 5′. . .TTGGGG-OH-3′, then the dark bands will correspond to TT and the light bands to GGGG. The pattern of two dark bands and four light bands is expected if pauses occur before the addition of dTTP. Consistent with this interpretation, in Figure 3B, when the dTTP concentration was raised to 100 μM, the intensity of the two darkest bands decreased (lane 6). Lane 1 of Figure 3B shows that the endogenous nucleotides in the crude extract allowed the synthesis of only a few repeats in the absence of added dTTP.

Final verification that $(TTGGGG)_n$ is the sequence synthesized in the extracts was obtained by a method analogous to that of Sanger for sequencing with dideoxynucleotides (Sanger et al., 1977). Each dideoxynucleoside triphosphate (ddNTP) was added in turn to the in vitro reactions in the presence of labeled dGTP and the other unlabeled dNTPs. The incorporation of a dideoxynucleotide terminates the chain at the site of addition, making it possible to deduce the sequence of the addition products. As shown in Figure 4A, the addition of ddGTP in a ratio of 1:1 with dGTP changed the pattern of banding such that the darker bands correspond to the middle four bands of the control reactions (lane 3). Higher ratios of ddGTP to dGTP markedly inhibited the reaction (lanes 4–7). The addition of ddTTP in a 1:1 ratio to dTTP accentuated the relative intensities of the two originally darkest bands (lane 9); higher ratios of ddTTP to dTTP inhibited the reaction (lanes 10–13). The addition of ddATP and ddCTP had no effect on the pattern of the elongation products (Figure 4B, lanes 1 and 2). Thus, all the data presented support the conclusion that telomeric repeat units with the sequence $(TTGGGG)_n$ are added to the oligomer primer in the cell free reactions.

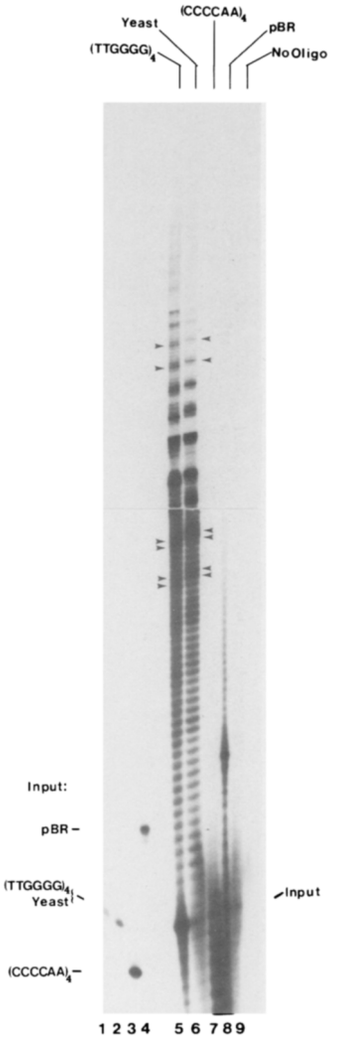

Figure 5. Primer Specificity of the Elongation Reaction

(A) Different synthetic single-stranded oligomers were added at 0.5 μM to reactions containing 1.25 μM α-^{32}P-dGTP and 50 μM unlabeled dTTP. $(TTGGGG)_4$ (lane 5), the yeast telomeric oligomer (lane 6), $(CCCCAA)_4$ (lane 7), a pBR322 sequence oligomer (lane 8), and no oligomer (lane 9) were added in turn to the reaction (see text for oligomer sequences). The 5′-end-labeled synthetic oligomers are shown in lanes 1–4 to indicate the sizes of the input oligomers: lane 1, $(TTGGGG)_4$; lane 2, yeast sequence; lane 3, $(CCCCAA)_4$; lane 4, pBR322 oligomer.

Tetrahymena $(TTGGGG)_n$ Repeats Are Added In Vitro to a Yeast Telomeric Oligomer

It was shown previously that Tetrahymena telomeres func-

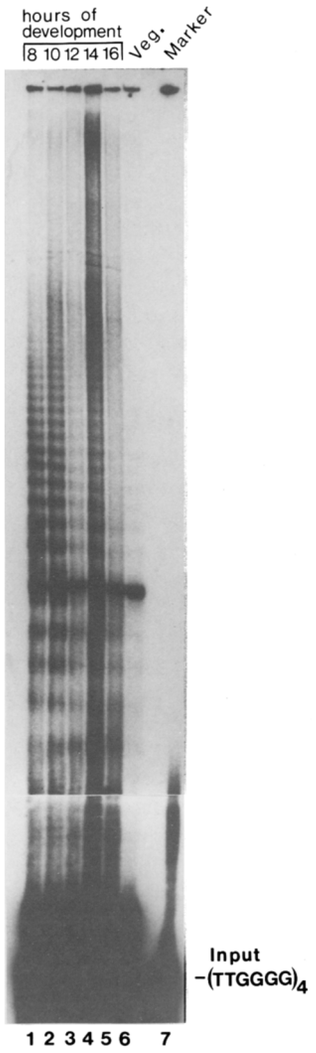

Figure 6. Developmental Time Course of Telomere Elongation Activity

Extracts made from mated cells at 8, 10, 12, 14, and 16 hr of macronuclear development (lanes 1–5) or from nonmated logarithmic phase cells (lane 6) were tested for their telomere elongation activity. Added to the reaction was 0.25 μM unlabeled $(TTGGGG)_4$ primer along with 1.25 μM of both α-^{32}P-dGTP and α-^{32}P-dCTP and 100 μM unlabeled dATP and dTTP. Extracts made from equivalent numbers of cells (4×10^4 per reaction) were added to each reaction. Lane 7 shows the end-labeled oligomer marker. The labeled material in this lane above the input 24 base primer is due to secondary structures formed by the $(TTGGGG)_4$ that migrate slowly in the 7 M urea, 6% polyacrylamide gel.

tion in yeast (Szostak and Blackburn, 1982), and that during maintenance in yeast, repeats of the yeast telomeric sequence $C_{1\text{-}3}A{\cdot}TG_{1\text{-}3}$ are added to the Tetrahymena ends (Shampay et al., 1984). In a reciprocal test, we determined whether a yeast telomere would function in a Tetrahymena cell free extract in our assay for telomere elongation. A synthetic single-stranded 24 base oligonucleotide consisting of the yeast telomeric sequence 5′-TGTGTGGGTGTGTGGGTGTGTGGG-OH-3′ (a sequence from a cloned yeast telomere; Shampay et al., 1984) was substituted for the $(TTGGGG)_4$ primer in the Tetrahymena extract. Figure 5 shows that this yeast sequence oligomer was efficiently used as a primer for the addition of the Tetrahymena TTGGGG repeats, while $(CCCCAA)_4$ and the pBR322 sequence oligomer were not. The banding pattern of addition to the yeast primer was shifted up by 1 base relative to the pattern obtained with the $(TTGGGG)_4$ primer (Figure 5, arrowheads). The Tetrahymena 24-mer ends in four G residues (5′. . .TTGGGG-OH-3′) whereas the yeast 24-mer ends in three G residues (5′. . .TGGG-OH-3′). The upward shift strongly suggests that an extra G is added to the yeast primer before the synthesis of the 6 base repeat pattern begins. This result provides further evidence that the novel activity in the extracts correctly synthesizes telomeric sequences onto an appropriate telomeric primer.

Elongation Activity Is Present in Both Newly Developing and Vegetative Tetrahymena Cells

The extracts used in the above experiments were made from synchronous cultures of mated Tetrahymena cells undergoing macronuclear development (see Experimental Procedures). To test when in development this activity is most abundant, we made extracts at several times after mixing cells to initiate mating. We also made extracts from nonmated vegetatively growing cells. Figure 6 shows that the activity is present in high amounts during macronuclear development and that nonmated cells contain an identical activity, although in lesser amount. Thus the TTGGGG repeat addition reaction is likely to be important for telomere growth and replication in vegetative cells as well as in cells undergoing macronuclear development. In Figure 6, six bases are added before the characteristic repeat pattern emerges. These addition products, like the product of the action of E. coli DNA polymerase I on $(TTGGGG)_4$ (see Figure 2), are due to a DNA repair activity in these extracts. We characterized the repair activity using double-stranded restriction fragments of known sequence, which were efficiently filled out by repair synthesis. In contrast to the sequence-specific repeat addition we describe, the repair activity was independent of input DNA sequence (data not shown).

Enzymatic Properties of Telomere Elongation

In preliminary characterization of the biochemical properties of the TTGGGG repeat addition reaction, incorporation of α-^{32}P-dGTP onto the oligomer primer was quantitatively assayed by counting ^{32}P-DNA adsorbed to Whatman DE81 paper (Maniatis et al., 1982). Figure 7A shows that at 30°C the incorporation of α-^{32}P-dGTP increased with time of incubation up to 100 min. Although background incorporation of ^{32}P in the absence of oligomer also increased with time, the overall incorporation of label was reproducibly lower in this reaction than in extracts to which $(TTGGGG)_4$ had been added. Label incorporation was eliminated by heating the extract to 90°C for 5 min (Figure 7A) or by treatment with proteinase K (Behring Diagnostics) (data not shown). The extent of α-^{32}P-dGTP incorporation was also measured as a function of increasing input oligomer concentration (Figure 7B). The steep rise in incorporation at input oligomer concentrations up to 0.50 μM suggests a high affinity for the oligomer primer. The

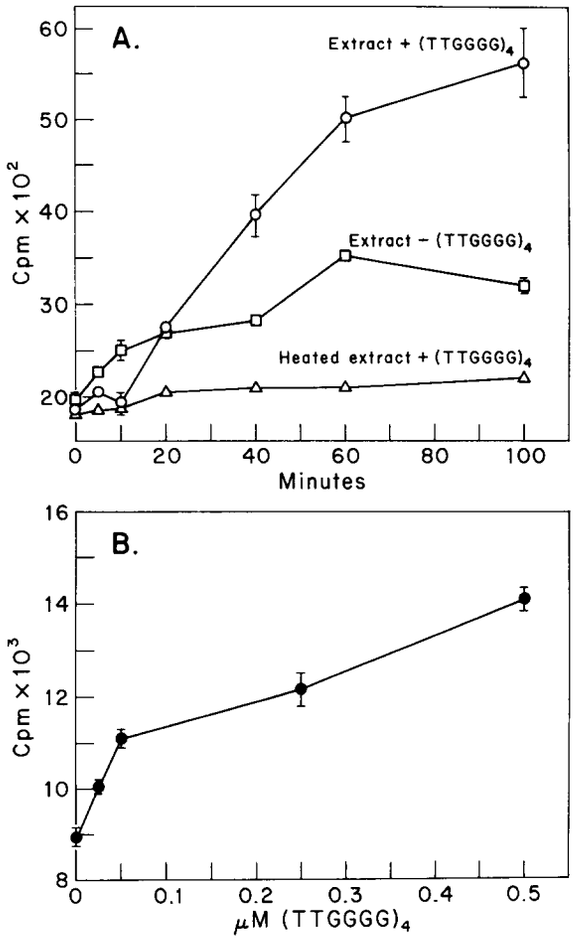

Figure 7. Time and Oligomer Dependence of the In Vitro Reaction

The incorporation of α-^{32}P-dGTP into DNA in reactions containing 1.25 μM α-^{32}P-dGTP and 50 μM dTTP was measured by adsorption to Whatman DE81 paper as described in Experimental Procedures. (A) Time dependence of reactions carried out by untreated extracts in the presence (circles) or absence (squares) of 0.25 μM $(TTGGGG)_4$ or in the presence of 0.25 μM $(TTGGGG)_4$ by extracts that were heat treated at 90°C for 5 min (triangles). (B) The effect of varying the concentration of the $(TTGGGG)_4$ oligomer on the incorporation of α-^{32}P-dGTP was assayed in 90 min reactions.

fact that the reaction is efficient at micromolar concentrations of dGTP and dTTP suggests a high affinity for the triphosphate substrates as well. This preliminary characterization provides evidence that the addition activity in the Tetrahymena extracts has the properties expected for an enzyme.

Discussion

The results reported here provide the first evidence for a terminal transferase-like activity that accurately adds telomeric sequence repeats onto synthetic telomere primers in cell free extracts. This addition reaction is highly specific to G+T-strand telomeric primers. We have shown by several independent methods that $(TTGGGG)_n$ repeats are synthesized in the extracts.

The accuracy of repeat synthesis suggested that the addition of preassembled telomere repeat units could take place; however, several kinds of experimental observations argue against this hypothesis. First, all six bands of the two dark, four light repeating pattern were clearly visible. Although such a pattern could be the result of a sequence-specific nuclease, no such pattern was observed when 5′-end-labeled $(TTGGGG)_4$ was incubated with the extract. Second, the pattern of bands changed in a manner consistent with the addition of single nucleotides when the concentrations of added dGTP and dTTP were varied (e.g. Figure 3B). Third, the shift in the banding pattern of the repeats added to the yeast telomere primer indicates a single dG residue was added to this primer to complete the GGGG sequence before the addition of TT. However, the most conclusive evidence for the addition of single nucleotides comes from the chain termination pattern upon addition of dideoxynucleotides. If preformed blocks of TTGGGG or TT and GGGG were added, chain termination would occur in blocks, not at single base residues as seen in Figure 4.

The ability of Tetrahymena and Oxytricha telomeres to stabilize linear plasmids in yeast argues that many features of telomere structure and function are highly conserved among lower eukaryotes. During maintenance of the Tetrahymena telomeres in yeast, $C_{1-3}A \cdot TG_{1-3}$ repeats are terminally added to the CCCCAA·TTGGGG tracts (Shampay et al., 1984). This can be explained by the de novo synthesis of $G_{1-3}T$ repeats onto the GGGGTT strand. We tested this explanation directly in Tetrahymena and found that a yeast telomeric sequence oligonucleotide allowed accurate addition of TTGGGG repeats in vitro, while $(CCCCAA)_4$ and two nontelomeric oligomers were unable to prime repeat synthesis. The specificity of the reaction for only the G+T-strand telomeric primers suggests that higher order structural characteristics of these sequences may be recognized for telomere elongation. We have shown that higher order interactions of $(TTGGGG)_4$ occur under our reaction conditions since the oligomer was able to self-associate and form a template-primer structure that was utilized by E. coli DNA polymerase I. This self-association of $(TTGGGG)_n$, depicted as a fold-back structure in Figure 1, may account for the observed inaccessibility of telomeric termini to end labeling (Blackburn, 1985).

Our data support the idea that the elongation activity is template independent. We have ruled out both template slippage of $(TTGGGG)_4$ and copying of endogenous $(CCCCAA)_n \cdot (TTGGGG)_n$ tracts as explanations of the repeat synthesis. In addition, the yeast telomeric sequence oligomer could not have supported $(TTGGGG)_n$ synthesis by self-association and template slippage. The untemplated addition of single deoxynucleotides to free DNA ends is similar to the properties of the enzyme terminal transferase (reviewed in Bollum, 1974). Furthermore, the addition of a specific sequence to a primer with a specific structure is reminiscent of the enzyme tRNA nucleotidyl transferase, which accurately adds the sequence CCA to the 3′ end of tRNA molecules in an untemplated manner (reviewed in Deutscher, 1982). These two enzymes provide a precedent for both the untemplated nature and sequence specificity of the activity we describe.

The in vitro primed $(TTGGGG)_n$ repeat synthesis has characteristics of an enzyme-catalyzed reaction, such as rapid increase in label incorporation with time, high affinity for substrates, and susceptibility to heat and proteinase K treatments. In addition, the developmental time course shown in Figure 6 argues that the activity is present in higher amounts per cell during macronuclear development, when a large number of telomeres are formed and undergo several rounds of replication, than during vegetative divisions. Although detailed kinetic studies await further purification of the telomere elongation activity, the efficiency with which up to 30 $(TTGGGG)_n$ repeats are added to the primer is noteworthy.

In vivo studies of telomere length regulation suggest that the repeat addition reaction we describe is closely regulated in the cell. The heterogeneous lengths of telomeres in many organisms, the observed net growth of telomeres in both dividing trypanosomes and Tetrahymena, and the recent finding of oversized telomeres in the developing macronuclear DNA of the ciliate Euplotes (Roth and Prescott, 1985) are all consistent with the presence of cellular activities that both lengthen and shorten telomeres. Although regulation of telomere size must involve dynamic interaction of the lengthening and shortening processes, these reactions are not always apparent in vivo. In yeast the mean length of telomeres does not increase, even when cells are kept in continuous logarithmic phase growth (Walmsley and Petes, 1985, J. Shampay, unpublished results). However, some data suggest that the balance between the lengthening and shortening processes can be perturbed. The presence of a multiple-copy linear plasmid in yeast results in a net shortening of all telomeres during logarithmic phase growth (J. Shampay, unpublished results). In addition, the telomeres of the temperature-sensitive yeast mutant *cdc17* are longer at permissive temperature than those of isogenic *CDC17* haploids, and a net increase in telomere length is seen when *cdc17* cells are grown at temperatures intermediate between permissive and restrictive (Carson and Hartwell, 1985).

Since the structures of all characterized telomeres are very similar, and several organisms have been shown to add telomeric repeats onto telomeres, it is likely that there is a common mode of telomere replication in eukaryotes. The model presented in Figure 1 is consistent with the current knowledge of telomere structure and function. The discovery we report here of a telomere terminal transferase activity in Tetrahymena cell free extracts provides strong evidence for this model.

Experimental Procedures

Cell Cultures

Tetrahymena thermophila strains B 1979 (mating type VI) and B 1868 (mating type IV) (provided by D. Nanney, University of Illinois) were grown to 2×10^5 cells/ml in 2% PPYS (2% proteose peptone, Difco; 0.2% yeast extract, Difco; 0.003% sequestrine, Ciba-Geigy). For matings, cells were washed twice in Dryls solution (1.7 mM Na-citrate, 1.2 mM NaH_2PO_4, 1 mM Na_2HPO_4, 2 μM $CaCl_2$) and starved for 24 hr in Dryls solution with gentle agitation. Equal numbers of cells of the two mating types were then mixed, split into several 100 ml cultures, and stood without shaking in 1 liter falcon T flasks to obtain a high ratio of surface area to volume. Mating cells were re-fed at 7 hr by addition of PPYS to 1% and were harvested for extract preparation at 8, 10, 12, 14, or 16 hr after mixing. Pair formation was monitored by microscopy and typical mating efficiencies were greater than 95% pairing.

Extract Preparation

Extracts were prepared by a modification of the method of Zaug and Cech (1980). Two hundred milliliters of mated or logarithmic phase cells (2×10^5 cells/ml) were washed twice in TMS (10 mM Tris [pH 8.0], 10 mM $MgCl_2$, 3 mM $CaCl_2$, 0.24 M sucrose) and brought to a final volume of 10 ml with TMS. The cells were then stirred rapidly on ice and lysed by the addition of NP40 to 0.2%. After 20 min of stirring on ice, 2.4 M sucrose was added to a final concentration of 0.6 M. This mixture was then layered over a 5 ml 2.4 M sucrose cushion in a 50 ml centrifuge tube and spun at 8000 rpm for 30 min at 4°C in an HB4 rotor. The material that collected at the interface of the 0.6 M and 2.4 M sucrose contained micronuclei, macronuclei, and newly developing macronuclear anlagen. This material was collected with a plastic pipette and aliquots were frozen in liquid nitrogen until use. Upon thawing and incubation at 30°C the nuclei were lysed, releasing the nuclear contents. The activity of the extract was stable for at least 6 months when stored in this manner. Except where otherwise indicated the extracts used were made either 8 or 10 hr after mixing cells to initiate mating.

Synthetic Oligomers

Specific DNA oligomers were synthesized on an Applied Biosystems DNA synthesizer. After synthesis and deprotection, the mixture of oligomers was dried down by vortex evaporation of the concentrated NH_4OH and resuspended in distilled water. To purify the 24-mer away from the smaller products, the oligomer was mixed 1:2 with an 80% formamide loading dye and run on a preparative 20% polyacrylamide, 7 M urea gel. After staining with ethidium bromide, the largest product band was cut out and the DNA eluted from the acrylamide as described by Maniatis et al. (1982). The crushed gel slice was incubated in 0.5 M NH_4OAc, 1 mM EDTA overnight at 37°C and the acrylamide was removed by centrifugation through a plastic filter disc in a disposable 5 ml polypropylene Quik-Sep column (Isolabs). The purified oligomer was then concentrated by several butanol extractions, brought to 2.5 M NH_4OAc, and precipitated with 3 volumes of 95% ethanol. After resuspension in TE (10 mM Tris-HCl (pH 7.5], 1 mM EDTA) the optical density at 260 nm was read and the concentration was determined assuming 1 OD unit to equal 20 μg/ml.

In Vitro Reaction Conditions

The in vitro reaction buffer was modified from that used by Zaug and Cech (1980). For each 40 μl reaction, 18 μl of extract in TMS and 2 μl of 400 Ci/mmol ^{32}P-dNTP (Amersham) were added to a 20 μl solution containing 0.5 μM oligomer, 100 mM Tris (pH 7.5), 20 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.2 mM spermidine, 2 mM spermine, 4 mM DTT, 1 mM of each rNTP, and varying concentrations of dNTPs. After incubation at 30°C for 90 min, the reaction was stopped by the addition of 10 μl of 200 mM EDTA and 90 μl TE. Each reaction was phenol extracted once and back extracted with 100 μl TE. Ten to twenty micrograms of E. coli tRNA was added as carrier and the samples were precipitated three to five times in 2.5 M NH_4OAc and 3 volumes of 95% ethanol to remove unincorporated nucleotides. The final pellets were dried in a vacuum desiccator.

Gel Electrophoresis

Samples were resuspended in an 80% formamide loading buffer containing 0.1% w/v xylene cyanol and loaded onto an 80 cm long 6% polyacrylamide 7 M urea sequencing gel. Electrophoresis was typically carried out at 2000 V for about 5 hr in 0.6× TBE buffer (1× TBE = 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0). After electrophoresis the gels were cut in half and dried down onto Whatman 3mm paper, and both halves were autoradiographed at −70°C with a Dupont Lightning-plus intensifying screen.

Quantitative Incorporation Assays

In vitro reactions were stopped by the addition of 10 μl 200 mM EDTA and 90 μl TE containing 1 mM dGTP. After phenol extraction, 10 μl samples were spotted onto duplicate 1 cm Whatman DE81 paper squares. The DE81 filters were washed in batches six times for 10–20 min each

with 0.5 M Na_2HPO_4 plus 22 mM sodium pyrophosphate, washed twice with water, and dried under a heat lamp. The radioactive incorporation was measured by liquid scintillation in Aquasol (NEN).

Preparation of S100 Fractions and Micrococcal Nuclease Digestion

Aliquots of cell extracts in TMS were centrifuged for 20 min at 55,000 rpm in a TLA 100.2 rotor in a Beckman TL100 tabletop ultracentrifuge. The S100 supernatant fraction was removed and used directly in an in vitro reaction. The pellet fraction was brought to a volume equal to that of the supernatant with TMS and was incubated for 10 min at 37°C in the presence or absence of 1 unit of micrococcal nuclease (Worthington). The micrococcal nuclease was then inactivated by the addition of EGTA to 10 mM to chelate the Ca^{2+}. $MgCl_2$ (10 mM) was added back to the extract, oligomer and reaction buffer were added, and the in vitro reactions were carried out as described above. To assay the action of micrococcal nuclease on telomeric sequences, a 276 base pair fragment containing the sequence $(CCCCAA)_{46} \cdot (TTGGGG)_{46}$ was isolated from the plasmid pCA2 (Blackburn et al., 1983), nick translated with α-^{32}P-dNTPs, and added to an extract before the addition of the micrococcal nuclease. After incubation at 37°C for 10 min, the mixture was phenol extracted, ethanol precipitated, run on a 6% polyacrylamide gel, and autoradiographed. Under these conditions >90% of the input $(CCCCAA)_{46} \cdot (TTGGGG)_{46}$ was reduced to fragments of less than 40 bases.

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