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Characterization of a Telomere-Binding Protein from *Physarum polycephalum*

JONATHON S. COREN,[1] EILEEN M. EPSTEIN,[2] AND VOLKER M. VOGT[2]*

Section of Genetics and Development,[1] and Section of Biochemistry, Molecular and Cellular Biology,[2] Cornell University, Ithaca, New York 14853

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We have partially purified a nuclear protein (PPT) from *Physarum polycephalum* that binds to the extrachromosomal ribosomal DNA telomeres of this acellular slime mold. Binding is specific for the $(T_2AG_3)_n$ telomere repeats, as evidenced by nitrocellulose filter binding assays, by gel mobility shift assays with both DNA fragments and double-stranded oligonucleotides, and by DNase I footprinting. PPT is remarkably heat stable, showing undiminished binding activity after incubation at 90°C. It sediments at 1.2S, corresponding to a molecular weight of about 10,000 (for a globular protein), and its binding activity is undiminished by incubation with RNase, suggesting that it is not a ribonucleoprotein. We hypothesize that PPT plays a structural role in telomeres, perhaps preventing nucleolytic degradation or promoting telomere extension by a telomere-specific terminal transferase.

Telomeres are specialized structures composed of repetitive DNA sequences that are found at the ends of linear chromosomes. They are required for both the proper replication and the stability of chromosomes. Removal of RNA primers during DNA synthesis leaves a 5′-terminal gap in one of the daughter molecules. Repetitive priming would result in a gradual loss of DNA from chromosome ends. Exonucleolytic degradation, which is observed with broken chromosomes (4), would also result in the loss of DNA at termini. Two mechanisms that offset this chromosomal shortening have been identified. The first is addition of telomeric repeats to the 3′ overhang by a telomere-specific terminal transferase. This telomere terminal transferase activity has been observed in the ciliates *Tetrahymena thermophila* (17, 18), *Oxytricha nova* (54), and *Euplotes crassus* (45) and in human cells (29). For example, up to 30 repeats of the *Tetrahymena* telomeric sequence, $(T_2G_4)_n$, were added to a $(T_2G_4)_4$ oligonucleotide in vitro by the *Tetrahymena* enzyme (which has been named telomerase). The second mechanism is recombination between telomeres. To date, this phenomenon has been reported only for *Saccharomyces cerevisiae* (51). Thus, there is a dynamic equilibrium between shortening and lengthening of the telomere. In yeast cells, mutations that perturb this equilibrium have been described (7, 25, 27). Functionally, telomeres appear to protect the ends of the chromosomes from the rapid recombination and degradation known to be associated with chromosome breaks (4, 55). This protection could result from the formation of a special DNA structure, for example, a hairpin formed by Hoogsteen-bonded guanine base pairs in the 3′ overhang region (20, 31, 42, 43, 52), or from the binding of proteins (3, 16, 33, 36, 37).

Telomeric sequences have been characterized in a wide range of organisms from lower eukaryotes to humans. They all consist of multiple simple repeat sequences of a C-rich strand and a G-rich strand; the G-rich strand always runs 5′ to 3′ toward the end of the molecule. Most telomeric sequences fit the consensus sequence 5′ $C_{1-8}(T/A)_{1-4}$ 3′ (for a recent review, see reference 55). The sequence 5′ T_2AG_3 3′ has been evolutionarily conserved among such diverse organisms as *Trypanasoma brucei* (2), *Neurospora crassa* (41), *Physarum polycephalum* (13), and humans (30).

Although diverse experiments suggest that specific proteins are bound to telomeres in vivo (3, 8, 16), only a few proteins that interact specifically with telomeric sequences in vitro have been purified or partially purified. The best studied of these are from the ciliates *Oxytricha* and *Euplotes* spp. and from yeast. The telomeres of these ciliates appear to be distinctive because the DNA sequence ends with a precise short overhang of the 3′ end, following the same short sequence as does double-stranded DNA, which then runs immediately into upstream nontelomeric DNA. In contrast, the telomeres of most other organisms are composed of heterogeneous ends containing large numbers of the characteristic telomeric repeat sequence. The *Oxytricha* protein is a 98-kDa heterodimer that binds tenaciously to the $(T_4G_4)_2$ 3′ overhang and in vitro protects the chromosome from degradation by the nuclease BAL 31 (16, 34–37). The *Euplotes* protein is a 50-kDa monomer that binds to the $T_4G_4T_4G_2$ overhang and also protects the chromosome from BAL 31 nuclease digestion (33). It has been speculated that these proteins function as chromosomal caps, thereby imparting stability to their respective chromosome termini. Several yeast proteins originally were reported to bind to yeast telomeric sequences (1, 6, 24, 48), but these have all turned out to be the same protein, RAP1, which binds to the upstream activating sequences of certain genes, the silencers at *HML* and *HMR*, as well as to telomeres. The role of RAP1 in telomere function has been addressed by altering its level of expression and observing the consequences. Underexpression of RAP1 reduces telomere length, while overproduction increases both telomere length and heterogeneity (9, 26). More recently, Liu and Tye (23a) have reported evidence for two new telomere-binding proteins in yeast cells. One, named TBFα, was shown to interact with T_2AG_3 sequences at the junction of the telomeric sequence proper, $(TG_{1-3})_n$, and subtelomeric X sequence DNA. TBFα also appears to recognize other telomeric sequences, such as the $(T_2G_4)_n$ DNA found in *Tetrahymena* cells.

In the acellular slime mold *Physarum polycephalum*, ribosomal DNA (rDNA) is present as several hundred extra-

* Corresponding author.

chromosomal DNA molecules (11, 12, 50). Each is a 60-kb palindrome that codes for two copies each of 19S, 5.8S, and 26S rRNA. Like those of mammals, *Physarum* rDNA telomeres consist of multiple copies of the sequence 5′ T_2AG_3 3′ (13). We have identified and partially purified a protein from *P. polycephalum* that binds to this sequence. This is the first example of a protein that specifically recognizes T_2AG_3 repeats.

MATERIALS AND METHODS

Preparation of nuclear extracts. Microplasmodia from the M_3C strain of *P. polycephalum* were grown in liquid shake cultures as previously described (10). Microplasmodia were collected by centrifugation at 1,000 × *g* in a clinical centrifuge, washed twice with distilled water, and resuspended in ice-cold lysis buffer (10 mM Tris-Cl [pH 7.5], 10 mM $CaCl_2$, 250 mM sucrose, 0.1% Triton X-100). The lysate was then homogenized for 1 min in an Omnimixer (Dupont) and centrifuged at 5,000 rpm in a Sorvall GSA rotor for 10 min. The pellet was resuspended in lysis buffer, homogenized, and centrifuged again in a Sorvall SS 34 rotor. The washing and centrifugation was repeated in 10 mM Tris-Cl (pH 7.5)–250 mM sucrose. The nuclei in the resulting pellet were resuspended in 10 mM Tris-Cl (pH 7.5)–2 M NaCl, placed on ice for 20 min, and centrifuged at 7,500 rpm for 10 min in an SS 34 rotor. The supernatant was placed on ice while the pellet was extracted with the 10 mM Tris-Cl (pH 7.5)–2 M NaCl solution a second time. Both supernatant fractions were pooled, and sucrose was added to a final concentration of 250 mM to generate the nuclear extract, which was frozen at −70°C.

Purification. Thawed nuclear extracts were dialyzed against 50 mM Tris-Cl (pH 7.8)–100 mM NaCl and centrifuged at 10,000 rpm for 15 min in a Sorvall SS 34 rotor to remove precipitated histones and other insoluble proteins. The supernatant was then heated at 90°C for 10 min, immediately placed into ice water, and centrifuged again to remove denatured proteins. Phenylmethylsulfonyl fluoride was added to the supernatant to a final concentration of 0.1 mM. The heated supernatant was then loaded onto a 15-ml heparin-agarose column (type I; Sigma), and proteins were eluted with a NaCl step gradient from 200 to 800 mM NaCl in 100 mM increments. Five 3-ml fractions were collected for each step. Glycerol was added to each fraction to a final concentration of 10%. The fractions were stored at −70°C for at least 6 months without loss of activity. Protein quantification was done by the method of Bradford (5).

Nitrocellulose filter binding. Nitrocellulose filter binding experiments (38) were performed with restriction endonuclease-cut, end-labeled rDNA that was incubated with a partially purified fraction in binding buffer (10 mM Tris-Cl [pH 7.5], 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol [DTT], and 25 μg of bovine serum albumin [BSA] per ml). rDNA was purified by isopycnic centrifugation as described previously (12). After incubation at room temperature for 30 min, the reactions were filtered over nitrocellulose (type HA, 0.45-μm pore size; Millipore), and the membranes were washed three times in washing buffer (10 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$, 0.1 mM EDTA, 100 mM NaCl). To release the bound DNA, the filters were incubated in TE (10 mM Tris-Cl [pH 7.5], 1 mM EDTA) plus 0.1% sodium dodecyl sulfate (SDS) for 2 h at 37°C. The DNA was then ethanol precipitated in the presence of 2 μg of carrier DNA and 0.3 M NaCl. The samples were resuspended in TE and electrophoresed on a 0.8% agarose gel. The gel was dried and subsequently exposed to Kodak XAR-5 film with an intensifying screen at −70°C.

Gel mobility shift assay. The standard binding assay was a modification of published protocols (14, 15, 21, 49). The fragment containing the sequence $(T_2AG_3)_{27}$ was obtained from the *Neurospora* plasmid pNC 50, a gift from M. Schechtman. pNC 50 DNA was linearized with *Hin*dIII and *Eco*RI, pUC 19 control DNA was linearized with either *Ava*II or *Taq*I, and the appropriate fragments were eluted from a 7.5% polyacrylamide gel. The 270-bp $(T_2AG_3)_{27}$ fragment was end labeled with [α-^{32}P]dATP, the 222-bp *Ava*II pUC 19 fragment was end labeled with [α-^{32}P]dGTP, and the 476-bp *Taq*I pUC 19 fragment was end labeled with [α-^{32}P]dCTP by incubating these fragments with the Klenow fragment of DNA polymerase I (27). HT-d, the double-stranded synthetic DNA molecule containing four copies of T_2AG_3, was a gift of Z. Liu. It was created by annealing the complementary oligonucleotides 5′ $TCGA(C_3TA_2)_4C$ 3′ and 5′ $TCGAG(T_2AG_3)_4$ 3′. HT-d was end labeled with [α-^{32}P] dTTP and the Klenow fragment of DNA polymerase I. The typical binding assay (20 μl) contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), 50 mM NaCl, 4 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM DTT, 1 mM EDTA, 10% glycerol, 1 to 4 μg of poly(dI-dC) (Boehringer Mannheim), 1 to 3 μl of protein extract (which contains the last four 700 mM fractions and the first two 800 mM fractions from the heparin-agarose column fractionation at a concentration of 0.47 μg/μl), and 1 to 2 ng of [^{32}P]DNA fragment. The assays were incubated at room temperature for 30 min and electrophoresed at 10 V/cm on a 7.5 or 15% (for HT-d) polyacrylamide gel that had been preelectrophoresed for 30 min at 10 V/cm. Gels were dried and exposed to Kodak XAR-5 film with an intensifying screen at −70°C.

Sedimentation analysis. Sedimentation analysis was carried out in a 10 to 30% linear glycerol gradient. PPT was mixed in the same tube with 50 μg each of BSA, carbonic anhydrase, and lysozyme. Centrifugation was carried out in a Beckman ultracentrifuge (SW 60 Ti rotor) at a centrifugal force of 260,000 × *g* for 30 h at 4°C. After centrifugation, 0.2-ml fractions were collected from the top of the tube and assayed for the marker proteins as well as for binding activity.

DNase I footprinting. The plasmid used for footprint probes was HT1Δ1, which consists of the sequence 5′ $(C_3TA_2)_3$CCCTA 3′ inserted into the *Sal*I site of pBluescript II KS (Stratagene). This plasmid was constructed by digesting the parent plasmid HT1 (a gift of Z. Liu) (which contains two tandem copies of the HT-d oligonucleotide in the same orientation inserted into the *Sal*I site of pBluescript II KS) with *Hin*dIII, treating the linearized DNA with exonuclease III (Pharmacia), and recircularizing the DNA. A portion of HT1Δ1 was then amplified between the reverse primer and the M13 −20 primer using polymerase chain reaction (PCR) methods (39), in a 50-μl reaction mixture containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 45 ng of reverse primer, 45 ng of M13 −20 primer, 0.6% Nonidet P-40, 0.6% Tween-20, 0.5 to 1 U of *Taq* polymerase (New England BioLabs), and 100 ng of HT1Δ1 DNA. This amplified 200-bp fragment was then used as template DNA in a modified PCR to selectively label either the C- or G-rich telomeric strand. The reverse primer was labeled with [γ-^{32}P]ATP and polynucleotide kinase and included in the reaction buffer in place of the unlabeled reverse primer to selectively label the C-rich telomere strand. Similarly, the M13 −20 primer treated with

kinase was used to label the G-rich strand. Various amounts of PPT (1 or 2 μl of the heparin-agarose pool) were incubated with the radioactive DNA fragments (1 ng per reaction, which is equivalent to 0.9 fmol of telomeric repeats) at room temperature for 20 min in a 50-μl reaction mixture containing 25 mM Tris-Cl (pH 8.0), 6.25 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, 0.5 mM DTT, 1 μg of poly(dI-dC), and 10% glycerol. The mixtures then were digested for 1 min at room temperature with 5 to 10 μl of a freshly diluted 5-mg/ml solution of DNase I (Sigma). The DNase I digestion reactions were terminated with 100 μl of a solution consisting of 200 mM NaCl, 20 mM EDTA, 100 μg of tRNA per ml, and 1% SDS. They were then phenol-chloroform extracted and precipitated with 2.5 volumes of ethyl alcohol, and the pellets were rinsed with 70% ethyl alcohol and dried before loading onto a 7% sequencing gel. The sequencing markers were made by annealing either the M13 −20 primer (G,T) or the reverse primer (A,C) to denatured HT1Δ1 and elongating the DNA with Sequenase (USB), using the dideoxy-chain termination method of Sanger (40).

RESULTS

Nuclear extracts contain a telomere binding activity. In our initial experiments, *Physarum* nuclear extracts were dialyzed against 50 mM Tris (pH 7.8)–100 mM NaCl, histones and other precipitated proteins were removed by centrifugation, and the nuclear extracts were fractionated by a step elution from phosphocellulose. Protein fractions eluting between 200 and 800 mM NaCl were examined for ability to bind specifically to rDNA fragments, by means of the classic nitrocellulose filter binding assay (38). The rDNA had been purified from *P. polycephalum*, digested with *Bst*EII, and end labeled. These experiments revealed that protein in the 700 mM NaCl fraction preferentially retained the terminal 4.49-kb *Bst*EII fragment as increasing amounts of competitor DNA were added to inhibit nonspecific binding proteins (Fig. 1A, lanes 3 to 5, and Fig. 1B). In Fig. 1A, the light intensity of this band results from the size heterogeneity of the terminal fragment, caused by the various amounts of telomeric repeats present on each rDNA molecule. Further experiments localized the binding to the terminal 2.7-kb *Bam*HI rDNA fragment (data not shown). This fragment is composed of several hundred base pairs of T_2AG_3 repeats plus subtelomeric sequences that have not been fully characterized. To determine whether the protein, which we call PPT for *P. polycephalum* telomere-binding protein, interacts specifically with the telomeric sequence $(T_2AG_3)_n$, we employed gel mobility shift assays. The reproducibility of these assays required further purification of the protein.

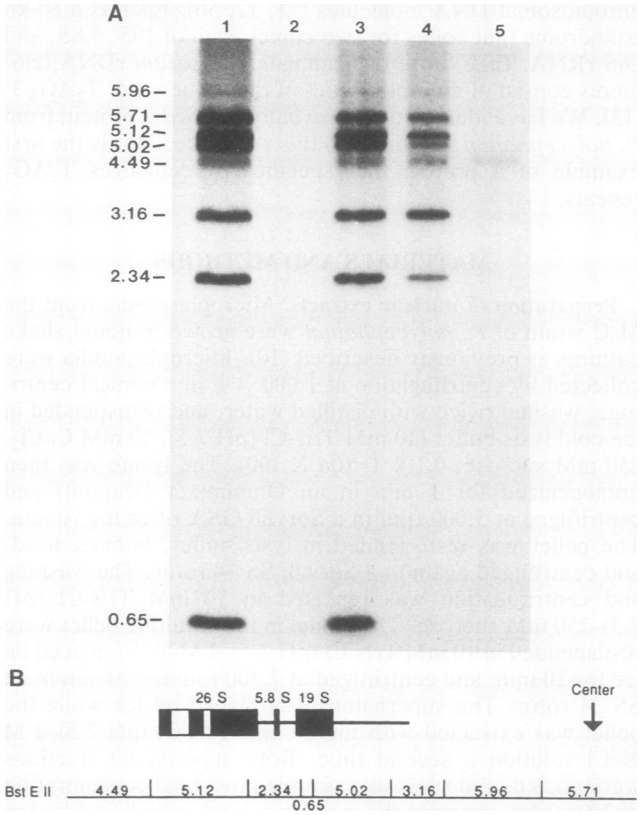

FIG. 1. Localization of telomere binding. (A) Nitrocellulose filter binding. *Bst*EII-digested and 5′-end-labeled rDNA was incubated with a protein fraction that had been purified over a phosphocellulose column. The mixture was filtered through a nitrocellulose membrane, and the retained DNA was analyzed by electrophoresis on a native 0.8% agarose gel. Increasing amounts of sonicated *E. coli* DNA were added as competitor. Lane 1, one-third of the input DNA; lane 2, no protein control; lane 3, no *E. coli* DNA; lane 4, 500 ng of *E. coli* DNA; lane 5, 1 μg of *E. coli* DNA. The no protein control lane demonstrates that no DNA fragments are retained on nitrocellulose unless bound to protein. (B) Diagram of the rDNA. The drawing shows a half palindrome of the 60-kb rDNA molecule. The exons are shown as black boxes, and the introns are shown as white boxes. Below the diagram is the restriction map of *Bst*EII-digested rDNA. The size (in kilobases) of each restriction fragment is shown and corresponds to the bands marked in panel A.

Purification of telomere-binding protein. We found that the telomere binding activity is extremely heat stable. Treatment of a partially purified protein fraction at 90°C for 10 min did not diminish binding (Fig. 2A, lane 7). This particular gel mobility shift assay used a 270-bp fragment that contains 27 copies of the $(T_2AG_3)_n$ telomeric repeat. Two nonspecific pUC 19 fragments were used as internal controls. Addition of protein resulted in the disappearance of the specific fragment, with no effect on the two control fragments. We interpret the absence of an obvious, discrete "shifted band" to be due to the many binding sites for PPT on this DNA fragment, resulting in the formation of a broad smear of shifted complexes. Heat treatment was incorporated into a new purification scheme. Nuclear extracts were heated at 90°C, chilled to 0°C, and then centrifuged at 8,000 × *g*; approximately 85% of the total protein was removed by this step. The supernatant was loaded onto a heparin-agarose column, from which proteins were eluted with a step gradient from 200 to 800 mM NaCl. At each step, the resulting material was subdivided into five fractions. Gel mobility shift assays on alternate fractions localized the specific binding activity to the last four 700 mM NaCl fractions and the first 800 mM NaCl fraction (Fig. 2B, lanes 13 to 15). Although there was a visible shift of the $(T_2AG_3)_{27}$ fragment in the first and third fractions of the 600 mM NaCl step (Fig. 2, lanes 10 and 11), there was also a concomitant shift in the 476-bp vector fragment in these same fractions, implying the presence of nonspecific binding proteins. Silver staining of an SDS polyacrylamide gel of a pool of fractions with PPT activity (the last four 700 mM NaCl fractions and the first two 800 mM NaCl fractions) showed approximately 10 major polypeptides, ranging in size from 10 to 100 kDa (data not shown). We estimate that purification to this stage resulted in a several hundred-fold enrichment of PPT, over crude

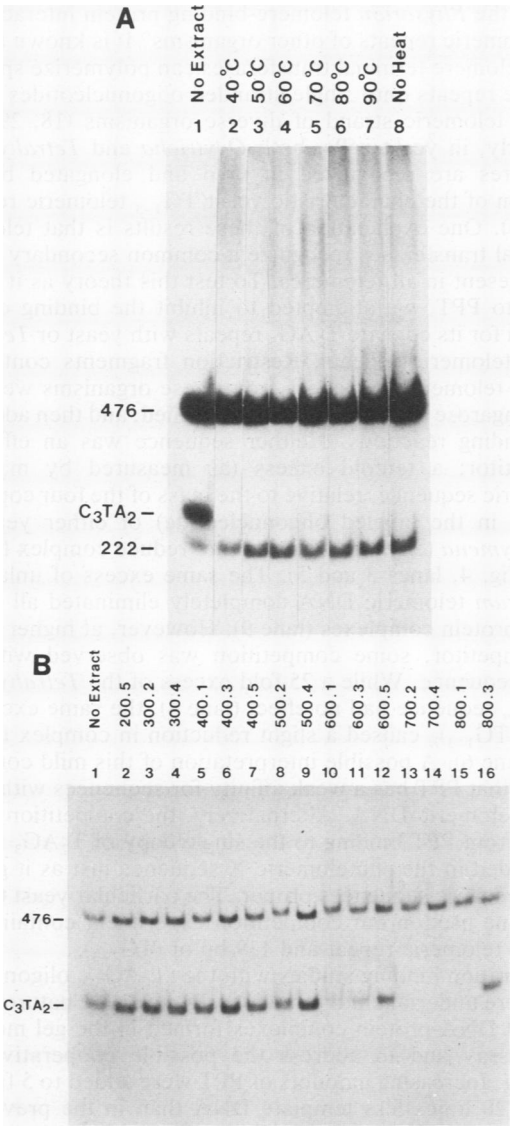

FIG. 2. Purification of PPT. (A) Heat stability. Protein from the heparin agarose step gradient was heated at different temperatures (40 to 90°C) for 10 min, allowed to cool to room temperature, and then assayed for telomere binding activity in a standard gel mobility shift assay. The template DNA is composed of three fragments: two nonspecific pUC 19 species of 222 and 476 bp and a 270-bp $(T_2AG_3)_{27}$ species. An autoradiogram of a native 7.5% polyacrylamide gel is shown. (B) Heparin agarose chromatography. Seven steps ranging from 200 to 800 mM NaCl, in 100 mM increments, were collected. The material at each step was subdivided into five fractions, and alternate fractions were assayed by the gel mobility shift assay in the presence of 4 μg of poly(dI-dC). The number in front of the decimal refers to the molarity of the NaCl step (in millimolar), and the number following the decimal indicates the particular fraction assayed.

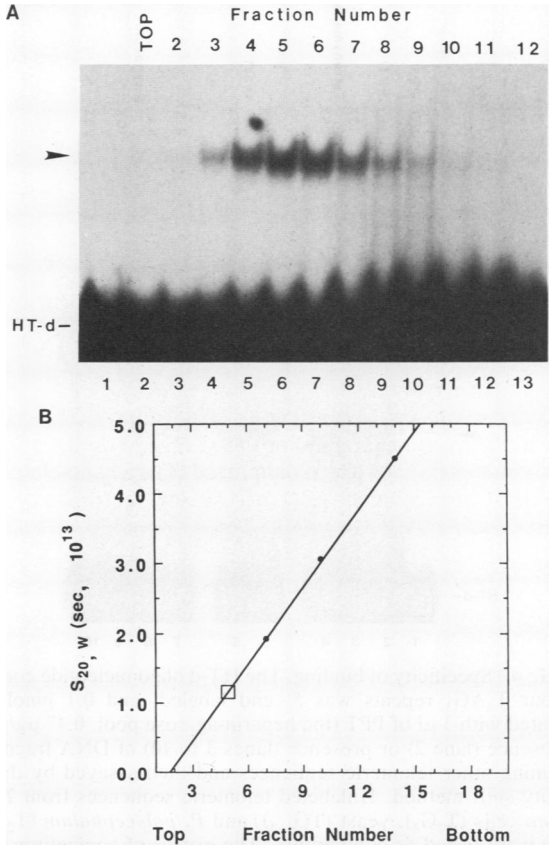

FIG. 3. Glycerol gradient sedimentation. (A) Gel mobility shift assay with oligonucleotide. End-labeled HT-d DNA was incubated with 20 μl of each glycerol gradient fraction in the presence of 4 μg of poly(dI-dC), and the complexes formed were visualized on a 15% acrylamide gel. Lane 1, input DNA; lane 2, top fraction. The arrowhead designates the peak of the DNA-protein complex. The autoradiogram was scanned by densitometry to determine the peak of PPT activity. (B) Sedimentation relative to standards. To identify the location of the protein standards, equal portions of the glycerol gradient fractions were subjected to electrophoresis on SDS-polyacrylamide gels, followed by Coomassie brilliant blue staining. Symbols: ●, protein standards, from left to right, lysozyme (1.91S), carbonic anhydrase (3.06S), and BSA (4.49S); □, PPT.

extracts before removal of nuclei, assuming no loss of activity. We do not know the actual purification factor, since PPT cannot be assayed readily in crude extracts.

We employed sedimentation in glycerol gradients to estimate the size of PPT. An aliquot of the pool of heparin-agarose fractions containing PPT activity was layered onto a glycerol gradient together with protein standards of known S values and then centrifuged at 260,000 × *g*. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis to locate the protein standards. In parallel, a gel mobility shift assay was employed to locate the telomere binding activity. The bulk of the binding activity was contained in fractions 4 to 6, with the peak in fraction 5 (Fig. 3A, lanes 5 to 7). Comparison with the sedimentation of the internal standards yielded a sedimentation coefficient of 1.2 (Fig. 3B). Assuming that the protein is globular, this S value would correspond to a molecular weight of about 10,000. This would make PPT the smallest known telomere-binding protein.

PPT binds to the telomeric repeats. To confirm the expectation that PPT binds to the telomeric repeats, we used a double-stranded oligonucleotide containing four copies of T_2AG_3. The heparin-agarose pool was incubated with 0.1 pmol of the end-labeled DNA, and the DNA-protein complexes were resolved on a native 15% polyacrylamide gel. Four separate complexes were observed (Fig. 4, lane 2). The specificity of binding to the labeled oligonucleotide is implied by competition with unlabeled oligonucleotide. A tenfold

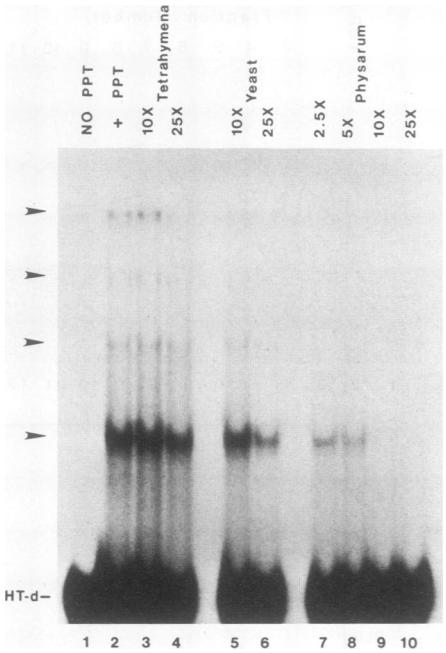

FIG. 4. Specificity of binding. The HT-d oligonucleotide containing four T_2AG_3 repeats was 5′ end labeled, and 0.1 pmol was incubated with 3 μl of PPT (the heparin-agarose pool, 0.47 μg/μl) in the absence (lane 2) or presence (lanes 3 to 10) of DNA fragments containing other telomeric sequences and then assayed by the gel mobility shift method. Unlabeled telomeric sequences from *Tetrahymena* cells (T_2G_4), yeast (TG_{1-3}), and *P. polycephalum* (T_2AG_3, HT-d) were tested as competitors. The excess of competitor DNA was calculated as the mass of telomeric sequences in the competitor divided by the mass of telomeric sequences in the labeled oligonucleotide, without regard to other DNA sequences present either in the HT-d oligonucleotide or in the competitor fragments. Lane 2, no competitor DNA; lanes 7 to 10, excess HT-d, as indicated above the lanes. The arrowheads mark the DNA-protein complexes formed.

excess of unlabeled HT-d DNA completely abolished all of the complexes (Fig. 4, lane 9). In the same assay, competition with 1,000-fold excess of the nonspecific competitor pUC 19 (which does not contain any T_2AG_3 sequences) did not affect the formation of complexes (data not shown). The simplest model to account for the four different complexes would be that the most rapidly migrating band results from the binding of a single PPT molecule to the DNA (monomers) and that the progressively larger complexes result from the binding of two, three, or four molecules of PPT. According to this model, PPT would be able to recognize a single T_2AG_3 sequence.

We attempted to address directly the possible ability of PPT to recognize a single telomeric repeat. A double-stranded oligonucleotide that contains the sequences 5′ TCGAGCGTTAGGGTG 3′ and 5′ TCGACACCCTAACGC 3′ was prepared. Note that the flanking base pair on each side of the telomeric repeat (underlined) is as would be found in the tandem repeats of native telomeric DNA. In a gel mobility assay, PPT did not interact detectably with this radioactive DNA under conditions where a parallel incubation yielded the usual pattern of four bands with the HT-d DNA that had been labeled to similar specific activity (data not shown). It appears from this experiment that the protein does not bind tightly to the single sequences GTTAGG, TTAGGG, and TAGGGT.

Can the *Physarum* telomere-binding protein interact with the telomeric repeats of other organisms? It is known that in vitro telomere terminal transferases can polymerize species-specific repeats onto single-stranded oligonucleotides of the G-rich telomeric strand of diverse organisms (18, 29, 45). Similarly, in yeast cells, both *Oxytricha* and *Tetrahymena* telomeres are recognized in vivo and elongated by the addition of the characteristic yeast TG_{1-3} telomeric repeats (32, 44). One explanation of these results is that telomere terminal transferases recognize a common secondary structure present in all telomeres. To test this theory as it might apply to PPT, we attempted to inhibit the binding of this protein for its cognate T_2AG_3 repeats with yeast or *Tetrahymena* telomeric repeats. Restriction fragments containing cloned telomeric sequences from these organisms were cut out of agarose or acrylamide gels, purified, and then added to the binding reactions. Neither sequence was an effective competitor; a tenfold excess (as measured by mass of telomeric sequence, relative to the mass of the four copies of T_2AG_3 in the labeled oligonucleotide) of either yeast or *Tetrahymena* telomere DNA did not reduce complex formation (Fig. 4, lanes 3 and 5). The same excess of unlabeled *Physarum* telomeric DNA completely eliminated all of the DNA-protein complexes (lane 9). However, at higher levels of competitor, some competition was observed with the yeast sequence. While a 25-fold excess of the *Tetrahymena* $(T_2G_4)_n$ sequence had no effect (lane 4), the same excess of yeast $(TG_{1-3})_n$ caused a slight reduction in complex formation (lane 6). A possible interpretation of this mild competition is that PPT has a weak affinity for sequences within the yeast telomeric DNA. Alternatively, the competition could result from PPT binding to the single copy of T_2AG_3 that is embedded in the subtelomeric X sequence just as it grades into telomeric sequences proper. The particular yeast telomere clone used in our competition experiment contains one T_2AG_3 telomeric repeat and 139 bp of $(TG_{1-3})_n$.

Saturation binding studies with the $(T_2AG_3)_4$ oligonucleotide were undertaken to provide insight into the nature of the several DNA-protein complexes formed in the gel mobility shift assay and to address the possible cooperativity of binding. Increasing amounts of PPT were added to 5 fmol of DNA (20 times less template DNA than in the previously described assays), and the formation of complexes was measured as described above. The lowest level of protein resulted in the formation predominantly of the fastest migrating band, with higher levels of PPT leading to increased formation of the more slowly migrating bands (Fig. 5, lanes 2 to 5). At the highest concentration of protein, most of the shifted DNA was observed in the largest complex (Fig. 5, lane 6), implying that at this concentration PPT is saturating with respect to the substrate DNA. The rapid increase in the large complex (ca. 100-fold) as the protein concentration is increased only slightly (~2-fold, compare lanes 4 and 5) suggests that binding is cooperative. However, a quantitative interpretation of these results is not possible without knowledge of the nature of the complexes formed.

To further confirm the binding specificity of PPT, we undertook DNase I footprinting studies. In order to maximize the saturation of binding sites by the protein, we first constructed a plasmid carrying only about four copies of the telomeric repeat. A 200-bp fragment containing this sequence was amplified by PCR (see Materials and Methods). The fragment was selectively end labeled on either the C- or G-rich telomeric strand by the inclusion of the appropriate end-labeled primer in the PCR amplification. After binding of PPT (1 or 2 μl of the heparin-agarose pool) to 1 ng of

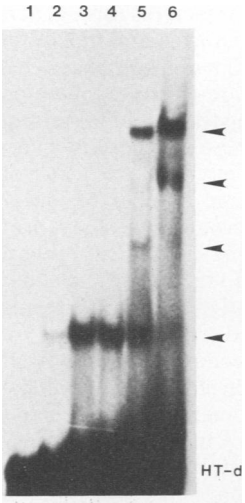

FIG. 5. Saturation of binding. The HT-d oligonucleotide was 5′ end labeled and 5 fmol was incubated with increasing amounts of PPT (1 to 10 μl of the heparin-agarose pool). Gel mobility shift assays were then performed. Lane 1, no PPT; lane 2, 1 μl of PPT; lane 3, 2 μl of PPT; lane 4, 4 μl of PPT; lane 5, 7 μl of PPT; lane 6, 10 μl of PPT. The arrowheads mark the DNA-protein complexes formed.

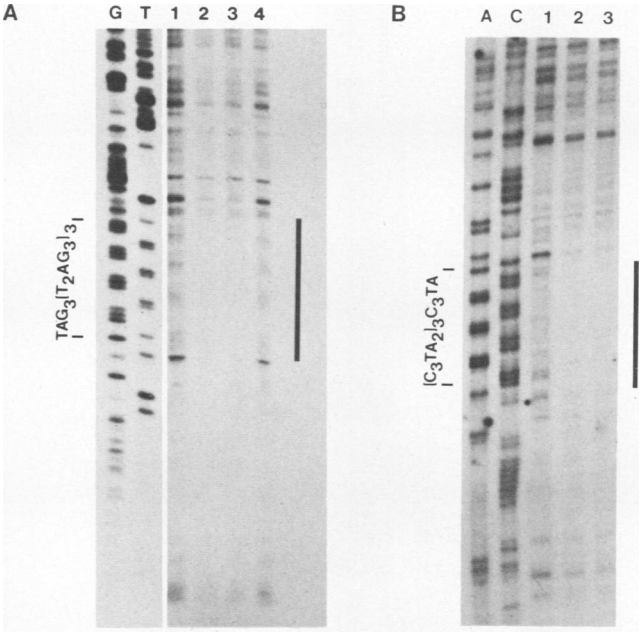

FIG. 6. Footprint analysis. (A) Strand containing T_2AG_3 repeats. The 200-bp probe was made by PCR amplification of HT1Δ1 between the reverse primer and the ^{32}P-labeled M13 −20 primer. Lanes G and T, HT1Δ1 sequencing markers; lane 1, DNase I digestion without PPT; lane 2, 1 μl of PPT added; lane 3, 2 μl of PPT added; lane 4, 2 μl of PPT and 5 pmol of HT-d oligonucleotide added. Each reaction contained approximately 0.9 fmol of telomeric repeats. The protected region is indicated with a bar. (B) Strand containing C_3TA_2 repeats. The 200-bp probe was made by PCR amplification of HT1Δ1 between the ^{32}P-labeled reverse primer and the M13 −20 primer. Lanes A and C, HT1Δ1 sequencing markers; lane 1, DNase I digestion without PPT; lane 2, 1 μl of PPT added; lane 3, 2 μl of PPT added. The protected region is indicated with a bar.

template DNA, protection from DNase I digestion was assessed by polyacrylamide gel electrophoresis by standard techniques (22). This amount of protein was sufficient to completely saturate all telomeric repeats, as inferred from gel mobility assays. The same primers that were used in the PCR were also used to generate dideoxy sequencing ladders to allow precise identification of the sequences bound by PPT. This experiment showed that the entire telomeric sequence 5′ $TAGGG(T_2AG_3)_3$ 3′ was protected, as evidenced by the lack of DNA fragments in this portion of the gel (Fig. 6A, lanes 2 and 3). Protection was abolished when the specific competitor oligonucleotide HT-d was included in the reaction (Fig. 6A, lane 4). Footprinting of the C-rich strand gave similar results; the entire telomeric sequence 5′ $(C_3TA_2)_3CCCTA$ 3′ was also protected (Fig. 6B, lanes 2 and 3). It is noteworthy that the PPT footprint extended over all four telomeric repeats on both strands, even though the fourth repeat is missing one nucleotide from the consensus sequence, the final A being replaced by a C. Thus, PPT may tolerate some sequence alteration of its binding site. Protein-protein interactions may also play a role in holding the protein over an imperfect binding site.

Further properties of PPT. Telomere terminal transferases contain a vital RNA component that acts as a template for addition of the characteristic species-specific telomeric repeats (18, 19, 29, 45, 46, 53, 54). We tested the possibility that PPT is also associated with an RNA. The heparin-agarose-purified protein pool was treated with increasing amounts of RNase A at room temperature and then incubated with $(T_2AG_3)_{27}$ DNA and two pUC 19 control fragments before gel mobility shift analysis. RNase A had no effect on the formation of telomeric DNA-protein complexes (Fig. 7, lanes 2 to 5). By contrast, incubation with proteinase K abolished binding activity (data not shown). These results, together with its small size, make it likely that PPT is a simple protein.

DISCUSSION

We have identified a small, very heat-stable *Physarum* protein called PPT, which binds to $(T_2AG_3)_n$ sequences in vitro. This sequence is found at telomeres of numerous organisms, including *P. polycephalum* and mammals. The specificity of the protein-DNA interaction was demonstrated by nitrocellulose filter binding with fragments of *Physarum* extrachromosomal rDNA, by gel mobility shift assays, and by DNase I footprinting. In gel mobility shift assays, PPT forms four major complexes with an oligonucleotide containing four tandem copies of T_2AG_3. Unlike the telomere terminal transferases, it appears not to contain an RNA component. It also differs greatly from telomere terminal transferases in size. For example, the prototypic *Tetrahymena* telomerase has a native size of 200 to 500 kDa (18). PPT does not bind with high affinity to any of the other telomeric sequences tested. It is the first reported example of a binding protein that is specific for telomeric DNA composed of T_2AG_3 repeats. Electron microscopic evidence for a *Physarum* protein bound to the extrachromosomal rDNA had been reported previously (8), but because of its location in the subtelomeric region this protein is unlikely to be identical to PPT.

An unanswered question that is raised by these results is, what is the minimum sequence that allows tight binding of PPT to telomeric DNA? On the one hand, the fact that PPT forms four complexes on a DNA with four repeats and the

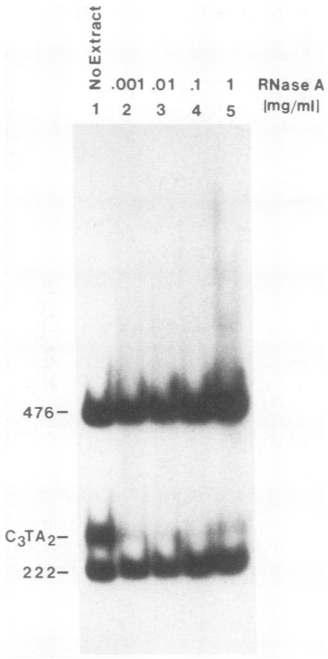

FIG. 7. RNase A insensitivity. PPT was treated with increasing amounts of RNase A (2 μl of a 1-, 10-, or 100-μg/ml solution or 1-mg/ml solution) for 15 min at room temperature and then added to end-labeled $(C_3TA_2)_{27}$ DNA and assayed by the gel mobility shift assay. Lane 1, No PPT; lanes 2 to 5, increasing amounts of RNase A (1 [lane 2], 10 [lane 3], and 100 [lane 4] μg/ml and 1 [lane 5] mg/ml).

fact that the larger complexes are concentration dependent suggest that one to four molecules of the protein are bound to these complexes and thus that one molecule recognizes a single repeat. This notion is consistent with the weak competition found for the yeast telomeric DNA clone, which carries a single TTAGGG sequence. On the other hand, the absence of complex formation observed with a short DNA containing the same TTAGGG sequence argues against tight binding to this isolated sequence. All of these results can be interpreted in multiple ways, however. For example, competition with the yeast DNA might result from a weak affinity of PPT for the bona fide yeast TG_{1-3} telomeric DNA. The single-copy sequence in the short DNA we constructed might not support binding because the sequence is not phased properly: only three of the six possible permutations of TTAGGG are represented in this oligonucleotide. Perhaps PPT can recognize and bind to a single repeat, but only in the context of multiple copies of that repeat or in the context of other telomeric DNA, as in the yeast clone, because of a DNA secondary structure. Also, it is possible that the four bands seen in the gel mobility shift assays correspond to more complicated protein-DNA complexes than suggested. Binding studies with DNAs containing other permutations of one, two, and three repeats will be necessary to define precisely the minimum strong binding site for PPT. No matter what the exact nature of the observed complexes, however, the concentration dependence of their formation suggests strongly that PPT binding to DNA is cooperative.

Several other proteins that interact specifically with telomeric DNA have been reported previously. The best studied are from the hypotrichous ciliates *Euplotes nova* and *Oxytricha crassa*. During formation of the macronucleus in these organisms, chromosomal DNA is cut into many small fragments, and new telomeres are generated for each end. Unlike those of mammals and most other species, *Euplotes* and *Oxytricha* telomeres are short, have a defined 14- or 16-bp 3′ overhang, respectively, with only a short stretch of contiguous double-strand telomeric sequence (23). Proteins bound to the telomeres of these two species in vivo that can rebind specifically in vitro have been isolated (33, 34, 36, 37). Because of the large number of chromosomal DNA fragments in the macronucleus, they are abundant proteins. They protect against exonuclease digestion in vitro and are thought to serve in vivo to cap the ends of DNA, preventing degradation of the DNA (16, 33). It might be expected that the highly repeated telomeric DNAs found in most eucaryotes also have specific and tightly associated proteins, but only indirect evidence for this notion has been reported. For example, the telomeric region of the extrachromosomal *Tetrahymena* rDNA molecule is not in a normal nucleosome structure (3), plausibly because of bound proteins that prevent their formation.

The ability of PPT to protect multiple repeats of TTAGGG in footprinting assays and the cooperativity of binding inferred from the gel mobility assays suggest to us that PPT may coat the entire double-stranded telomeric DNA in vivo. This coating could extend to the single-stranded $(T_2AG_3)_n$ 3′ overhang characteristic of all telomeres, since in preliminary studies we have found that PPT can bind to the single-stranded $(T_2AG_3)_4$ oligonucleotide (9a). The possible function of binding to telomeric DNA remains a matter of speculation. It could lead to protection of the telomere from degradation by nucleases, thus giving PPT a function similar to that suggested for the *Oxytricha* and *Euplotes* telomeric capping proteins. Or it might set up a chromatin complex that facilitates the actions of telomere terminal transferase or DNA primase and the replicating machinery. Another possibility is that PPT promotes attachment of telomeres to the nuclear matrix, as has been suggested for vimentin (47). Some of these possibilities will be experimentally testable once the gene for PPT is isolated, allowing preparation of highly purified protein in large quantities and the generation of antisera.

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