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Telomere Terminal Transferase Activity from *Euplotes crassus* Adds Large Numbers of TTTTGGGG Repeats onto Telomeric Primers

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A telomere terminal transferase activity was identified in developing macronuclear extracts from *Euplotes crassus*. The activity was essentially unregulated in vitro: up to 50 tandem repeats of the *Euplotes* telomeric repeat sequence TTTTGGGG were added onto synthetic telomeric oligonucleotide primers. Both the structure of the telomere substrate and its 3′-terminal sequence were recognized. The activity was destroyed by low concentrations of RNase A.

Replication of telomeres, the essential G+C-rich structures at the ends of eucaryotic chromosomes (reviewed in references 1 and 3), is thought to involve a telomere terminal transferase (telomerase) that extends the 3′ end of the G-rich strand by de novo synthesis of telomeric repeats (4, 14). Such a telomerase activity has been identified in *Tetrahymena thermophila* extracts (4). Unregulated in vitro, the *Tetrahymena* telomerase adds more than 1,000 TTGGGG repeats onto telomeric primers (E. H. Blackburn, C. W. Greider, E. Henderson, M. Lee, J. Shampay, and D. Shippen-Lentz, Genome, in press). Further characterization of the activity showed that it is a ribonucleoprotein enzyme containing a single essential RNA species (5, 6).

The highly conserved structures of telomeres among different eucaryotes imply that the mechanism of telomere elongation may also be conserved. Besides *T. thermophila*, other potentially good sources of telomerase activity are the hypotrichous ciliates *Euplotes* and *Oxytricha* spp., which contain approximately 10^7 chromosomal termini per cell (reviewed in reference 7). Analysis of the telomerase enzyme in these organisms may be particularly enlightening since their telomeric repeat sequence (TTTTGGGG) is different from the *Tetrahymena* repeated sequence (TTGGGG) (2, 8). Moreover, telomere length is regulated differently in the two types of ciliates. In *Tetrahymena thermophila*, telomeres typically consist of 50 to 70 repeats; however, length fluctuations occur under certain physiological conditions (9). In contrast, telomere length in the hypotrichous ciliates is tightly regulated during vegetative growth. Macronuclear telomeres in vegetative *Euplotes crassus* consist of a 28-base-pair duplex region plus a 14-base single-stranded tail (8, 11). During *Euplotes* macronuclear development, oversized telomeres are added onto DNA molecules immediately after chromosomal fragmentation and are subsequently trimmed back to their stable vegetative lengths (13).

A telomerase activity has recently been identified in vegetative macronuclear extracts from *Oxytricha nova* (15). Unlike the *Tetrahymena* telomerase, the *Oxytricha* activity added only five to seven TTTTGGGG repeats onto telomeric primers. In this study, we analyzed telomerase activity from the developing macronuclei of another hypotrichous ciliate, *E. crassus*, to determine whether this apparent difference in telomerase activity reflects the comparatively shorter telomeres of the hypotrichs.

E. crassus was cultured under nonsterile conditions, using the alga *Dunaliella salina* as the live food source (12). After 1 to 2 days of starvation, cell mating was initiated by mixing starved cultures of two mating types, cc 51(mt3 mt1:III) and cc 55(mt1 mt1:I). Developing macronuclei were isolated 60 h after mating (12), the time when chromosomal fragmentation and telomere addition occur under our culture conditions (S. Tausta and L. Klobutcher, personal communication). Cells from 20 liters of mated cultures were collected on 15-μm-pore-size Nitex filters (Tetko), concentrated to 200 ml, and centrifuged at 100 × *g* for 3 min in an IEC clinical centrifuge. The cell pellet was suspended in 10 mM Tris (pH 7.5)–0.05% spermidine phosphate–1 mM phenylmethylsulfonyl fluoride—10 μM pepstatin–40 U of RNasin (Promega Biotech, Milwaukee, Wis.) per ml. After addition of Triton X-100 to a final concentration of 0.5%, the sample was mixed for approximately 5 s and then transferred to a sterile Dounce homogenizer. Cells were broken at room temperature with 5 to 10 strokes, and lysis was monitored by microscopy. After filtration through a 25-μm-pore-size Nitex filter to remove cell ghosts and unlysed cells, the sample was purified through a 25 to 50% Percoll (Sigma Chemical Co., St. Louis, Mo.)-sucrose step gradient. The gradient was spun at 4,000 × *g* for 10 min at 4°C in an HB-4 rotor of a Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). Fractions containing developing macronuclei were collected by dilution 1:1 with 5% sucrose–10 mM Tris (pH 7.5)–0.05% spermidine phosphate and centrifugation as described above. The pellet was suspended in 10 to 20 volumes of TMG buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM $MgCl_2$, 10% glycerol) plus 1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin, and 40 U of RNasin per ml, frozen, and stored in liquid nitrogen. The final concentration of developing nuclei was approximately 10^5/ml.

Extracts were assayed as described previously (5) except that additional $MgCl_2$ and spermine were omitted from the reaction cocktail. Incubation of the extract in the presence of [α-^{32}P]dGTP, cold dTTP, and a synthetic single-stranded telomeric DNA oligonucleotide resulted in elongation of the primer by the addition of many 8-base repeats (Fig. 1). The reaction worked equally well at 22°C, the optimum temperature for *Euplotes* growth, or at 30°C, the temperature at which *Tetrahymena* telomerase was assayed (Fig. 1A). Telomerase activity was found exclusively in the nuclei and did not appear to leak out; however, hypotonic lysis of the nuclei released all of the activity into the supernatant fraction (data not shown). Although the products of the *Tetrahymena* telomerase reaction (Fig. 1A and C) appeared much

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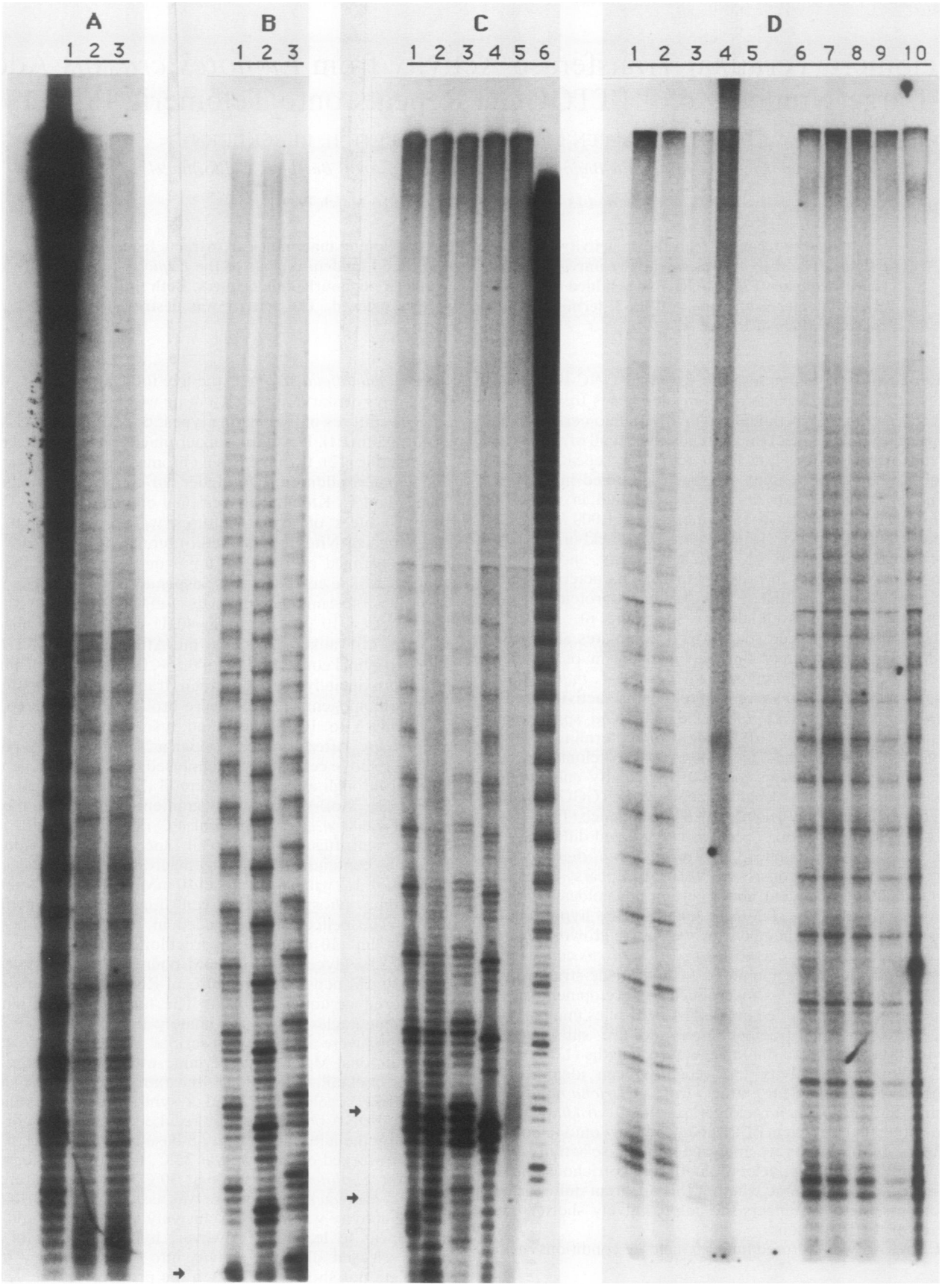
A
1 2 3
B
1 2 3
C
1 2 3 4 5 6
D
1 2 3 4 5 6 7 8 9 10

more abundant than the *Euplotes* products, the *Tetrahymena* preparation contained the equivalent of 10^8 nuclei per ml, as opposed to only 10^5/ml for the *Euplotes* extract. Therefore, the level of telomerase activity in mated *Euplotes* nuclear extracts was comparable to that found in whole-cell S-100 preparations from mated *Tetrahymena* cells.

To determine the total number of TTTTGGGG repeats added, the *Euplotes* telomerase products were assayed on an alkaline agarose gel (10). Products with lengths of up to 400 nucleotides (50 repeats) were detected, although the major products ranged from approximately 75 to 300 bases (Fig. 2). These data indicate that the *Euplotes* telomerase is highly regulated in vivo, since telomeres at this stage of development are approximately 80 base pairs long and are subsequently shortened and stably maintained at 28 base pairs plus a 14-base 3′ protruding tail during vegetative divisions (13).

The substrate specificity for the *Euplotes* telomerase activity was similar to that of the *Tetrahymena* telomerase (5); both the structure of the telomere substrate and its 3′-terminal sequence were recognized. Primers that differed in their 3′-end sequences were tested to determine whether the permutation of the input oligonucleotide was perceived. The pattern of bands repeated with an 8-base periodicity in the elongation products was relatively shifted up or down, depending on the 3′ sequence of the three input telomeric primers of identical lengths and repeat units (Fig. 1B and 3). Oligonucleotide primers ending with less than four G residues (5′-GTTGGGGTTGGGGTTGGG-3′) or less than four T residues (5′-GGGGTTGGGGTTGGGGTT-3′) were elongated by the appropriate number of nucleotides to create a complete *Euplotes* telomeric repeat, GGGGTTTT, or TTT TGGGG before additional repeats were added. These results show clearly that the *Euplotes* telomerase activity, like that of *T. thermophila* (5), adds both G and T residues one nucleotide at a time to the 3′ terminus of the telomeric primer rather than adding them as a single block.

The *Euplotes* activity also recognized the structure of the telomeric substrate. G-rich telomeric sequences from a variety of organisms were elongated by the addition of many 8-base repeats (Fig. 1C).

Like the *Tetrahymena* telomerase, the *Euplotes* activity contained an essential RNA component (Fig. 1D). The difference between the telomeric sequences synthesized by these two activities may be specified by the RNA moiety, since a potential templating sequence has been identified in the *Tetrahymena* telomerase RNA species (6). Preliminary experiments to identify the *Euplotes* telomerase RNA gene by cross-hybridization with *Tetrahymena* telomerase RNA indicate that the primary sequence of the telomerase RNA is not highly conserved.

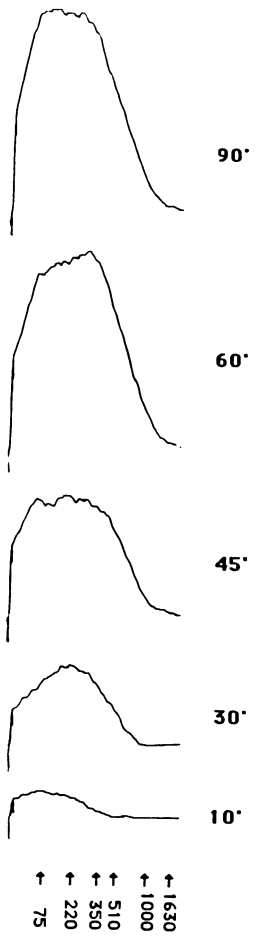

FIG. 2. Scanning densitometry analysis of *Euplotes* telomerase products. Assays were carried out at 30°C as described in the text, using a $(TTTTGGGG)_4$ primer. The reactions were terminated at the time points indicated, and the products were resolved on an alkaline 1.5% agarose gel (10). The autoradiogram was scanned by a LKB 2202 Ultroscan laser densitometer. Arrows below the scans indicate positions of the 1-kilobase-ladder size markers.

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FIG. 1. (A) Temperature dependence of the *Euplotes* telomerase reaction. Extracts from *T. thermophila* or *E. crassus* were incubated in reaction cocktail containing a synthetic DNA oligonucleotide, $(TTGGGG)_4$ for the *Tetrahymena* telomerase assay or $(TTTTGGGG)_4$ for the *Euplotes* telomerase assay, at 22 or 30°C for 60 min. The reaction products were resolved on a 6% polyacrylamide sequencing gel. Lanes: 1, *Tetrahymena* telomerase incubated at 30°C; 2, *Euplotes* telomerase incubated at 22°C; 3, *Euplotes* telomerase incubated at 30°C. (B) Recognition of the 3′-terminal sequence of the primer by *Euplotes* telomerase. Extracts were assayed at 30°C with synthetic oligonucleotide primers having differing 3′-terminal sequences: TTGGGGTTGGGGTTGGGG (lane 1); GGGGTTGGGGTTGGGGTT (lane 2); and GT TGGGGTTGGGGTTGGG (lane 3). The arrow indicates the migration position of a 32-base oligonucleotide marker, $(TTTTGGGG)_4$. (C) Recognition of telomeric sequences from different organisms. Extracts were assayed at 30°C in the presence of oligonucleotides representing the telomeric repeat sequences from *E. crassus* [$(TTTTGGGG)_4$; lane 1], *T. thermophilia* [$(GGGGTT)_4$; lane 2], *Saccharomyces cerevisiae* [$(TG)_3G_2(TG)_3G_2(TG)_3G_2$; lane 3], and *Dictyostelium discoideum* [$AG_7AGAG_6AG_6$; lane 4]. Lane 5, No oligonucleotide; lane 6, products of a *Tetrahymena* telomerase reaction primed by $(GGGGTT)_4$. Arrows indicate migration positions of the 24-base [$(GGGGTT)_4$] and 32-base [$(TTTTGGGG)_4$] markers. (D) RNase sensitivity of the *Euplotes* telomerase. Extracts were preincubated for 10 min at 22°C with various concentrations of either RNase A (Sigma) alone, RNase A in the presence of RNasin, or oxidized RNase A. After preincubation, RNasin was added to the samples that did not have it. Telomerase reaction cocktail and $(TTTTGGGG)_4$ primer were added to all tubes, and the samples were assayed for telomerase activity at 30°C. Lanes: 1, no RNase A; 2 to 5, 0.1, 1.0, 5.0, and 10.0 μg of RNase A per ml, respectively; 6 to 9, 0.1, 1.0, 5.0, and 10 μg of RNase A per ml, respectively, plus 5,000 U of RNasin per ml; 10, 10 μg of oxidized RNase A per ml.

Input Oligonucleotide	Primer Sequence	Sequence Added	Position Shift
$(T_2G_4)_3$	TTGGGGTTGGGGTTGGGGttttgggg**g**ttttgggg**g**...		0
$G(T_2G_4)_2T_2G_3$	GTTGGGGTTGGGGTTGGG**g**ttttgggg**g**ttttggg...		1
$(G_4T_2)_3$	GGGGTTGGGGTTGGGGTTttgggg**g**ttttgggg**g**tt...		2

FIG. 3. Recognition of 3'-terminal sequence permutations. Uppercase letters indicate the input oligonucleotide sequence; lowercase letters indicate the sequence added by the telomerase. The telomerase pauses before adding the first T residue in the repeat sequence, as indicated by the letters in boldface type.

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LITERATURE CITED

1. **Blackburn, E. H.** 1984. Telomeres: do the ends justify the means? Cell **37**:7–8.
2. **Blackburn, E. H., and J. C. Gall.** 1978. A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*. J. Mol. Biol. **120**:33–53.
3. **Blackburn, E. H., and J. W. Szostak.** 1984. The molecular structure of centromeres and telomeres. Annu. Rev. Biochem. **53**:163–194.
4. **Greider, C. W., and E. H. Blackburn.** 1985. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell **43**:405–413.
5. **Greider, C. W., and E. H. Blackburn.** 1987. The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell **51**:887–898.
6. **Greider, C. W., and E. H. Blackburn.** 1989. A telomeric sequence in the RNA of *Tetrahymena* telomerase is required for telomere repeat sequence. Nature (London) **337**:331–337.
7. **Klobutcher, L. A., and D. M. Prescott.** 1986. The special case of the hypotrichs, p. 111–154. *In* J. Gall (ed.), The molecular biology of ciliated protozoa. Academic Press, Inc., Orlando, Fla.
8. **Klobutcher, L. A., M. T. Swanton, O. Donini, and D. M. Prescott.** 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. USA **78**:3015–3019.
9. **Larson, D. D., E. Spangler, and E. H. Blackburn.** 1987. Dynamics of telomere length variation in *Tetrahymena thermophila*. Cell **50**:477–487.
10. **McDonell, M., M. Simon, and F. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights of electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119–146.
11. **Pluta, A. F., B. P. Kaine, and B. B. Spear.** 1982. The terminal organization of macronuclear DNA in *Oxytricha fallax*. Nucleic Acids Res. **10**:8145–8154.
12. **Roth, M., M. Lin, and D. Prescott.** 1985. Large scale synchronous mating and the study of macronuclear development in *Euplotes crassus*. J. Cell Biol. **101**:79–84.
13. **Roth, M., and D. Prescott.** 1985. DNA intermediates and telomere addition during genome reorganization in *Euplotes crassus*. Cell **41**:411–417.
14. **Shampay, J., J. W. Szostak, and E. H. Blackburn.** 1984. DNA sequences of telomeres maintained in yeast. Nature (London) **310**:154–157.
15. **Zahler, A. M., and D. M. Prescott.** 1988. Telomere terminal transferase activity in the hypotrichous ciliate *Oxytricha nova* and a model for replication of the ends of linear DNA molecules. Nucleic Acids Res. **16**:6953–6972.