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Identification and Characterization of a Putative Telomere End-Binding Protein from *Tetrahymena thermophila*

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Telomeric DNA of *Tetrahymena thermophila* consists of a long stretch of $(TTGGGG)_n$ double-stranded repeats with a single-stranded $(TTGGGG)_2$ 3′ overhang at the end of the chromosome. We have identified and characterized a protein that specifically binds to a synthetic telomeric substrate consisting of duplex DNA and the 3′ telomeric repeat overhang. This protein is called TEP (telomere end-binding protein). A change from G to A in the third position of the TTGGGG overhang repeat converts the substrate to a human telomere analog and reduces the binding affinity approximately threefold. Changing two G's to C's in the TTGGGG repeats totally abolishes binding. However, permutation of the *Tetrahymena* repeat sequence has only a minor effect on binding. A duplex structure adjacent to the 3′ overhang is required for binding, although the duplex need not contain telomeric repeats. TEP does not bind to G-quartet DNA, which is formed by many G-rich sequences. TEP has a greatly reduced affinity for RNA substrates. The copy number of TEP is at least 2×10^4 per cell, and it is present under different conditions of cell growth and development, although its level varies. UV cross-linking experiments show that TEP has an apparent molecular mass of ~65 kDa. Unlike other telomere end-binding proteins, TEP is sensitive to high salt concentrations.

Telomeres are the natural ends of eukaryotic chromosomes. They protect chromosomes from nuclease degradation and from end-to-end ligation, ensure complete replication of chromosomes, and are involved in chromosome organization and nuclear architecture (2, 22, 34, 47). Telomeres typically contain an array of short (5- to 8-bp) sequence repeats which are G rich in the strand that extends to the 3′ end of the chromosome (2, 22, 34, 47). In those cases studied in molecular detail, it has been shown that the G-rich strand forms a 3′ single-stranded overhang of 12 to 16 nucleotides at the chromosomal terminus (20, 25, 32). Most telomeric sequences fit the consensus $C_{1-8}(T/A)_{1-4}$ (2, 22, 34, 47).

It is important to characterize proteins that bind to telomeres because they are intimately involved in telomere-mediated chromosome stabilization. Moreover, telomere-binding proteins must interact with telomerase, an enzyme involved in telomere replication and maintenance, whose activity is implicated in both cancer and aging (10). Telomeric DNA is associated with two types of proteins in vivo. Internal telomere-binding proteins interact with the duplex region of telomeric repeats. These include PPT, identified in *Physarum polycephalum*, and RAP1, identified in the yeast *Saccharomyces cerevisiae* (3, 4, 8, 9, 27). PPT is a 10-kDa heat-stable protein that binds specifically to the duplex region of the telomeric sequence $(T_2AG_3)_n$ and is thought to cover the length of the telomere (8). RAP1 is a multifunctional protein that, in addition to binding telomeric repeats in yeast cells, binds to the upstream activating sequences of many genes and to silencer elements. Underexpression of RAP1 reduces telomere length, whereas overproduction increases both telomere length and heterogeneity (7, 28, 41). Another internal duplex telomere-binding protein has been characterized in extracts of mammalian cells and may bind along the length of mammalian telomeres (48).

A second type of telomere-binding protein binds specifically to the duplex and 3′ overhang structures at the telomeric terminus. Telomere end-binding proteins have been isolated from *Euplotes crassus* and *Oxytricha nova* and have recently been identified in *Xenopus* egg extracts (5, 15, 16, 33, 35). The proteins from the ciliate species bind specifically to the T_4G_4 or T_4G_2 repeats at the 3′ overhang and protect the telomeric DNA from chemical modification and *Bal* 31 nuclease digestion (15, 16, 33, 35, 36). These protein-telomeric DNA complexes are resistant to high concentrations of salt (e.g., 2 M NaCl or 6 M CsCl) (33, 35). The *Oxytricha* telomere end-binding protein is a 98-kDa heterodimer containing subunits of 56 kDa (α subunit) and 41 kDa (β subunit). Although both subunits are required for maximal binding activity, the binding domain is located entirely in the α subunit. The DNA binding activity is stabilized when the β subunit is present. The *Euplotes* end-binding protein has a single subunit of 51 kDa (33), which is homologous to the α subunit of the *Oxytricha* protein (42).

To further characterize the interaction between telomeric DNA and telomere-binding proteins, we have identified a protein (telomere end-binding protein [TEP]) from *Tetrahymena thermophila* that binds specifically to the 3′ overhang telomeric repeats of synthetic telomeres. Our results show that both the $(TTGGGG)_2$ telomeric overhang sequence and the duplex structure adjacent to it are necessary for TEP binding activity. This protein is distinct from previously identified telomere end-binding proteins in that its binding is salt sensitive. Purification of TEP will provide an excellent opportunity to further investigate the interaction between a telomere end-binding protein and telomerase (17) in *T. thermophila*, the organism in which telomerase is best characterized.

MATERIALS AND METHODS

Preparation of cell extracts. Mating-cell extracts were prepared as described previously (18), with several modifications. Briefly, strains C3V and C3rmm1 were grown to mid-log phase (3×10^5 to 5×10^5 cells per ml) at 30°C. Cells were then washed twice with 10 mM Tris-Cl (pH 7.5) and resuspended in an equal volume of the wash buffer. After starvation for 24 h, cells from the two strains were mixed together and incubated for 9 h without shaking. Pairing efficiency was greater than 90%. Cells were harvested by centrifugation and resuspended in 5

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volumes of TMG buffer (10 mM Tris-Cl [pH 7.5], 1 mM $MgCl_2$, 10% glycerol, 10 μg of pefabloc [Boehringer Mannheim] per ml, 1 μg of pepstatin per ml, 1 μg of leupeptin per ml, 10 mM β-mercaptoethanol). One-tenth volume of 2% Nonidet P-40 in TMG was added immediately to lyse the cells, and the mixture was stirred at 4°C for 30 min. The lysate was then subjected to ultracentrifugation at 100,000 × *g* for 1 h at 4°C. The supernatant, termed S100, was quickly frozen in liquid nitrogen and stored at −80°C. Protein concentration was determined by the Bradford method. As a control for the mating-cell extract, starved cells from the two strains were mixed together and harvested immediately.

Mid-log-phase cell extract was prepared when the cell densities reached 5×10^5 cells per ml (for C3V cells) and 3×10^5 cells per ml (for C3rmm1 cells). Stationary-phase cell extract was prepared after cells had been grown to 1.5×10^6 cells per ml (C3V) and 8×10^5 cells per ml (C3rmm1). For preparation of extracts from starved cells, cells were grown to mid-log phase and starved for 33 h before harvesting.

Preparation of DNA substrates. DNA oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems) and purified by electrophoresis in 20% denaturing (7 M urea) polyacrylamide gels. DNA bands were visualized by UV shadowing, cut from the gel, and eluted by shaking in Tris-EDTA (pH 7.5) overnight. Gel-purified oligonucleotides were then desalted by C_{18} (Waters) column chromatography. Oligonucleotides were 5′ end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and were purified again on 12% denaturing (7 M urea) polyacrylamide gels. Usually the G-rich strands were end labeled and annealed to unlabeled complementary strands. Duplexes were formed by boiling a labeled G strand with a 5- to 10-fold molar excess of unlabeled complementary strand for 2 min in the presence of 10 mM Tris-Cl (pH 7.5) and 100 mM LiCl (to minimize G-DNA formation [45, 46]) followed by cooling in a 41°C heat block for 1 h. The amount of labeled oligonucleotide in duplex form was determined by running the renatured sample on a 20% nondenaturing polyacrylamide gel. For experiments described in this report, approximately 95% of G strands were present in the duplex form.

Preparation of RNA substrates. The RNA oligonucleotide ST(S)R was derived from the in vitro transcription of a specific DNA template containing a T7 RNA polymerase promoter sequence and the complementary sequence of the anticipated RNA product.

Two DNA oligonucleotides (A [5′ TAATACGACTCACTATAG 3′] and B [3′ ATTATGCTGAGTGATATCTTTGAGCTGATCACGTAGCTGAACCCCA ACCCC 5′]) were synthesized and purified as described above. The oligonucleotides were allowed to anneal by boiling together for 2 min in 10 mM Tris-Cl (pH 8)–100 mM LiCl and slowly cooling to room temperature. The resultant DNA template contained a duplex region corresponding to the 17-bp conserved promoter sequence for T7 RNA polymerase and a single-stranded region representing the complementary sequence of ST(S)R, which was generated and radiolabeled by in vitro transcription as described previously (31). Typical reaction mixtures contained 40 mM Tris-Cl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 1 mM dithiothreitol (DTT), 1.3 U of RNasin (Promega) per μl, 1 mM rATP, rGTP, and rCTP, 40 μM rUTP, 6 μM [α-^{32}P]rUTP, 40 nM DNA template, and 30 U of T7 RNA polymerase. The reaction mixture was incubated at 37°C for 2 h and resolved on a denaturing (7 M urea) 20% polyacrylamide gel. The location of full-length ST(S)R was determined by using an RNA molecular size marker generated by in vitro transcription of *Not*I-linearized pBluescript SK II (Stratagene), using T3 RNA polymerase. The full-length radiolabeled ST(S)R was cut out from the gel, eluted overnight with 400 μl diethylpyrocarbonate-treated water at room temperature, ethanol precipitated, and resuspended with diethylpyrocarbonate-treated water. The nonspecific RNA competitor (51 nucleotides) used in the electrophoretic mobility retardation assay (EMRA) (see below) was derived from the in vitro transcription of *Hin*dIII-linearized pBluescript SK II, using T7 RNA polymerase.

EMRA. Radiolabeled DNA probe (0.06 to 0.08 pmol) was incubated with crude cell extract (S100, containing 2 to 5 μg of protein) in 20 μl of EMRA buffer {10 mM Tris-Cl (pH 7.5), 100 to 150 mM KCl or LiCl, 5 mM $MgCl_2$, 1 mM DTT, 4 to 10% glycerol, 25 to 50 ng of poly[d(I-C)] per μl}. After incubation for 20 to 30 min at room temperature, the mixture was separated on a 5% polyacrylamide gel (Protogel; National Diagnostics) in 0.6× TBE (54 mM Tris-borate [pH 8.3], 1 mM EDTA) at 10 V/cm for 1.5 h. Gels were dried under vacuum at 80°C, and protein-DNA complexes were visualized by autoradiography or analyzed with a PhosphorImager (Molecular Dynamics). For experiments using RNA as a probe or competitor, the gel box and glass plates were treated with a 1:250 dilution of diethylpyrocarbonate to inactivate RNases. In addition, 1 μl (26 U) of RNasin (Promega) was added to each reaction mixture before S100 was added.

Salt stability. To test the salt stability of the DNA-TEP complex, binding assays were carried out at various salt (LiCl) concentrations. LiCl was used in the assay because it decreases guanine quadruplex formation, whereas Na^+ and K^+ can facilitate the formation of that DNA structure (46). In addition, two procedures were used to ensure that each binding reaction occurred under defined conditions. First, glycerol (up to 10%) was added to increase the viscosity of the reaction mixture just before loading, so that the salt conditions of the binding reactions would not be easily changed by mixing with the gel running buffer during loading. Second, EDTA was added to a final concentration of 10 mM just before the samples were loaded onto a running gel. This inhibited further binding because Mg^{2+}, which is required for DNA-TEP interaction (data not shown), was chelated by the excess EDTA. For salt stability studies, the reaction mixtures were incubated on ice for 30 min before loading. Gel electrophoresis and autoradiography were performed as described for the EMRA procedure.

TABLE 1. Oligonucleotide probes and competitors

Oligonucleotide[a]	Sequence
ST (S) 4	5′ AAAACTCGACTAGTGCATCGACTTGGGGTTGGGG
ST (D) 4	5′ AAAACTCGACTAGTGCATCGACTTGGGGTTGGGG
	3′ TTTTGAGCTGATCACGTAGCTG
SI (S) 4	5′ AAAACTCGACTTGGGGTTGGGGTAGTGCATCGAC
SI (D) 4	5′ AAAACTCGACTTGGGGTTGGGGTAGTGCATCGAC
	3′ TTTTGAGCTGAACCCCAACCCC
NS (S)	5′ AAAACTCGACTAGTGCATCGACCTCAAGAACTCA
NS (D)	5′ AAAACTCGACTAGTGCATCGACCTCAAGAACTCA
	3′ TTTTGAGCTGATCACGTAGCTG
ST (D) GC	5′ AAAACTCGACTAGTGCATCGACTTGCGCTTGCGC
	3′ TTTTGAGCTGATCACGTAGCTG
ST (D) 1	5′ AAAACTCGACTAGTGCATCGACGGGTTGGGGTTG
	3′ TTTTGAGCTGATCACGTAGCTG
ST (D) 2	5′ AAAACTCGACTAGTGCATCGACGGTTGGGGTTGG
	3′ TTTTGAGCTGATCACGTAGCTG
ST (D) 3	5′ AAAACTCGACTAGTGCATCGACGTTGGGGTTGGG
	3′ TTTTGAGCTGATCACGTAGCTG
ST (D) 0	5′ AAAACTCGACTAGTGCATCGACGGGGTTGGGGTT
	3′ TTTTGAGCTGATCACGTAGCTG
ST (D) H	5′ AAAACTCGACTAGTGCATCGACTTAGGGTTAGGG
	3′ TTTTGAGCTGATCACGTAGCTG
TeLOOP	A^ATTGGGGTTGGGGTTGGGGTTGGGG 3′
	A_6AACCCCAACCCC 5′
ST (D) R	5′ GAAACUCGACUAGUGCAUCGACUUGGGGUUGGGG
$(U_2G_4)_4$	5′ UUGGGGUUGGGGUUGGGGUUGGGG

[a] ST, specific tail; SI, specific internal; (D), double stranded; (S), single stranded; NS, nonspecific sequence; H, human telomere sequence (TTAGGG); GC, mutated version (TTGCGC) of the telomere sequence; R, RNA version (UUGGGG) of the telomere sequence; 0 to 4, number of G's at the end of the telomeric sequences.

Estimation of TEP abundance. In a series of binding assays, various amounts of crude cell extract (S100) were incubated with a constant amount of ST(D)4 probe (Table 1). The mixtures were subsequently loaded onto a gel as described above. Radioactivity in specific bands on the gel was quantitated with a PhosphorImager (Molecular Dynamics). The number of DNA molecules bound by TEP was calculated from the quantitated bound/total ratio and the total number of probe molecules used in each reaction. Assuming that each TEP molecule binds one DNA molecule, the number of bound TEP molecules should be the same as that of the bound DNA molecules. The abundance of TEP in *Tetrahymena* cells was then estimated from the number of TEP molecules and the number of cells that gave rise to the amount of S100 extract present in each binding assay.

UV cross-linking. For UV cross-linking in solution, 0.1 pmol of radiolabeled oligonucleotides [ST(D)4, ST(S)4, and NS(D); Table 1] was incubated with ~6 μg of S100 cell extract at room temperature for 10 min in the presence or absence of competitors [ST(D)4, NS(D), ST(S)4, and NS(S); Table 1]. Each reaction mixture contained 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and 100 ng of poly[d(I-C)] per μl in a 10-μl volume. After the incubation, the reaction mixtures were irradiated with 254-nm light for 30 min and separated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE). Gels were dried under vacuum at 80°C, and protein-DNA complexes were visualized by autoradiography.

For UV cross-linking in situ, radiolabeled ST(D)4 probe (0.8 pmol) was incubated with S100 cell extract (5 μg) in 20 μl of reaction buffer containing 10 mM Tris-Cl (pH 7.5), 100 mM LiCl, 5 mM $MgCl_2$, 1 mM EDTA, 4% glycerol, and 4 μg of poly[d(I-C)]. After incubation at 4°C for 20 min, the mixture was separated on 5% polyacrylamide gel as described above. Cross-linking of DNA and TEP was carried out by exposing the wet gel to 254-nm light for various times. Shifted DNA-TEP complexes were visualized on an X-ray film that had been exposed to the UV-irradiated gel for 4 h at 4°C. The UV cross-linked complexes were excised from the gel and denatured by boiling for 5 min in 100 μl of sample buffer (1% SDS, 3 mM DTT, 125 mM Tris-Cl [pH 6.8]). As a control, an equivalent gel slice that contained free DNA probe was also excised from the gel and subjected to the same denaturation treatment. The gel slices were then placed side by side between two glass gel plates about 1 cm from the top edge and were polymerized directly into the stacking gel. Protein size markers were loaded in an adjacent well. SDS-PAGE was performed as described above.

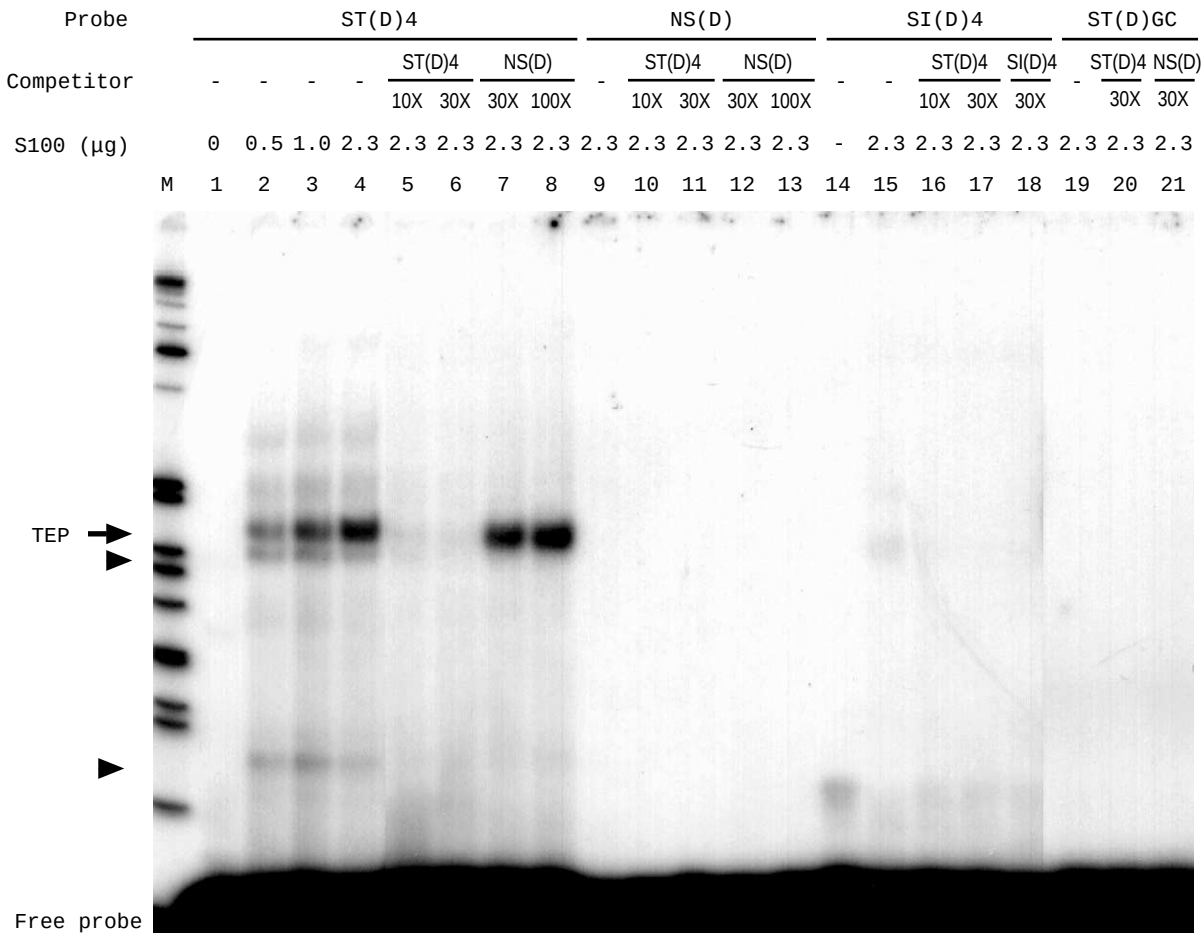

FIG. 1. Identification of TEP. EMRA (see Materials and Methods) was performed with ST(D)4 [duplex oligonucleotide with a specific $(T_2G_4)_2$ overhang and nonspecific internal sequence; lanes 1 to 8], NS(D) (duplex oligonucleotide with nonspecific internal and overhang sequences; lanes 9 to 13), SI(D)4 [specific $(T_2G_4)_2$ internal sequence and nonspecific overhang; lanes 14 to 18], or ST(D)GC [a mutated version of ST(D)4; lanes 19 to 21] as a probe and increasing amounts of crude cytoplasmic extract (S100) from mating cells (lanes 2 to 4). The amount of protein used in the assay is indicated above each lane. In competition experiments, unlabeled ST(D)4 (lanes 5, 6, 10, 11, 16, 17, and 20), or NS(D) (lanes 7, 8, 12, 13, and 21), or SI(D) (lane 18) was used as the competitor. The molar excesses of the competitors are indicated above the lanes. One microgram of unlabeled poly[d(I-C)] as a nonspecific competitor was present in all experiments. The arrow marks the DNA-TEP complex. The arrowheads indicate nonspecific complexes.

RESULTS

Identification of TEP. EMRAs were used to identify complexes between cellular proteins and a synthetic DNA probe that mimics the structure found at natural telomeres. This probe, ST(D)4 (see Table 1 for names and description of all probes used in this study), has a duplex portion of random sequence and a 3′ extension consisting of two telomeric repeats, $(TTGGGG)_2$. EMRA analysis of S100 extract from mating cells by using ST(D)4 as the probe revealed a specific DNA-protein complex that migrated more slowly than the free probe (Fig. 1, lanes 1 to 4). A 10-fold and a 30-fold molar excess of unlabeled ST(D)4 effectively competed with the labeled probe for complex formation (Fig. 1, lanes 5 and 6). In contrast, a 30-fold and a 100-fold molar excess of a nonspecific competitor with a similar structure but an altered sequence in the 3′ overhang [NS(D); Table 1] did not compete with ST(D)4 (Fig. 1, lanes 7 and 8). Quantitation of the results from several experiments showed that a 10-fold molar excess of unlabeled ST(D)4 competed for more than 90% of the binding activity (Fig. 1, lane 5; see also Fig. 3, lane 2), whereas a 100-fold molar excess of unlabeled NS(D) competed for less than 20% (Fig. 1, lane 8; see also Fig. 3, lane 4). When radiolabeled NS(D) was used as the binding substrate under conditions identical to those used for ST(D)4, no shifted band was observed (Fig. 1, lanes 9 to 13). ST(D)4 and NS(D) have exactly the same sequence in their double-stranded portions, differing only in the 3′ overhang, which is $(TTGGGG)_2$ in ST(D)4 but a random sequence in NS(D). Other experiments indicated that binding did not occur if the telomeric sequence was located internally in the duplex region of the probe (see below). Taken together, these results suggest that the binding observed with ST(D)4 is dependent upon the telomeric repeat sequence in the 3′ overhang, as would be expected for a telomere end-binding protein. TEP activity can be attributed to a protein(s), since proteinase K or heat treatment (75°C) of S100 extracts could abolish its binding with ST(D)4 (data not shown). In addition, complex formation between TEP and ST(D)4 was not altered in the presence of RNase A (data not shown), suggesting that RNA is not a component of TEP.

In addition to the specific DNA-TEP complex, two faster-migrating complexes (arrowheads in Fig. 1) were also detected. Neither their appearance nor their susceptibility to competi-

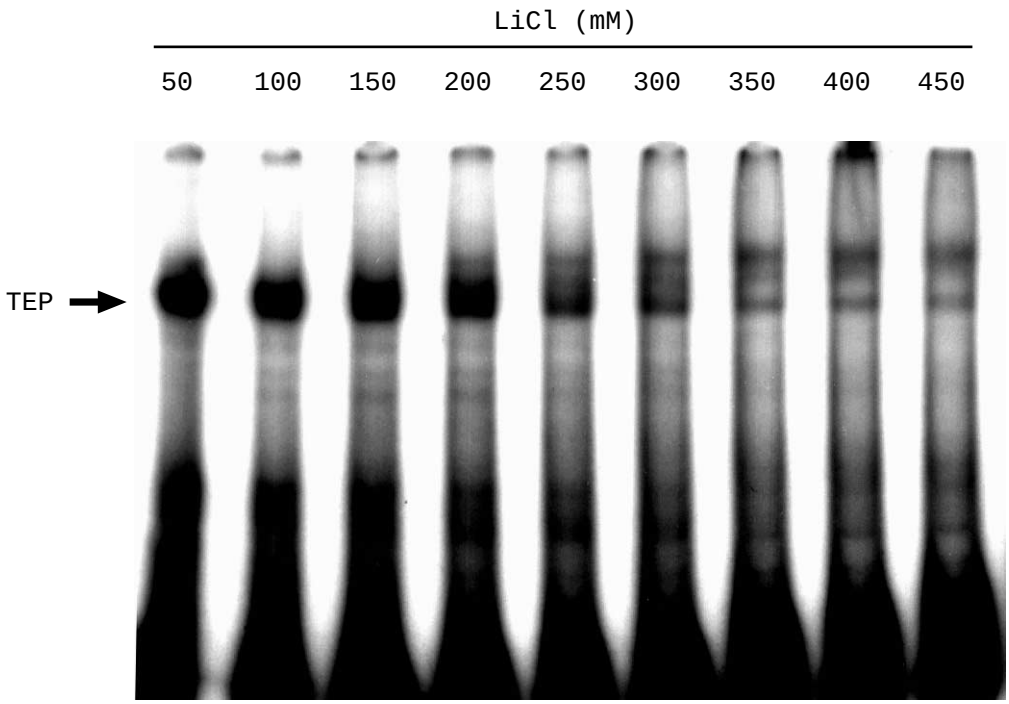

FIG. 2. TEP is salt sensitive. ST(D)4 was used as a probe in the presence of 5 μg of S100 protein from mating cells in each binding assay. The salt (LiCl) concentration is indicated above each lane. More glycerol (up to 10%) was added just before the samples were loaded onto a running gel (see Materials and Methods). One microgram of unlabeled poly[d(I-C)] as a nonspecific competitor was present in all experiments. The arrow marks the DNA-TEP complex.

tion in binding experiments was reproducible, suggesting that they probably resulted from nonspecific DNA-protein interactions.

TEP binding activity is sensitive to high salt concentrations. Previous studies have shown that telomere end-binding proteins found in *O. nova*, *E. crassus*, and *Xenopus* egg extracts are resistant to high salt concentrations (e.g., 2 M NaCl) (4, 33, 35). In contrast, the *Tetrahymena* DNA-TEP complex is salt sensitive (Fig. 2). Optimal binding for TEP occurred at 0 to 50 mM LiCl, and the binding activity decreased at higher salt concentrations. More than 90% of the binding activity was lost when the salt concentration reached 450 mM (Fig. 2). This result explains why high-salt extraction protocols that were successfully used to purify telomere end-binding proteins in other systems were not successful with *T. thermophila* (data not shown).

TEP specifically recognizes the 3′ overhang of DNA substrates. If TEP is a telomere end-binding protein with properties similar to those found in other species, it would be expected to show specificity with regard to both sequence and arrangement of the two repeats in the 3′ overhang. To investigate the sequence specificity, a DNA substrate with an overhang containing the human telomere repeat sequence TTA GGG [ST(D)H; Table 1] was used in competition experiments with radiolabeled ST(D)4 as the probe. Figure 3 shows that the human telomeric sequence competed somewhat less effectively than its *Tetrahymena* counterpart: ST(D)H required at least a 30-fold molar excess to achieve the same level of competition as was observed with a 10-fold molar excess of unlabeled ST(D)4 (Fig. 3; compare lane 2 with lanes 5 to 7). When the 3′ overhang of the radiolabeled probe was changed from $(TTGGGG)_2$ to $(TTGCGC)_2$, no shifted band was detected (Fig. 1, lanes 19 to 21). Thus, TEP can distinguish telomeric from nontelomeric sequences but has only slightly reduced affinity for telomeric sequences from phylogenetically distant species.

To determine whether permutation of the *Tetrahymena* G-strand sequence alters the binding efficiency of TEP, a series of oligonucleotides with the same length of 3′ overhang but different arrangements of G's within the $(TTGGGG)_2$ sequence were tested in competition experiments (Fig. 3). The relative ability of these oligonucleotides to compete for TEP was ST(D)4 ≈ ST(D)3 ≈ ST(D)2 ≈ ST(D)1 > ST(D)0 ⩾ NS(D). Therefore, the permutation of the telomere repeat is not critical as long as G's are present at the 3′ end.

TEP does not bind G-DNA. G-rich telomeric and nontelomeric oligonucleotides are capable of forming unusual structures that are extremely stable (G-DNA, a four-stranded arrangement stabilized by G tetraplexes [39]). Therefore, it was of interest to determine whether the double-stranded DNA substrates used in this study could form such structures under the assay conditions used. Various combinations of oligonucleotides were allowed to interact with one another under the binding assay conditions (except that S100 was not present in the mixture) and examined on 12% nondenaturing polyacrylamide gels previously shown to reveal structural variability in telomeric oligonucleotides (21, 46). Under these conditions, DNA bands were observed only in positions expected for Watson-Crick duplexes; no aberrantly migrating species were detected (data not shown). Furthermore, when Li^+, known to decrease the stability of G-quartet structures, was used as the monovalent cation in binding assays, complex formation was not impaired (Fig. 2 and 4). Finally, when ST(S)4 was induced to form an intermolecular G-quartet DNA complex in the presence of K^+, TEP did not bind it (data not shown). Taken together, these results demonstrate that TEP does not require a G-DNA structure for binding.

TEP requires a duplex structure adjacent to the 3′ overhang. Complexes formed between S100 proteins and G-rich single-stranded substrates [e.g., ST(S)4 and SI(S)4] were nonspecific in nature and therefore probably unrelated to TEP

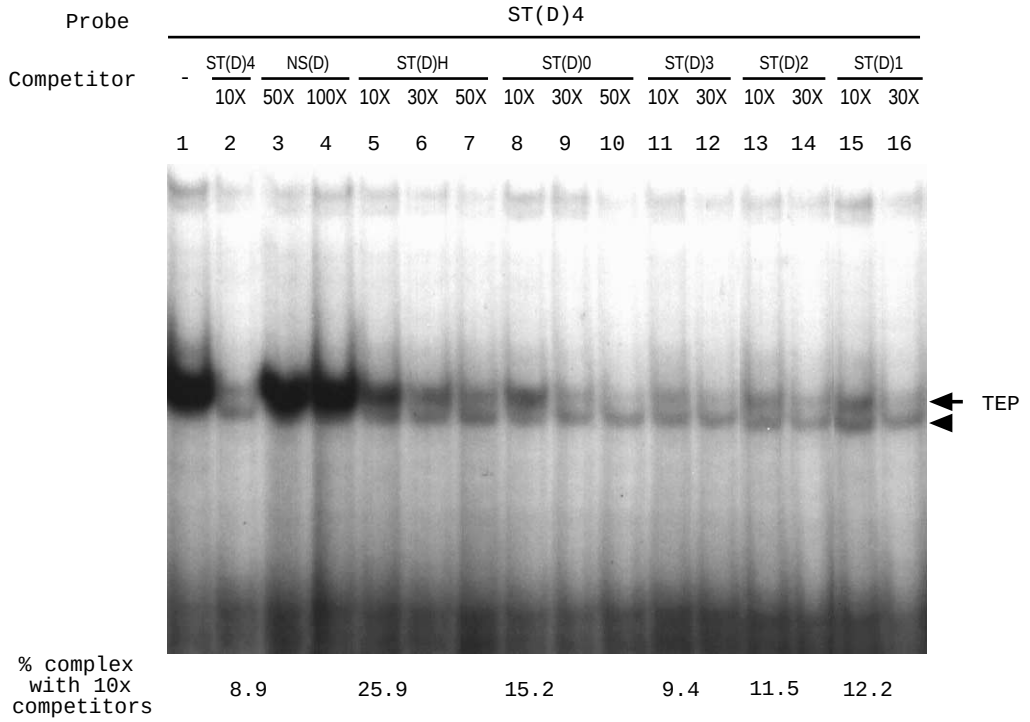

FIG. 3. Effect of alteration and permutation of G-strand overhang sequence on TEP binding. Radiolabeled ST(D)4 (0.06 pmol) was incubated with S100 extract (2.3 μg) from mating cells in the presence of different competitors. Lane 1, no competitor; lane 2, ST(D)4; lanes 3 and 4, NS(D); lanes 5 to 7, ST(D)H; lanes 8 to 10, ST(D)0; lanes 11 and 12, ST(D)3; lanes 13 and 14, ST(D)2; lanes 15 and 16, ST(D)1. All of these oligonucleotides have the same duplex sequence (Table 1). The molar excess of the competitor is indicated above each lane. The arrow marks the DNA-TEP complex. The arrowhead indicates a nonspecific complex. The numbers below the lanes indicate the residual DNA-TEP complex (compared with lane 1) in the presence of a 10-fold molar excess of indicated competitors. One microgram of unlabeled poly[d(I-C)] as a nonspecific competitor was present in all experiments.

(Fig. 4 and data not shown). In these experiments, the complexes that formed did not migrate to the same position as that of TEP (Fig. 4A, lane 16; Fig. 4B, lane 6). The nonspecific nature of the complexes was further demonstrated by binding competition experiments in which complex formation could be altered to the same extent by specific and nonspecific competitor oligonucleotides (Fig. 4A, lanes 17 and 18; Fig. 4B, lanes 7 and 8). In these experiments, the single-stranded probe was not limiting since excess free probe is evident at the bottom of the gel (Fig. 4). A TEP band is seen in competition between NS(D)4 and radiolabeled ST(S)4 but not in the competition between ST(D)4 and ST(S)4 (Fig. 4, lanes 9 and 10, respectively). This is explained as follows. Preparation of the unlabeled duplex competitors required addition of excess complementary strand to ensure 100% duplex formation with regard to the overhang strands [ST(S)4 and NS(S)4]. The excess complementary strand complexed with the radiolabeled ST(S)4 probe, forming radiolabeled ST(D)4. In lane 9, in which there was a 30-fold molar excess of unlabeled ST(D)4, the radiolabeled ST(D)4 that formed was effectively competed for, and no TEP band was visible. However, in lane 10, the unlabeled competitor, NS(D)4, although present in 30-fold molar excess over the labeled probe, was unable to compete for TEP binding with the small amount of radiolabeled ST(D)4 that formed, and therefore a TEP band is visible. Taken together, these data strongly indicate that TEP requires both the duplex and overhang portions of the substrate for binding, as would be expected for a telomere end-binding protein (15, 16, 33, 35, 42).

To investigate the sequence requirements in the duplex domain for TEP binding, an oligonucleotide containing the sequence $(TTGGGG)_2$ in the duplex portion near a random sequence 3′ overhang, SI(D)4, was used as a probe in the binding assay. As shown in Fig. 1, lanes 14 to 18, and Fig. 4, lanes 11 to 14, no complex formation was observed under the conditions used. Furthermore, when an oligonucleotide containing both telomeric duplex and overhang regions was used in competition experiments, it competed only marginally better than ST(D)4 (data not shown). This oligonucleotide (Te-LOOP; Table 1) contained a tetraloop sequence that minimized formation of slipped structures which would lack the desired overhang and confuse interpretation of competition data. Thus, it appears that TEP binding specificity is dictated by the G-strand overhang and an adjacent duplex, but that the duplex sequence requirements are quite relaxed and telomeric repeats are not required.

TEP prefers a free 3′ end. To test whether the presence of a free 3′ end is important for TEP binding, several DNAs in which two single-stranded TTGGGG repeats occupy internal positions were used as competitors. The competitors contained duplexes at either end or both ends. As shown in Fig. 5, none of these three molecules competed as efficiently as ST(D)4, which contains the two single-stranded TTGGGG repeats at the 3′ end. These results indicate a strong preference of TEP for substrates with single-stranded TTGGGG repeats at a free 3′ end, as would be expected for a telomere end-binding protein.

TEP does not efficiently bind to RNA oligonucleotides. To investigate the affinity of TEP for RNA analog of telomeric sequence, an RNA oligonucleotide [ST(S)R] with the same sequence as that of ST(S)4 (except one nucleotide at the 5′ end) was generated by in vitro transcription (see Materials and Methods). The integrity of the RNA was verified electro-

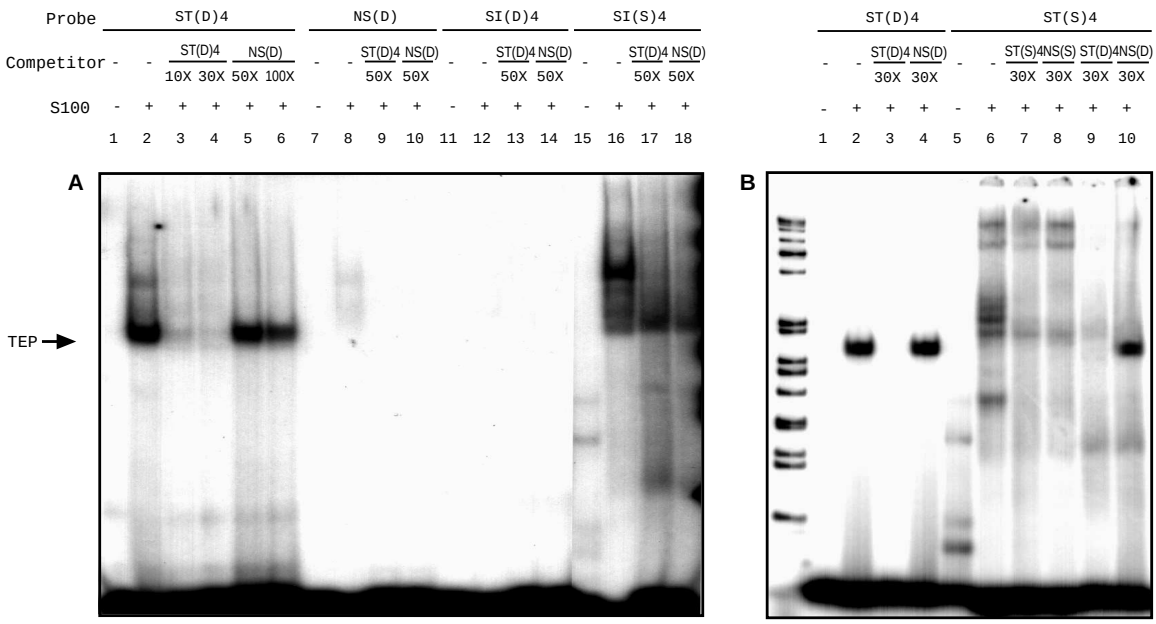

FIG. 4. Both the $(TTGGGG)_2$ overhang and the adjacent duplex are required for TEP binding. (A) Various probes were incubated in the presence (+) or absence (−) of 5 μg of S100 protein from mating cells. LiCl was present at 150 mM in all binding assays. Lanes 1 to 6, ST(D)4; lanes 7 to 10, NS(D); lanes 11 to 14, SI(D)4; lanes 15 to 18, SI(S)4. For the competition experiments, unlabeled ST(D)4 (lanes 3, 4, 9, 13, and 17) or NS(D) (lanes 5, 6, 10, 14, and 18) was used as a competitor. The molar excess of the competitor is indicated above each lane. Binding assays in lanes 1, 2, 7, 8, 11, 12, 15, and 16 did not contain any competitor. The arrow marks the position of the DNA-TEP complex. (B) Radiolabeled ST(D)4 (lanes 1 to 4) or ST(S)4 (lanes 5 to 10) was incubated with or without 6 μg of S100 extract from starved cells and examined by EMRA (as described in Materials and Methods except that KCl or LiCl was omitted and 1 mM EDTA was present). Lane 1, 2, 5, and 6, no competitors; lanes 3 and 9, specific competitors; lanes 4 and 10, nonspecific competitors; lanes 7 and 8, single-stranded specific and nonspecific competitors. The molar excess of each competitor is indicated below the competitor designation. The position of the DNA-TEP complex is aligned with that in panel A. One microgram of unlabeled poly[d(I-C)] as a nonspecific competitor was present in all experiments.

phoretically before it was used in the binding assays (data not shown). When ST(S)R was incubated with S100 extract in the presence of a 50-fold molar excess of nonspecific single-stranded RNA, only one radioactive complex was detected (Fig. 6A). It is unlikely that TEP was responsible for this RNA-protein complex, because the latter moved much faster than TEP-DNA complex on the same gel (Fig. 6A, lanes 1 and 3). Moreover, binding competition assays revealed that the RNA-protein interaction is nonspecific in nature, since ST(D)4 (specific) and NS(D) (nonspecific) competitors had equivalent effects on the complex formation (Fig. 6A, lanes 3 to 5). Furthermore, when a $(UUGGGG)_4$ RNA oligonucleotide $[(U_2G_4)_4$;

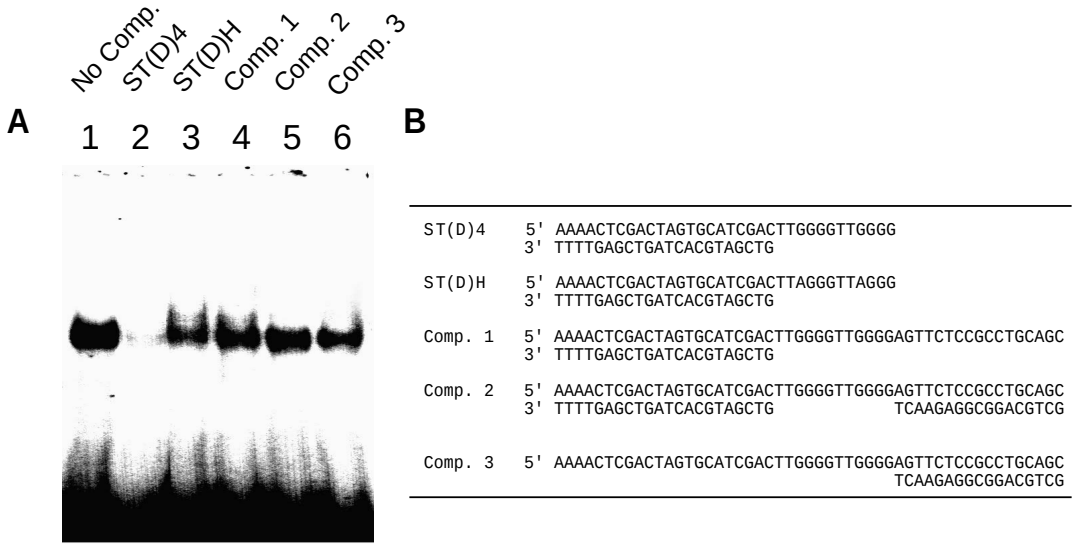

FIG. 5. TEP prefers $(TTGGGG)_2$ repeats at a free 3′ end. (A) Radiolabeled ST(D)4 (0.06 pmol) was incubated with S100 extract (5 μg) from mating cells in the presence of a 30-fold molar excess of different competitors. The competitor used is indicated above each lane. (B) The sequences and arrangements of the competitors used in the experiment shown in panel A are listed. One microgram of unlabeled poly[d(I-C)] as a nonspecific competitor was present in all experiments.

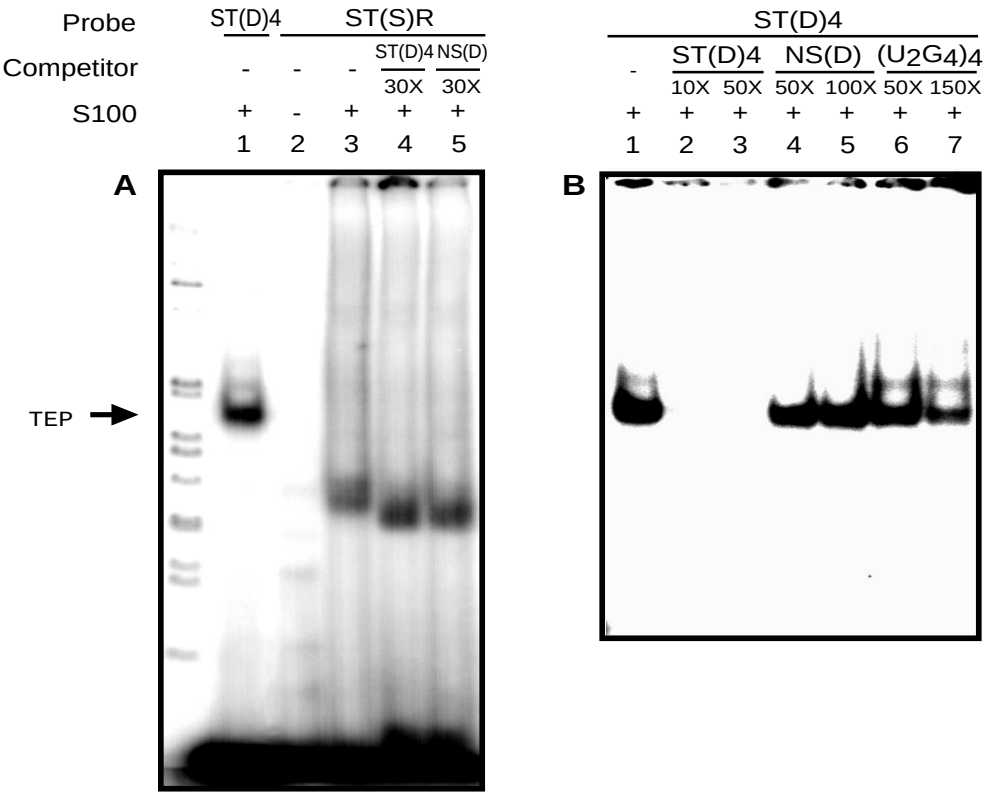

FIG. 6. TEP has higher affinity for DNA than for RNA substrates. (A) Radiolabeled ST(D)4 (lane 1) or ST(S)R (lanes 2 to 5) was used as the probe in EMRA in the presence and absence of 6 μg of S100 extract from starved cells. A 50-fold molar excess of nonspecific RNA competitor transcribed from pBluescript SK II (see Materials and Methods) was present in all reactions. Lanes 1 to 3, no DNA competitors; lanes 4 and 5, specific and nonspecific DNA competitors at a 30-fold molar excess. The DNA-TEP complex is indicated by an arrow. (B) Radiolabeled ST(D)4 was incubated with 5 μg of S100 protein from mating cells in the presence or absence of different competitors. Lane 1, no competitor; lanes 2 and 3, 10- and 50-fold molar excesses of unlabeled ST(D)4; lanes 4 and 5, 50- and 100-fold molar excesses of unlabeled NS(D); lanes 6 and 7, 50- and 150-fold molar excesses of unlabeled $(UUGGGG)_4$. The DNA-TEP complex is aligned with the DNA-TEP complex in panel A. One microgram of unlabeled poly[d(I-C)] as a nonspecific competitor was present in all experiments.

Table 1) was used to compete with radiolabeled ST(D)4 for TEP binding, a 50-fold and a 150-fold molar excess of $(U_2G_4)_4$ caused only 40 and 62% reduction in TEP binding to ST(D)4, whereas a 10-fold molar excess of ST(D)4 abolished 90% of the DNA-TEP complexes under the same conditions (Fig. 6B). Thus, the RNA analog of the G-strand telomeric sequence has a much lower affinity for TEP than the DNA duplex/overhang structure.

TEP activity varies as a function of cell growth and development. To investigate the possibility that TEP activity varies as a function of cell growth and/or development, protein extracts were made from mid-log-phase cells, stationary-phase cells, starved cells, and mating cells. TEP was detected in every case, although the relative activity levels differed (Fig. 7). The activity was highest in starved cell extracts and stationary-phase cell extracts and lower in mid-log-phase cell extracts and mating-cell extracts. This trend was reproducible, although the absolute activity levels varied in different trials. In an attempt to ensure that most of the TEP was released from the cellular DNA during extract preparation, and thus available for extraction, the salt concentration was adjusted to 300 mM before the cells were lysed in a control experiment. No significant change in the amount of TEP in S100 extracts was detected in binding assays (data not shown).

TEP has a copy number of at least 2×10^4 per cell and a molecular mass of approximately 65 kDa. The abundance of TEP in *Tetrahymena* cells was estimated by quantitation of EMRA gels like the one shown in Fig. 1 (see Materials and Methods); on the basis of the calculation, there are at least 2×10^4 TEP molecules per mating cell. This value roughly corresponds to the number of telomeres per cell in *T. thermophila* ($\sim 4 \times 10^4$).

The molecular mass of TEP was estimated by UV cross-linking experiments both in solution and in situ (see Materials and Methods). UV cross-linking in solution gave rise to only one prominent protein that was specifically UV cross-linked to ST(D)4 (Fig. 8, lanes 1 to 4). An ST(D)4-protein complex of similar gel migration rate was also evident by UV cross-linking in situ (data not shown). No specific DNA-protein complex was observed when NS(D) or ST(S)4 was used as the probe (Fig. 8, lanes 5 to 14). This result is perfectly consistent with those obtained from EMRAs (e.g., Fig. 4), strongly suggesting that TEP is responsible for the specific DNA-protein complex in lanes 2 and 4 of Fig. 8. Prestained protein size markers run on the same gel were used to estimate the approximate molecular mass of TEP. Previous work has shown that under the conditions used, protein-DNA complexes usually migrate with the same electrophoretic mobility as the protein alone (44), although this is not always true (23). The apparent molecular mass of TEP is ~65 kDa.

DISCUSSION

Identification and characterization of telomere-binding proteins from a wide variety of eukaryotes will facilitate our understanding of how telomeres function. In this paper, we re-

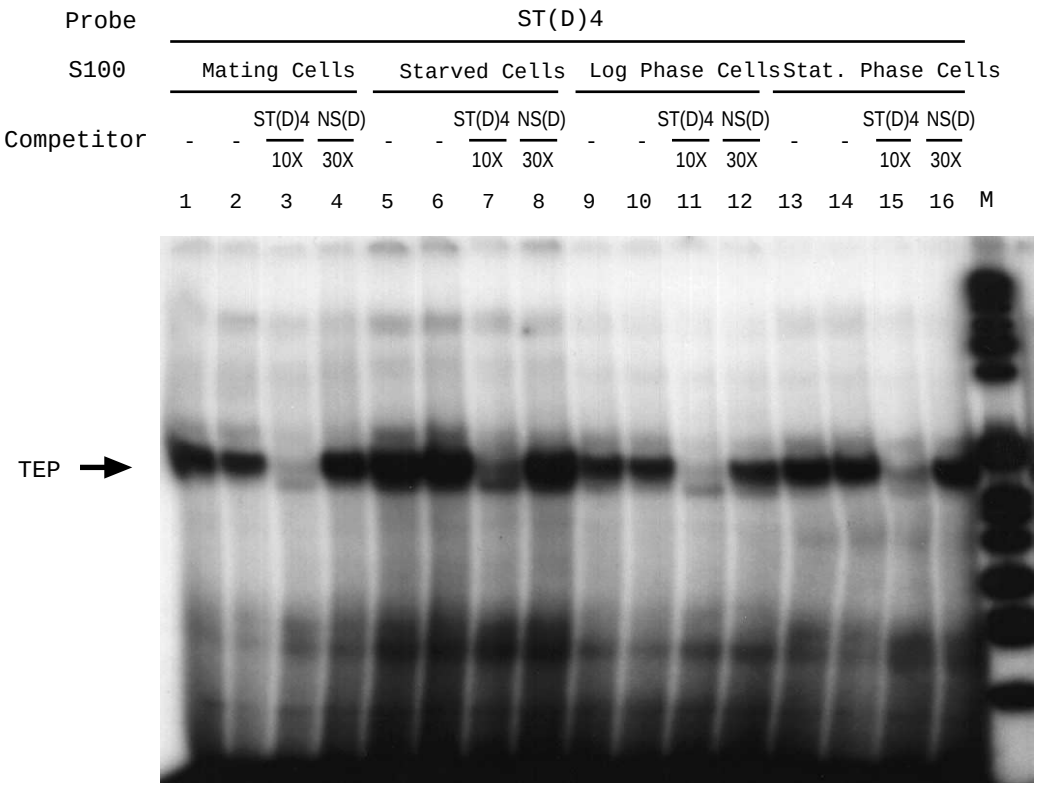

FIG. 7. Levels of TEP vary with different growth stages and conditions. S100 abstracts (8 μg) prepared from different stages of cell growth and development were tested for TEP activity. Lanes 1 to 4, mating cells; lanes 5 to 8, starved cells; lanes 9 to 12, logarithmic-phase cells; lanes 13 to 16, stationary-phase cells. Radiolabeled ST(D)4 (0.06 pmol) was used as probe in all binding assays, with unlabeled ST(D)4 (lanes 3, 7, 11, and 15) or unlabeled NS(D) (lanes 4, 8, 12, and 16) as the competitor. No specific competitor was used in lanes 1, 2, 5, 6, 9, 10, 13, and 14. The molar excesses of competitors are indicated above the lanes. One microgram of unlabeled poly[d(I-C)] as a nonspecific competitor was present in all experiments.

port the identification of a relatively salt-sensitive protein from *T. thermophila* which specifically binds to the $(TTGGGG)_2$) sequence present in the 3′ overhang of synthetic telomeres. This protein, TEP, binds to synthetic telomeres having two repeats of the *Tetrahymena* G-strand telomeric sequence as long as this sequence is adjacent to duplex DNA. However, TEP does not exhibit a strong requirement for a telomere sequence in the duplex region. This is consistent with TEP being an end-binding factor, with other proteins being responsible for binding to the exclusively duplex region. TEP showed roughly equal binding affinity to all possible permutations of the *Tetrahymena* G-strand sequence. This could be due to insensitivity to subtle differences in our assay or reflect the presence of ragged ends at natural telomeres in *T. thermophila*. Finally, it is curious that TEP binding is decreased only about threefold upon changing the *Tetrahymena* sequence to that found in human and other telomeres (TTAGGG), suggesting that the A residue in the altered sequence is not critical for complex formation. Thus, TEP is a good candidate for a telomere end-binding protein analogous to those characterized in other eukaryotes (11, 15, 16, 33, 35, 42) but with somewhat relaxed sequence and structural requirements for its substrate.

Unusual DNA structures stabilized by G tetrads are formed by many telomeric G-strand repeat sequences and other G-rich sequences. This form of DNA, G-DNA, was originally characterized with guanine derivatives (1, 19) and later characterized in telomeric G-strand sequences (21, 38–40, 45, 46). G-DNA's role at telomeres or elsewhere in the chromosome remains unclear. Nonetheless, several proteins have been identified in various organisms, including *T. thermophila*, that specifically bind to G-quartet DNA, suggesting that it does have a physiological role (6, 12, 13, 26, 37, 43). Recently, the β subunit of the telomere end-binding protein from *O. nova* and RAP1 in *S. cerevisiae* have been shown to facilitate G-DNA formation in vitro (13, 14). In contrast to these proteins, TEP does not bind to G-DNA.

Unlike other telomere end-binding proteins, TEP binding is sensitive to salt concentration. The salt-resistant nature of telomere-binding proteins in other ciliates facilitated their identification and purification (5, 15, 33, 35). As more is learned about TEP, it will be of interest to determine what differences between its mode of binding and that of its putative homologs in other species give rise to its salt-sensitive characteristic.

Several vertebrate DNA-binding factors with affinity for single-stranded telomeric TTAGGG repeats were recently identified in nuclear extracts of murine and HeLa cells (24, 29, 30). Subsequently these factors were shown to be components of heterogeneous nuclear ribonucleoproteins. These factors bind more tightly to single-stranded RNA oligonucleotides having r(UUAGGG) repeats than to DNA of the same sequence. We tested the RNA version of the DNA G-strand sequence used in this study and found binding to be greatly reduced. Additional studies using $(UUGGGG)_4$ as a binding substrate corroborated this observation. Therefore, telomeric DNA, rather than RNA containing telomeric sequences, is likely to be the natural binding substrate of TEP.

TEP was detected in cells grown under a variety of physiological conditions including log phase, stationary phase, star-

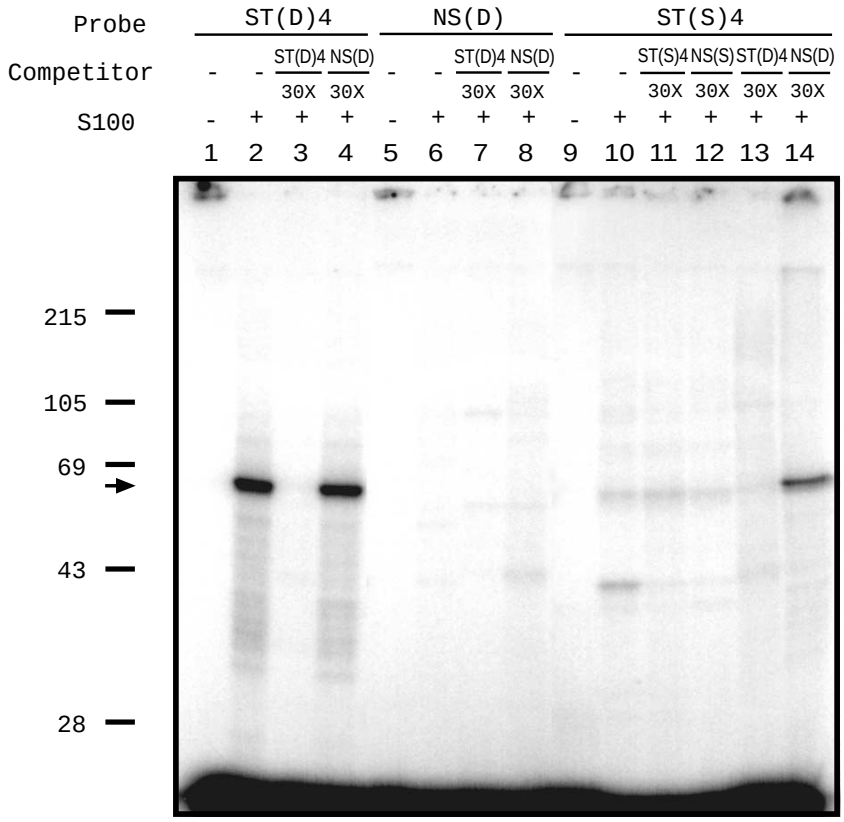

FIG. 8. UV cross-linking indicates a molecular mass for TEP of ~65 kDa. Radiolabeled ST(D)4 (lanes 1 to 4), NS(D) (lanes 5 to 8), or ST(S)4 (lanes 9 to 14) was incubated alone (lanes 1, 5, and 9) or allowed to bind TEP (6 μg of S100 extract from starved cells in lanes 2 to 4, 6 to 8, and 10 to 14) in the presence and absence of a 30-fold molar excess of various types of oligonucleotide competitors as indicated above the lanes. The reaction mixtures were irradiated with UV light and separated by SDS-PAGE. Protein size markers are indicated at the left in kilodaltons. The specific DNA-TEP complex revealed by SDS-PAGE is marked by an arrow. One microgram of unlabeled poly[d(I-C)] as a nonspecific competitor was present in all experiments.

vation, and conjugation. It was somewhat surprising that starved cells and stationary-phase cells had the highest levels of TEP since it might be predicted that TEP would be up-regulated following mating or during log-phase growth, when new telomeres are being generated at a rapid rate. A possible explanation for this observation is that the proportion of TEP in starved cell and stationary-phase cell extracts is higher because of a reduction in the concentration of other cellular proteins. Therefore, at a given total protein concentration in starved or stationary-phase cell extracts, the TEP activity would appear to be elevated relative to that in nonstarved and log-phase cells. Additional studies will be necessary to understand the regulation of TEP expression during cell growth and development in *T. thermophila.*

Purification and further characterization of TEP should provide insight into the mechanism by which telomeres function. Moreover, since telomerase, the enzyme responsible for telomere replication and maintenance, has been best characterized in *T. thermophila*, the interplay of this fascinating enzyme with other telomere-binding factors can be further investigated in this well-studied system.

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