DNA Recognition and Binding by the *Euplotes* Telomere Protein†

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Received June 2, 1992; Revised Manuscript Received August 18, 1992

ABSTRACT: The 51-kDa telomere protein from *Euplotes crassus* binds to the extreme terminus of macronuclear telomeres, generating a very salt-stable telomeric DNA–protein complex. The protein recognizes both the sequence and the structure of the telomeric DNA. To explore how the telomere protein recognizes and binds telomeric DNA, we have examined the DNA-binding specificity of the purified protein using oligonucleotides that mimic natural and mutant versions of *Euplotes* telomeres. The protein binds very specifically to the 3′ terminus of single-stranded oligonucleotides with the sequence $(T_4G_4)_{\geq 3}T_4G_2$; even slight modifications to this sequence reduce binding dramatically. The protein does not bind oligonucleotides corresponding to the complementary C_4A_4 strand of the telomere or to double-stranded C_4A_4·T_4G_4-containing sequences. Digestion of the telomere protein with trypsin generates an N-terminal protease-resistant fragment of ~35 kDa. This 35-kDa peptide appears to comprise the DNA-binding domain of the telomere protein as it retains most of the DNA-binding characteristics of the native 51-kDa protein. For example, the 35-kDa peptide remains bound to telomeric DNA in 2 M KCl. Additionally, the peptide binds well to single-stranded oligonucleotides that have the same sequence as the T_4G_4 strand of native telomeres but binds very poorly to mutant telomeric DNA sequences and double-stranded telomeric DNA. Removal of the C-terminal 15 kDa from the telomere protein does diminish the ability of the protein to bind only to the terminus of a telomeric DNA molecule.

Telomeres consist of the most terminal DNA sequence on a chromosome and the associated telomere-binding proteins [reviewed in Zakian (1989), Blackburn (1991), and Biessmann and Mason (1992)]. The DNA component of telomeres from organisms as diverse as plants, mammals, and unicellular eukaryotes consists of a tandemly repeated 6–8 bp sequence. In most (but not all) organisms, these telomeric repeats contain clusters of G or C residues and exhibit segregation of the G's and C's to opposite strands of the telomere (Price, 1992; Blackburn, 1991; Muller et al., 1991). Although the total length of the telomeric DNA varies dramatically from species to species (from 36 bp to >15 kb), there are well-defined upper and lower limits for each species, indicating that telomere length is regulated (Larson et al., 1987; Lundblad & Szostak, 1989; Harley et al., 1990; Biessmann & Mason, 1992). Telomeres that are particularly short and which exhibit strict length regulation are found in the hypotrichous ciliates *Euplotes*, *Oxytricha*, and *Stylonychia* (Klobutcher et al., 1981). The sequence and organization of *Euplotes* telomeres are shown below. The extension of the 3′ strand is a characteristic of telomeres from a number of organisms and may be a general feature of telomere structure (Klobutcher et al., 1981; Henderson & Blackburn, 1989).

nnnnnnnnnCCCCAAAACCCCAAAACCCCAAAACCCC - 5′
nnnnnnnnnGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG - 3′

To date, two groups of telomere-binding proteins have been isolated. These include proteins that bind along internal stretches of telomeric DNA and proteins that bind only to the extreme end of the telomere. Proteins that bind internal stretches of telomeric DNA have been isolated from *Physarum* (PPT) and yeast (TBFα and RAP1) (Coren et al., 1991; Liu & Tye, 1991; Shore & Nasmyth, 1987; Buchman et al., 1988; Longtine et al., 1989). PPT is a small (10 kDa) heat-stable protein that binds specifically to the $(T_2AG_3)_n$·$(A_2TC_3)_n$ telomeric sequence and is thought to coat the length of the telomere. TBFα may bind the junction between the $G_{1-3}T$ repeats and the subtelomeric X sequence. RAP1 is a multifunctional protein that binds a consensus sequence which occurs within the telomeric $G_{1-3}T$ repeats as well as in the upstream activating elements and silencer elements of some genes. The role played by RAP1 in telomere length regulation appears to be complex as mutation or overexpression of RAP1 deregulates telomere length control (Conrad et al., 1990; Lustig et al., 1990; Sussel & Shore, 1991). There is some evidence that RAP1 may affect telomere length by altering the telomeric chromatin structure.

Proteins that bind specifically to the end of the telomeric DNA have been isolated from the ciliates *Oxytricha nova* and *Euplotes crassus* (Gottschling & Zakian, 1986; Price, 1990). The proteins from both ciliates bind with great specificity to the T_4G_4-containing extension on the 3′ strand of the telomere and protect the telomeric DNA from chemical modification and nuclease digestion (Price & Cech, 1987; Price, 1990). Although the proteins from both ciliates bind telomeric DNA noncovalently, the association is tenacious. The telomere protein–DNA complexes are completely resistant to high concentrations of salt (e.g., 2 M NaCl, 6 M CsCl), and dissociation has only been achieved by denaturing the proteins or by digesting away the DNA (Price & Cech, 1989; Price, 1990).

The *Oxytricha* telomere protein is a 98-kDa heterodimer with subunits of 56 and 41 kDa (referred to as the α and β subunits). Binding of the purified protein to telomeric DNA sequences reconstitutes essentially all the sequence-specific interactions observed in vivo (Raghuraman et al., 1989; Raghuraman & Cech, 1989). Because the two subunits are

† This research was supported by the National Institutes of Health (Grant GM41803), by a Basil O'Connor Starter Scholar Research Award from the March of Dimes Birth Defects Foundation (C.M.P.), and by an American Cancer Junior Faculty Research Award (C.M.P.).

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0006-2960/92/0431-10835$03.00/0

Table I: Sequences of the Oligonucleotides and Synthetic Telomeres Used To Examine the DNA-Binding Specificity of the Telomere Protein[a]

oligonucleotide	DNA sequence	DNA-binding capacity	
		50-kDa protein	35-kDa fragment
GT_{52}·CA_{38}	CGGCTTAAGCGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG GCCGAATTCGCCCCAAAACCCCAAAACCCCAAAACCCC	***[b]	***
GT_{52}·CA_{52}	CGGCTTAAGCGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG GCCGAATTCGCCCCAAAACCCCAAAACCCCAAAACCCCAAAACCCCAAAACC	–[c]	–
GT_{52}·CA_{46}	CGGCTTAAGCGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG GCCGAATTCGCCCCAAAACCCCAAAACCCCAAAACCCCAAAACCCC	TR[d]	TR
GT_{52}·CA_{30}	CGGCTTAAGCGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG GCCGAATTCGCCCCAAAACCCCAAAACCCC	***	***
G→C·CA_{38}	CGGCTTAAGCGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTCCCCTTTTCC GCCGAATTCGCCCCAAAACCCCAAAACCCCAAAACCCC	–	*
GT_{46}·CA_{30}	CGGCTTAAGCGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG GCCGAATTCGCCCCAAAACCCCAAAACCCC	**	***
act1·act2	ATCCCACACGCCATTGAGAAAATCCTGCAGGAGTCTTGCAGGAAGAG TAGGGTGTGCGGTAACTCTTTTAGGACGTCCTC	–	–
CA_{38}	GCCGAATTCGCCCCAAAACCCCAAAACCCCAAAACCCC	–	–
GT_{14}	TTTTGGGGTTTTGG	–	–
GT_{22}	TTTTGGGGTTTTGGGGTTTTGG	TR	*
GT_{30}	TTTTGGGGTTTTGGGGTTTTGGGGTTTTGG	**	***
GT_{42}	GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG	***	ND[e]
GT_{52}	CGGCTTAAGCGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG	***	***
GT_{46}	CGGCTTAAGCGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG	*	***
TGpermut	GGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTT	–	–
T→U	CGGCTTAAGCGGGGTTTTGGGGTTTTGGGGUUUUGGGGUUUUGGGG	*	***
GA_{32}	AAAAGGGGAAAAGGGGAAAAGGGGAAAAGGGG	–	ND

[a] The relative affinities of the native protein and the 35-kDa N-terminal fragment are shown to the right of each oligonucleotide or synthetic telomere. [b] ***, most efficient binding. [c] –, none detected. [d] TR, trace. [e] ND, not determined.

very tightly associated, it only became possible to examine the DNA-binding properties of the individual subunits when the corresponding genes were cloned and expressed separately in *Escherichia coli* (Hicke et al., 1990; Gray et al., 1991). It is now apparent that contact with the DNA is predominantly via the α subunit, although both subunits are required to form the full DNA-binding site.

The *Euplotes* telomere protein has been isolated as a single subunit of 51 kDa (Price, 1990). Cloning and sequencing of the gene encoding the 51-kDa protein revealed that the protein is homologous to the α subunit of the *Oxytricha* telomere protein (Wang et al., 1992). Much of the sequence conservation is located in the N-terminal two-thirds of each protein. In this paper, we examine the DNA-binding properties of the *Euplotes* telomere protein. We show that the protein has the capacity to recognize and bind oligonucleotides that mimic the sequence and structure of a natural telomere. We also show that the N-terminal ~35 kDa of the protein can be isolated as a discrete structural domain. This domain retains the ability to bind telomeric DNA in a sequence-specific and salt-stable manner.

MATERIALS AND METHODS

Culturing of Euplotes crassus and Isolation of Macronuclei. *Euplotes crassus* was grown in septic culture using *Dunaliella salina* as a food source (Roth et al., 1985; Price, 1990). Macronuclei were isolated as described previously (Roth et al., 1985; Price, 1990) except that 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM Tris, pH 7.5, 0.5% Triton X-100, 0.1 mM PMSF, and 0.1 mM TPCK were used to lyse the cells. Where necessary, macronuclei were released from the burst cells by homogenization with a dounce homogenizer. The *Euplotes* were bred to obtain strains that lysed readily and gave clean macronuclei. Cells of opposite mating types were mixed together, and mating was allowed to occur. Individual exconjugates were isolated 48–72 h after mating and grown in culture for 50 or more divisions (Roth et al., 1985). Each clonal strain was then tested for its susceptibility to lysis.

Oligonucleotide Purification and Hybridization. Oligonucleotides were synthesized using an Applied Biosystems PCR Mate synthesizer and purified by electrophoresis through 8% polyacrylamine/8 M urea gels. Following elution from the gel, the oligonucleotide concentration was determined from the absorbance at A_{260}. The extinction coefficient for each oligonucleotide was determined from the base composition. The sequences of the oligonucleotides used in this study are given in Table I; all but act1 and act2 are permutations of the natural telomeric DNA sequence. Act1 is a PCR primer which corresponds to nucleotides 605–628 of the *Euplotes* actin gene plus a *Pst*I restriction site. Act2 is the complement of act1 plus an additional 14 bases at the 3′ end.

Fifty to one hundred picomoles of each oligonucleotide was 5′ end-labeled with T_4 polynucleotide kinase and [γ-^{32}P]ATP. After the extent of labeling was determined, the concentration and specific activity of each oligonucleotide were adjusted to 0.1 pmol/μL and 1×10^5 cpm/pmol by dilution and addition of cold oligonucleotide. To form the synthetic telomeres, equimolar amounts of complementary 5′ end-labeled oligonucleotides were combined, boiled for ≥2 min, and allowed to anneal for 15 min at 5 °C below the T_m for the duplex followed by 15 min at 10 °C below the T_m. The T_m for each duplex was calculated using the equation described by Maniatis (Maniatis et al., 1982). To ensure correct alignment within the duplex, the oligonucleotides were designed with a 10-base nontelomeric DNA sequence at the flush end of the duplex [the 5′ end of the G-rich strand in GT_{52}·CA_{52} (see Table I)]. The annealing temperatures were chosen so that complementary oligonucleotides would only anneal if they were correctly aligned at the end with the nontelomeric sequence.

The proportion of each oligonucleotide incorporated into the duplex form was assessed by running the duplex sample on a nondenaturing gel next to the two component oligonu-

cleotides. If one oligonucleotide was then observed to be in excess over the other, more of the less abundant oligonucleotide was added to the sample, and the sample was boiled and allowed to reanneal. In most cases, over 90% of each oligonucleotide was present in the duplex form. One exception was the duplex G→C·Ca_{38} where the CA_{38} was in ~2-fold excess over the G→C. However, close examination of the sample by nondenaturing gel electrophoresis revealed that essentially all the G→C strand was in the duplex form.

Isolation of Euplotes Telomere Protein for DNA-Binding Studies. Euplotes telomere-binding protein was isolated by the CsCl purification protocol described by Raghuraman and Cech (1990). Macronuclei [(5–10) × 10^7] were suspended in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA) plus 1 g/mL CsCl, any insoluble material was removed by centrifugation at 10 000 rpm for 10 min, and the DNA–telomere protein complexes were then separated from other macronuclear proteins by centrifugation at 57 000 rpm in a 70.1 Ti rotor for 21 h. The gradient was fractionated and the CsCl removed from DNA–telomere protein containing fractions by dialysis against 10 mM Tris, pH 7.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol, and 0.5 M KCl. The protein was then released from the DNA by digestion with micrococcal nuclease (1 unit/9 A_{260} units of DNA–protein complex) for 1 h at 37 °C in the presence of 5 mM $CaCl_2$. EGTA was then added to a final concentration of 15 mM and calf thymus DNA to a concentration of 40 μg/10 A_{260} units of DNA–protein complex. The KCl was removed either by dialysis against 10 mM Tris, pH 7.0, 1 mM EDTA, and 30% glycerol or by dilution with the same buffer followed by concentration in a Centricon 30 (Amicon). The sample was then concentrated to 100–200 μL in a Centricon 30, and any precipitated protein was removed by centrifugation. The calf thymus DNA was heat-denatured and sheared to an average size of about 150 bp by autoclaving.

Protease Digestion of the Telomere Protein. Macronuclear DNA–telomere protein complexes were purified by centrifugation in a CsCl gradient as described above. The complexes were dialyzed against 10 mM Tris, pH 8.0, 1 mM EDTA, and 50 mM KCl to remove the CsCl. Various amounts of trypsin were added to the DNA–protein complexes and incubated at 37 °C for 75 min. Digestion was stopped by addition of tosyllysine chloromethyl ketone (TLCK) to 0.1 mM. A 10-fold variation in the sample volume (0.1–1.0 mL) had no effect on the amount of protease digestion.

Separation of DNA-Bound and Dissociated Telomere–Protein Fragments by Gel Filtration. Samples of trypsin-cleaved DNA–telomere protein complexes were adjusted to 50 mM, 500 mM, or 2 M KCl and applied to a 50-mL BioGel A15M gel filtration column that had been equilibrated with the same concentration of KCl. The DNA-containing fractions were identified by reading the absorbance at 260 nm. The peak fractions were pooled, TLCK was added to inhibit any residual trypsin, the salt concentration was reduced to ~50 mM by dialysis, and the DNA was degraded by digestion with 0.5–1.0 unit of micrococcal nuclease for 3 h at 37 °C. Any residual protein was concentrated by precipitation with trichloroacetic acid and examined by SDS–polyacrylamide gel electrophoresis.

Mobility Shift Assays. 5′ end-labeled oligonucleotides (0.1–1 pmol) were boiled to remove any structure and incubated with 1–8 μL of telomere protein in a final volume of 10 μL, for 1 h at 37 °C. One-half volume of sample buffer was added to each sample (50% glycerol, 0.01% bromophenol blue, and 0.01% xylene cyanol), and the samples were loaded on a 10% acrylamide gel that had been prerun for at least 30 min at 15 mA. The gels contained 55:1 acrylamide/bis-(acrylamide) and 0.5× Tris–borate buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA).

Nitrocellulose Filter-Binding Assay. 5′ end-labeled oligonucleotides (0.05 pmol) were incubated with 0.25–5 μL of telomere protein in a final volume of 10 μL, for 1 h at 37 °C. The binding reaction was applied to nitrocellulose filters (24-mm circles, 0.45-μm pore size) that had been soaked in TE buffer. Each filter was washed 10 times with 1 mL of TE and the amount of bound DNA determined by liquid scintillation counting.

Methylation Interference Assay. The oligonucleotides GT_{52} and GT_{42} (Table I) were 5′ end-labeled and randomly methylated by treatment with 10 mM dimethyl sulfate (DMS) at 25 °C for 10 min (Raghuraman & Cech, 1989). The modified oligonucleotide was incubated with telomere protein as described above; the DNA–protein complexes were then separated from free DNA by electrophoresis through a 10% mobility shift gel. Regions of the gel containing free DNA or the DNA–protein complexes were excised, and the DNA was eluted by incubating the crushed gel in 10 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl, and 0.1% SDS for ≥14 h. The DNA was extracted with phenol and chloroform, ethanol-precipitated, and cleaved at methylated guanines with piperidine. The DNA fragments were then separated on 8 or 12% sequencing gels using a 0–1 M sodium acetate gradient to compress the separation between the faster migrating fragments (Ausubel et al., 1988).

RESULTS

The DNA Sequence and Structure Specificity of Telomere Protein Binding. The telomere-binding protein was isolated from *Euplotes* macronuclei as a DNA–protein complex by centrifugation in a CsCl density gradient (Raghuraman & Cech, 1990). The high concentration of CsCl dissociated essentially all other chromosomal proteins from the DNA, yielding extremely pure preparations of telomere protein (Price, 1990). The protein was released from the macronuclear DNA by extensive digestion with micrococcal nuclease.

A mobility shift assay was used to examine the affinity of the telomere protein for wild-type or mutant versions of *Euplotes* macronuclear telomeres. Synthetic telomeres that differed in the length or sequence of the extension on the 3′ strand were prepared by annealing together pairs of oligonucleotides (shown in Table I). When the telomere protein was incubated with synthetic telomeres that had the natural telomere sequence and structure (GT_{52}·CA_{38}), the protein bound efficiently and gave rise to band shifts in the mobility shift assay (shown in Figure 1). The protein also bound quite efficiently to a telomere with a 22-base 3′ extension corresponding to the natural 14-base extension plus an extra T_4G_4 repeat (GT_{52}·CA_{30}), and to the single-stranded oligonucleotide GT_{52}. However, the protein bound very poorly to the C_4A_4-containing oligonucleotide (CA_{38}). When the length of the 3′ T_4G_4 extension on the synthetic telomere was reduced from 14 to 6 bases (GT_{52}·CA_{46}), the binding efficiency of the protein dropped dramatically. All binding was eliminated when the telomere had a blunt 3′ end (GT_{52}·CA_{52}). The contribution of the T_4G_4 sequence to protein binding was examined using telomeres that retained the 14-base extension on the 3′ strand but either had the G's changed to C's (G→C·CA_{46}) or consisted entirely of nontelomeric DNA sequences (act1·act2). In both cases, protein binding was completely eliminated. Digestion of the telomere protein preparation with proteinase K prior

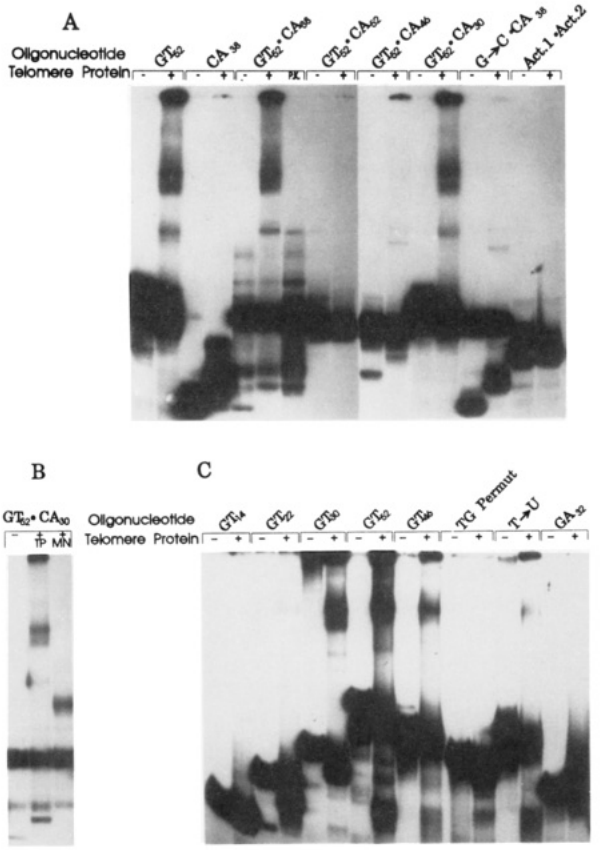

FIGURE 1: Mobility shift gels showing binding of the telomere protein to wild-type or mutant telomeres. Following incubation of the telomere protein with 5′ end-labeled oligonucleotide, the DNA–protein complexes were separated from free DNA by electrophoresis through nondenaturing gels. The sequence of each oligonucleotide is shown in Table I. (A) Synthetic telomeres incubated with (+) and without (–) telomere protein or proteinase K treated telomere protein (PK). (B) Synthetic telomeres incubated with high concentrations of micrococcal nuclease (+MN), telomere protein (+TP), or no protein (–). (C) Single-stranded telomeric oligonucleotides incubated with (+) and without (–) telomere protein.

to addition of the synthetic telomeres eliminated band shifting (Figure 1A).

Although the telomere protein preparations contained micrococcal nuclease, the band shifts shown in Figure 1A are not caused by binding of the nuclease. When present at very high concentrations, micrococcal nuclease does bind the synthetic telomeres, but the resulting DNA–nuclease complex migrates much faster than the DNA–telomere protein complexes (Figure 1B). The much lower concentrations of nuclease present in the telomere protein preparations give rise to negligible amounts of DNA–nuclease complex.

Many of the DNA–telomere protein complexes are retained in the wells of the gels shown in Figure 1. The amount of material in the well relative to the amount of material in faster migrating bands varies between protein preparations (compare panels A–C of Figure 1) and appears to result from aggregation of the telomere protein during concentration in low-salt buffer. As discussed later, this aggregation can be eliminated by removal of the C-terminus of the protein.

The telomere protein has a strong tendency to aggregate and precipitate from solution when removed from the macronuclear DNA. The purification protocol outlined under Materials and Methods has been developed to minimize this problem. When the macronuclear DNA is removed by digestion with micrococcal nuclease in the presence of 0.5 M KCl, the bulk of the protein remains unaggregated and

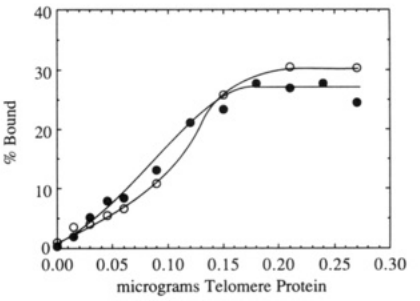

FIGURE 2: Affinity of the telomere protein for wild-type synthetic telomeres (GT_{52}·CA_{38}) as compared to the single-stranded oligonucleotide GT_{52}. Increasing amounts of telomere protein were incubated with 0.05 pmol of oligonucleotide; a nitrocellulose filter-binding assay was then used to determine the relative amount of DNA–telomere protein complexes formed (percent counts bound).

sediments as a monomer in a glycerol gradient (data not shown). Monomer-containing fractions from such a glycerol gradient give rise to proportionately more of the faster migrating DNA–protein complexes relative to the complexes that remained in the wells. However, the high concentration of KCl in these protein preparations causes some G-rich oligonucleotides to form folded "G-quartet" structures (Williamson et al., 1989; Sundquist & Klug, 1989; Henderson et al., 1990). It was important to avoid the formation of such folded structures when trying to compare the binding efficiency of the telomere protein to different telomeric oligonucleotides because the *Oxytricha* telomere protein is known to bind very poorly to oligonucleotides that are folded into G-quartets (Raghuraman & Cech, 1990). Consequently, it was necessary to perform all the binding studies with preparations of telomere protein that contained low concentrations of monovalent cation.

Despite the aggregation, it is clear that the telomere protein binds DNA in a sequence- and structure-specific manner. The main feature of the telomere that appears to be recognized by the protein is the T_4G_4-containing extension on the 3′ end of the DNA. In fact, the mobility shift experiments suggest that the C_4A_4 strand may not be important for binding as the protein appears to bind the single-stranded oligonucleotide corresponding to the 3′ strand of the telomeric DNA (GT_{52}) as efficiently as the native telomeric DNA structure (GT_{52}·CA_{38}). This premise was confirmed using a quantitative nitrocellulose filter-binding assay to determine the relative affinity of the protein for the single-stranded oligonucleotide GT_{52} versus the duplex GT_{52}·CA_{38}. Increasing amounts of telomere protein were incubated with a constant amount (0.05 pmol) of single-stranded GT_{52} or synthetic telomere GT_{52}·CA_{38}; the amount of DNA–protein complex formed in each binding reaction was then assayed by nitrocellulose filter binding. As shown in Figure 2, the protein bound to the two DNA substrates with roughly equal affinity. The total percent of DNA bound to the filters is lower in the experiments with the GT_{52}·Ca_{38} duplexes because the preparations contained a slight excess of 5′ end-labeled CA_{38} over GT_{52} to ensure that most of the GT_{52} existed in the duplex form.

Due to the tendency of the telomere protein to aggregate, it was not possible to use the data from the nitrocellulose filter-binding assay to determine either the stoichiometry of the telomere protein–DNA complex or the dissociation constant. Although a large molar excess of protein to DNA was required to obtain saturation of the DNA, this probably reflected inactivation of the protein during aggregation rather than the actual stoichiometry of the complex. Data obtained during purification of the protein from macronuclei suggest that the stoichiometry is roughly one molecule of protein per macronuclear telomere (data not shown).

Since the telomere protein binds single-stranded oligonucleotides as efficiently as the native telomeric structure, it was possible to use single-stranded oligonucleotides to extend our studies of the length and sequence requirements for telomere protein binding. Mobility shift assays were performed with a range of oligonucleotides that were variations of the $(G_4T_4)_nG_2$ sequence that is the natural 3′ end of the telomere. As shown in Figure 1C (and summarized in Table I), the length of the T_4G_4-containing oligonucleotide is extremely important. The protein did not bind to the oligonucleotide GT_{14} that mimics the 14-base extension on the 3′ strand. However, increasing the oligonucleotide length by addition of T_4G_4 repeats resulted in a concomitant increase in protein binding (see GT_{22}, GT_{30}, and GT_{52}). Thus, the protein requires significantly more than 14 bases of the sequence $(G_4T_4)_nG_2$ for binding, although only 14 bases at the 3′ end of the DNA have to be single-stranded.

The specificity of the protein for a T_4G_4-containing sequence was further examined using the oligonucleotides GT_{46}, TG permut, T→U, and GA_{32}. As illustrated in Figure 1A,B, the protein does require a T_4G_4-containing sequence for binding, although some close variants such as dU_4G_4 can be tolerated. Interestingly, the protein shows a reduced affinity for the T_4G_4-containing oligonucleotide GT_{46} that ends in a G_4 instead of the natural G_2 and did not bind a T_4G_4-containing sequence that ends in T (TG permut) instead of G (compare GT_{52}·CA_{38}, GT_{46}·CA_{30}, GT_{52}, and GT_{46}). While Figure 1A–C illustrates the results from individual mobility shift experiments, the relative affinities summarized in Table I reflect the data gathered from multiple experiments performed with at least four different protein preparations.

An N-Terminal Fragment of the Telomere Protein Retains the Capacity To Bind DNA. Since the DNA sequences recognized by the *Euplotes* and *Oxytricha* telomere proteins are quite similar, it seemed likely that the two proteins would bind DNA using a conserved DNA-binding motif. The extensive amino acid sequence identity between the N-terminal two-thirds of the *Euplotes* protein and the α subunit of the *Oxytricha* protein (Wang et al., 1992) further suggested that this portion of each protein might comprise a DNA-binding domain. We decided to test this hypothesis by using partial proteolysis to physically separate the *Euplotes* telomere protein into structural domains. We were then able to determine whether one of the domains retained the ability to bind DNA.

Euplotes telomere protein, still in the form of a protein–macronuclear DNA complex, was digested with various amounts of trypsin. The DNA was then removed by micrococcal nuclease digestion and the protein loaded on an SDS–polyacrylamide gel. As shown in Figure 3A, trypsin digested the 51-kDa telomere protein to intermediates of 38–40 kDa and finally to a protease-resistant fragment of ~35 kDa. The residual 10–12 kDa of the protein was not released as a discrete fragment. Rather, the trypsin appeared to digest the end(s) of the protein, generating many small peptides in addition to the 35-kDa trypsin-resistant fragment. The small peptides could be seen as a smear below the band of micrococcal nuclease (18 kDa) when the digestion products were separated on a high-percentage (20%) polyacrylamide gel (Figure 3B).

The location of the trypsin-resistant 35-kDa peptide within the native protein was determined by sequencing the N-terminus of the isolated fragment. Sequence was obtained from six amino acids. These amino acids, AAKKDH, are identical to the predicted protein sequence found between amino acids 6 and 12 at the N-terminus of the native protein (Wang et al., 1992). Thus, our findings indicate that the conserved

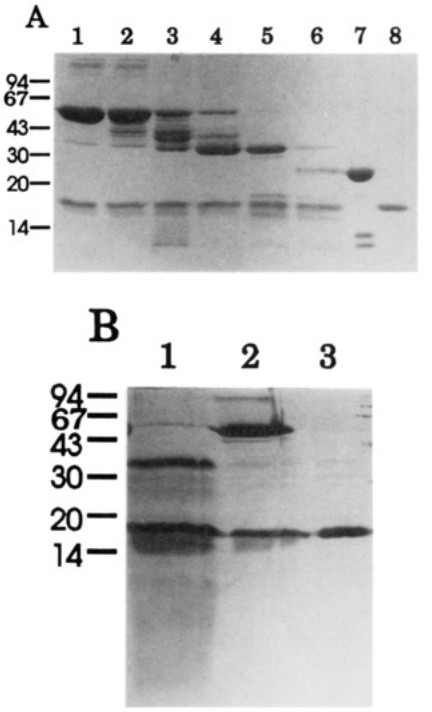

FIGURE 3: Trypsin digestion of the telomere protein. (A) The Coomassie blue-stained 12% SDS–polyacrylamide gel shows the cleaved telomere protein after digestion of DNA–telomere protein complexes with varying amounts of trypsin: lane 1, purified and undigested telomere protein representing 10 A_{260} units of macronuclear DNA; lanes 2–6, cleaved telomere protein obtained after treating 10 A_{260} units of DNA–telomere protein complex with 5×10^{-4} μg of trypsin (lane 2), 5×101^{-3} μg of trypsin (lane 3), 5×10^{-2} μg of trypsin (lane 4), 5×10^{-1} μg of trypsin (lane 5), and 5 μg of trypsin (lane 6); lane 7, 20 μg of trypsin; lane 8, micrococcal nuclease. The positions and molecular weights ($\times 10^{-3}$) of the standard proteins are indicated at the side. (B) The Coomassie blue-stained 20% SDS–polyacrylamide gel shows the prominent 35-kDa fragment and the faint smear of peptide fragments ≤12 kDa that were generated by trypsin digestion of DNA–telomere protein complexes: lane 1, cleaved telomere protein obtained after treating 10 A_{260} units of macronuclear DNA with 0.275 μg of trypsin; lane 2, purified and undigested telomere protein bound to 10 A_{260} units of macronuclear DNA; lane 3, micrococcal nuclease.

N-terminal region of the *Euplotes* telomere protein comprises a discrete protease-resistant domain of ~35 kDa.

Experiments were performed to determine whether the 35-kDa domain retained the capacity to bind DNA. Telomere protein, that was still bound to macronuclear DNA, was digested with trypsin to produce the 35-kDa peptide. Any DNA-bound peptides were then separated from dissociated peptides by gel filtration on a Bio-Gel A15M column. The 35-kDa peptide generated by the trypsin cleavage eluted from the column very rapidly and was found in the same fractions as the macronuclear DNA (Figure 4, lane 3). By comparison, BSA (67 kDa) eluted much more slowly and was not found in any of the DNA-containing fractions. From these results, we conclude that the 35-kDa peptide remained bound to the macronuclear DNA. In contrast, the small peptides from the C-terminus (≤12 kDa) were removed from the DNA-containing fractions by gel filtration.

Native telomere protein is not dissociated from DNA by high concentrations of salt. To determine whether the 35-kDa domain of the protein retains this unusual DNA-binding characteristic, the gel filtration experiment was repeated following addition of 0.5 or 2 M KCl to the DNA–35-kDa peptide complexes. At both salt concentrations, the 35-kDa peptide eluted from the Bio-Gel column in the same fractions

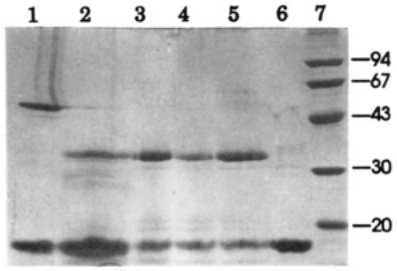

FIGURE 4: Binding of the 35-kDa telomere protein fragment to macronuclear DNA in low and high salt. The Coomassie blue-stained 12% SDS–polyacrylamide gel shows the relative amounts of trypsin-digested telomere protein that remained bound to 20 A_{260} units of macronuclear DNA following gel filtration in the presence of 50 mM, 500 mM, or 2 M KCl. Lane 1, purified and undigested telomere protein bound to 10 A_{260} units of macronuclear DNA. Lane 2, total digested telomere protein obtained after treating 10 A_{260} units of DNA–telomere protein complexes with trypsin. Lane 3, amount of cleaved telomere protein that remained bound to 20 A_{260} units of macronuclear DNA following gel filtration in the presence of 50 mM KCl. Lane 4, amount of cleaved telomere protein that remained bound to 20 A_{260} units of macronuclear DNA following gel filtration in the presence of 500 mM KCl. Lane 5, amount of cleaved telomere protein that remained bound to 20 A_{260} units of macronuclear DNA following gel filtration in the presence of 2 M KCl. Lane 6, micrococcal nuclease. Lane 7, marker proteins. The sizes ($\times 10^{-}$) of marker proteins are shown at the side.

as the macronuclear DNA (Figure 4, lanes 4 and 5). From this, we conclude that the 35-kDa domain encompasses the portion of the telomere protein that is responsible for salt-stable binding to telomeric DNA.

DNA-Binding Specificity of the 35-kDa Domain of the Telomere Protein. A mobility shift assay was used to determine whether the 35-kDa domain of the telomere protein has the same sequence and structure specificity of DNA binding as the native protein. More concentrated and DNA-free preparations of the 35-kDa peptide were prepared by digesting away the macronuclear DNA with micrococcal nuclease and concentrating the protein in a Centricon 30 (Amicon). The degree to which the 35-kDa peptide aggregated and precipitated during this procedure was monitored by centrifuging the protein preparation and examining any pelleted material by SDS–polyacrylamide gel electrophoresis. The 35-kDa peptide showed a dramatic decrease in aggregation relative to whole telomere protein isolated from the same batch of nuclei (data not shown). However, the partially degraded fragments released from the C-terminus precipitated almost quantitatively. Consequently, the resulting preparations contained primarily pure 35-kDa peptide.

The DNA-binding specificity of the 35-kDa peptide was examined by mobility shift assay using the same assortment of wild-type and mutant telomeric DNA sequences that were employed to assay the binding specificity of the native telomere protein. As shown in Figure 5 and summarized in Table I, the DNA sequence and structure requirements for binding are similar for the 35-kDa peptide and the native telomere protein. The peptide binds only to the T_4G_4-containing strand of the telomere and recognizes the single-stranded T_4G_4-containing oligonucleotide GT_{52} as well as the native telomeric sequence and structure (GT_{52}·CA_{38}). Like the native protein, the peptide requires over 14 bases of T_4G_4-containing sequence for binding, and the most 3′-terminal 14 bases must be single-stranded.

However, there are differences between the DNA-binding specificities of the 35-kDa peptide and the native telomere protein suggesting that the specificity of the 35-kDa peptide is somewhat more relaxed than that of the native protein.

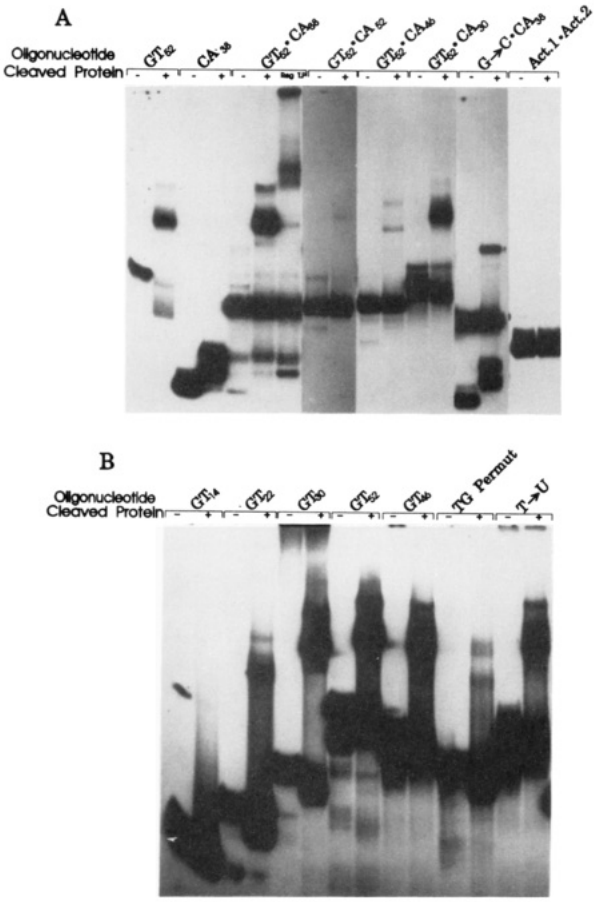

FIGURE 5: Mobility shift gels showing binding of the 35-kDa N-terminal telomere protein fragment to wild-type or mutant telomeres. Following incubation of the telomere protein with 5′ end-labeled oligonucleotide, the DNA–protein complexes were separated from free DNA by electrophoresis through nondenaturing gels. The sequence of each oligonucleotide is shown in Table I. (A) Synthetic telomeres incubated with (+) and without (–) telomere protein. (B) Single-stranded telomeric oligonucleotides incubated with (+) and without (–) cleaved telomere protein or with the native 51-kDa telomere protein (REG TP).

Specifically, the relative affinity of the peptide for G→C·CA_{38} and T→U is greater. The 35-kDa peptide also appears to better tolerate an oligonucleotide that ends in G_4 as opposed to the natural G_2 sequence (see GT_{46}·CA_{30} and GT_{46} in Figures 1 and 5).

One striking difference between the mobility shift gels performed with the 35-kDa peptide versus the whole telomere protein is the position of the shifted bands within the gel. The aggregation observed in the native protein is not observed with the cleaved protein, and essentially all of the DNA–peptide complexes enter the gel. It appears that many of the aggregation problems encountered with the native telomere protein are caused by the highly charged C-terminus.

Recognition of the DNA 3′ Terminus by the Telomere Protein. Whether or not the native telomere protein recognizes and binds specifically to the extreme 3′ terminus of telomeric DNA is of great interest given the current debate over how the end of the telomere is generated. Natural *Euplotes* telomeres end very precisely with a 14-base $T_4G_4T_4G_2$ extension on the 3′ strand. This indicates that there is a very accurate mechanism for generating the terminus of the chromosome, and it has been suggested that the telomere protein is involved in this process. However, it is difficult to suggest models for how the telomere protein might participate in generating the correct terminus without knowing whether

the protein binds preferentially to terminal or internal T_4G_4-containing sequences.

Data from the mobility shift experiments presented in Figure 1 indicate that the native telomere protein has a preference for oligonucleotides that end in a G_2 (e.g., GT_{52}) as opposed to a G_4 (e.g., GT_{46}). This suggests that the protein may bind specifically to the 3′ terminus of the DNA rather than to internal T_4G_4 repeats. In contrast, the 35-kDa peptide shows less of a preference for the natural terminus, suggesting that removal of the C-terminal 15 kDa of the protein may decrease its ability to discriminate between terminal and internal T_4G_4 sequences. To investigate this point further, a methylation interference assay was used to determine whether the native telomere protein and the 35-kDa peptide display a specificity for the 3′ terminus of a long T_4G_4-containing oligonucleotide.

Native or trypsin-cleaved telomere protein was allowed to bind randomly methylated, 5′ end-labeled oligonucleotide GT_{52} or GT_{42}. The resulting DNA–protein complexes were separated from free DNA by electrophoresis through a nondenaturing gel. The complexes, detected as mobility shifted bands, were recovered from the gel. The DNA was purified and cleaved at methylated guanines, and the fragments were separated on a sequencing gel. When compared to control DNA, the DNA fragments bound preferentially by the native telomere protein showed reduced cleavage at three out of the four guanine residues nearest the 3′ end of the DNA molecule (G_2, G_7, and G_8, marked with an asterisk in Figure 6A). The amount of cleavage at G_1 cannot be determined as this band is obscured by the band containing uncleaved oligonucleotide. The frequency of cleavage at the remaining 16 guanines was essentially the same for the protein-bound DNA and the control DNA. These results indicate that methylation of the guanines at the 3′ end of the oligonucleotide inhibits formation of the DNA–telomere protein complex but methylation of the remaining guanines has little effect on complex formation. Thus, the telomere protein appears to bind preferentially to the 3′ terminus of the oligonucleotide rather than to internal T_4G_4 repeats.

The pattern of methylation interference obtained with the telomere protein is quite similar to the methylation protection of G_2, G_7, G_8, and G_{10} that is observed when living cells or isolated nuclei are treated with dimethyl sulfate (Price, 1990). It was not possible to use the methylation protection assay with the telomere protein–oligonucleotide complexes because DMS methylation of the purified telomere protein seems to cause the protein to denature so that it will no longer remain bound to the DNA in a mobility shift gel. In cells, the telomere protein may be protected from denaturation because it is associated either with other proteins or with more internal stretches of macronuclear DNA (Price, 1990; Wang et al., 1992).

When the trypsin-digested telomere protein was used in the methylation interference assay, no obvious interference pattern was observed. The frequency of cleavage at each guanine was very similar for the DNA fragments bound by the 35-kDa peptide and the control DNA (Figure 6B). This result indicates that methylation of the guanines at the 3′ end of the DNA does not have much effect on complex formation and hence the 35-kDa peptide has less specificity for the 3′ terminus than does the native protein. Thus, it appears that the C-terminal domain of the telomere protein contributes to the specificity of the protein for the 3′ terminus of telomeric DNA. In Figure 6B, the reduced methylation of the middle guanine residues in the free DNA sample probably reflects the presence of some folded oligonucleotide during the DMS methylation

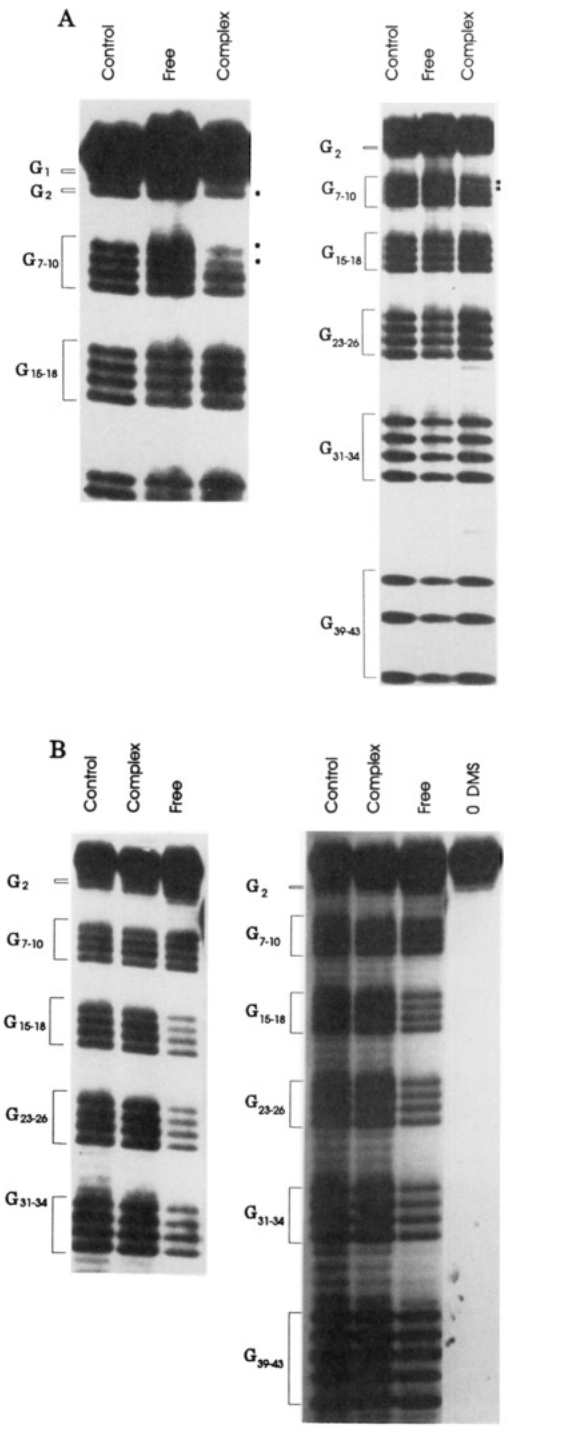

FIGURE 6: Methylation interference assay showing the specificity of the native and trypsin-cleaved telomere protein for the 3′ terminus of GT_{52} or GT_{42}. 5′ end-labeled control DNA, DNA bound by telomere protein, and DNA unbound by telomere protein were isolated and cleaved at methylated guanine residues by treatment with piperidine. The DNA fragments were then separated on 8% or 12% sequencing gels. The positions of the guanines relative to the 3′ end of the oligonucleotide are shown to the side of each panel. (A) Methylation interference pattern obtained with the native telomere protein. Control, control DMS-treated oligonucleotide; Complex, oligonucleotide from DNA–telomere protein complexes; Free, DNA that was not bound by the telomere protein. The gel on the left contained 8% polyacrylamide and the gel on the right 12%. Most of the guanines ran off the 8% gel. (B) Methylation interference pattern obtained with the 35-kDa peptide. Control, control DMS-treated oligonucleotide; Complex, oligonucleotide from DNA–35-kDa peptide complexes; Free, DNA that was not bound by the 35-kDa peptide; 0 DMS, DNA treated with piperidine but not DMS. Both gels contained 12% polyacrylamide; the gel on the left was run for a longer period of time.

step. Folding the telomeric DNA into G-quartets can cause methylation protection of guanines (Williamson et al., 1989; Sundquist & Klug, 1989; Henderson et al., 1990). Reduced methylation of the middle guanine residues in the free DNA sample was not observed in other experiments (data not shown).

DISCUSSION

Over the past few years, a number of proteins have been identified that bind with great sequence specificity to single- but not double-stranded DNA (Lannigan & Notides, 1989; Feavers et al., 1989; Rajavashisth et al., 1989; Collick et al., 1991; Grey et al., 1991). Most of these proteins are still poorly characterized, and very little is known about how they recognize and interact with single-stranded DNA. The telomere proteins from *Euplotes crassus* and *Oxytricha nova* are particularly interesting members of this group of single-strand sequence-specific DNA-binding proteins because they bind only to the extreme terminus of a DNA molecule and they resist dissociation by high concentrations of salt. These properties, together with the lack of sequence homology to other DNA-binding proteins, suggest that their DNA-binding site may be quite unique. In an attempt to learn more about how the *Euplotes* telomere protein recognizes and binds DNA, we have determined which features of telomeric DNA sequence and structure are required for binding. We have also shown that the N-terminus of the protein can be isolated as a discrete structural domain. This domain appears to comprise the DNA-binding site of the protein as it retains most of the DNA-binding characteristics of the native protein. However, our results suggest that the C-terminus of the protein does modify the DNA-binding specificity of the N-terminal domain. When the C-terminus is removed, the ability of the protein to specifically recognize the 3′ end of the telomeric DNA is diminished.

DNA-Binding Properties of the Euplotes Telomere Protein. We have shown that the *Euplotes* telomere protein binds very specifically to T_4G_4-containing oligonucleotides that mimic the 3′ strand of native macronuclear telomeres. The protein recognizes the extreme 3′ terminus of the DNA and requires that the terminal 14 bases be single-stranded. Overall, more than 22 bases of T_4G_4 sequence are required for efficient binding; however, it does not matter whether the more internal T_4G_4 repeats are single- or double-stranded. This requirement for an extended length of telomeric DNA for protein binding is consistent with data from earlier methylation protection experiments which demonstrated in vivo interactions between protein and guanine residues in the double-stranded region of the telomeric DNA. In this respect, the *Euplotes* protein differs from the *Oxytricha* telomere protein. The *Oxytricha* protein does not give rise to methylation protection of guanine residues in the double-stranded region of the telomere, and it binds quite efficiently to a 13-base oligonucleotide that mimics the 3′ terminus of the T_4G_4 strand (Price, 1990; C. Price, unpublished results).

Initially it was somewhat surprising to isolate the *Euplotes* telomere protein as a single 51-kDa subunit when the equivalent *Oxytricha* protein is a heterodimer. We have since determined that proteins equivalent to the β subunit of the *Oxytricha* protein probably do exist in *Euplotes* but they do not seem to be premanently associated with the 51-kDa telomere protein (Wang et al., 1992). It is unlikely that these proteins contribute much to telomere protein DNA recognition and binding because, as we show here, the 51-kDa protein exhibits most of the DNA-binding properties observed with the *Oxytricha* heterodimer. In fact, both the characteristic sequence specificity and salt stability of telomere protein binding are contributed solely by the 35-kDa N-terminal domain of the *Euplotes* protein.

Telomere Protein Aggregation. Aggregation of the native telomere protein upon removal of the telomeric DNA has been a persistent problem that has hampered characterization of the protein. The aggregation can be reduced by increasing the salt concentration, while inclusion of nonionic detergents promotes aggregation. The most effective way to prevent aggregation is to remove the C-terminal 15 kDa of the protein. In hindsight, these observations are explained by the amino acid composition of the C-terminal region. This region is highly charged as it contains a high proportion of basic amino acids (~18% Lys, 3% Arg, and 3% His, depending on the exact site of trypsin cleavage) and has an isoelectric point of ~10.6 (Wang et al., 1992).

While the tendency of the telomere protein to aggregate may be a complete artifact of the purification protocol, it is also possible that this property reflects a biological activity. There are several lines of evidence which suggest that the telomeres of hypotrichous ciliates associate in vivo via protein–protein interactions. The first line of evidence comes from dimethyl sulfate footprinting of *Oxytricha* DNA–telomere protein complexes. Like the *Euplotes* telomere protein, the *Oxytricha* protein has a tendency to form high molecular weight aggregates. The aggregated *Oxytricha* DNA–telomere protein complexes give a DMS footprint that differs from the footprint of monomeric DNA–telomere protein complexes (Raghuraman & Cech, 1989). The footprint of the aggregated complexes resembles the footprint observed with telomeres from living cells (Price & Cech, 1987). Thus, it is suspected that the native telomeres undergo similar protein–protein associations (Raghuraman & Cech, 1989).

Additional evidence for the association of telomers via protein–protein interactions comes from electron microscopy of spread preparations of nuclei from the ciliate *Holosticha* sp. (Prescott, 1983; Klobutcher & Prescott, 1986). When macronuclei from this organism were lysed in distilled water at pH 9.3, treated briefly with protease, and spread on an aqueous surface, the chromatin appeared as rosettes with the DNA molecules radiating out from a central point. The telomeres of the DNA molecules appeared to be held into the center of the rosettes by protein, suggesting that telomere protein–telomere protein associations tethered the ends of the macronuclear DNA molecules.

Generation of the 3′-Terminal T_4G_2 Repeat. The length of hypotrich telomeres is very precisely regulated, and in *Euplotes*, the vast majority of the telomeres have exactly 28 base pairs of C_4A_4·T_4G_4 sequence plus a 14-base 3′ extension (Klobutcher et al., 1981; C. Price and J. Vermeesch, unpublished results). It is of great interest that the telomere ends in the sequence T_4G_2 as this does not correspond to the end of the template region of the telomerase RNA which has the sequence 5′CAAAACCCCAAA3′ (Shippen-Lentz & Blackburn, 1990). To generate this G_2 end of the telomere, either the telomerase has to stop in the middle of a repeat or the telomere has to be trimmed back following telomerase activity. One way that a telomere of the correct length could be generated is if one molecule of telomere protein binds to the single-stranded T_4G_4 repeats immediately adjacent to the double-stranded C_4A_4·T_4G_4 region. If the protein protected exactly 14 bases of T_4G_4 sequence from nuclease digestion, subsequent trimming of the DNA by a nuclease could then generate the 3′ $(T_4G_4)_nT_4G_2$ extension. However, it has been shown quite clearly that not only the *Euplotes* protein but

also the *Oxytricha* telomere-binding protein binds preferentially to the most terminal telomeric repeat (Raghuraman & Cech, 1989). Thus, the above model for generating the correct 3′ end of the telomere could only be correct if the binding specificity of the protein is somehow altered during different stages of the cell cycle.

ACKNOWLEDGMENT

We thank Mark Griep, Paul Blum, and Myron Braake for their helpful suggestions about how to prevent protein aggregation. We thank J. Berman and D. Shippen-Lentz for reading the manuscript and making helpful comments.

REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. E., Smith, J. A., & Struhl, K., Eds. (1988) *Current Protocols in Molecular Biology*, Unit 7.4.13, John Wiley & Sons, New York.

Biessmann, H., & Mason, J. M. (1992) *Adv. Genet.* (in press).

Blackburn, E. (1991) *Nature 350*, 569–573.

Buchman, A. R., Kimmerly, W. J., Rine, J., & Kornberg, R. D. (1988) *Mol. Cell. Biol. 8*, 210–225.

Collick, A., Dunn, M. G., & Jeffreys, A. J. (1991) *Nucleic Acids Res. 19*, 6399–6404.

Conrad, M. N., Wright, J. H., Wolf, A. J., & Zakian, V. A. (1990) *Cell 63*, 739–750.

Coren, J., Epstein, E., & Vogt, V. (1991) *Mol. Cell. Biol. 11*, 2282–2290.

Feavers, I. M., McEwan, I. J., Liang, H., & Jost, J.-P. (1989) *J. Biol. Chem. 264*, 9114–9117.

Gottschling, D. E., & Zakian, V. A. (1986) *Cell 47*, 195–205.

Gray, J. T., Celander, D. W., Price, C. M., & Cech, T. R. (1991) *Cell 67*, 807–814.

Harley, C. B., Futcher, A. B., & Greider, C. W. (1990) *Nature 345*, 458–460.

Henderson, E. R., & Blackburn, E. H. (1989) *Mol. Cell. Biol. 9*, 345–348.

Henderson, E., Moore, M., & Malcolm, B. (1990) *Biochemistry 29*, 732–737.

Hicke, B., Celander, D., Macdonald, G., Price, C., & Cech, T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1481–1485.

Klobutcher, L. A., & Prescott, D. M. (1986) in *The Molecular Biology of Ciliated Protozoa* (Gall, J. G., Ed.) p 144, Academic Press, San Diego, CA.

Klobutcher, L. A., Swanton, M. T., Donini, P., & Prescott, D. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3015–3019.

Lannigan, D. A., & Notides, A. C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 863–867.

Larson, D. D., Spangler, E. A., & Blackburn, E. H. (1987) *Cell 50*, 477–483.

Liu, Z., & Tye, B. (1991) *Genes Dev. 5*, 49–59.

Longtine, M. S., Wilson, N. M., Petracek, M. E., & Berman, J. (1989) *Curr. Genet. 16*, 225–239.

Lundblad, V., & Szostak, J. W. (1989) *Cell 57*, 633–643.

Lustig, A. J., Kurtz, S., & Shore, D. (1990) *Science 250*, 549–553.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular cloning: a laboratory manual*, pp 388–389, Cold Spring harbor Laboratory Press, Cold Spring Harbor, NY.

Müller, F., Wichy, C., Spicher, A., & Tobler, H. (1991) *Cell 67*, 815–822.

Prescott, D. M. (1983) *Mod. Cell Biol. 2*, 329–352.

Price, C. M. (1990) *Mol. Cell. Biol. 10*, 3421–3431.

Price, C. M. (1992) *Curr. Opin. Cell Biol.* (in press).

Price, C. M., & Cech, T. R. (1987) *Genes Dev. 1*, 783–793.

Price, C. M., & Cech, T. R. (1989) *Biochemistry 28*, 769–774.

Raghuraman, M. K., & Cech, T. R. (1989) *Cell 59*, 719–728.

Raghuraman, M. K., & Cech, T. R. (1990) *Nucleic Acids Res. 18*, 4543–4552.

Raghuraman, M. K., Dunn, C. J., Hicke, B. J., & Cech, T. R. (1989) *Nucleic Acids Res. 17*, 4235–4253.

Rajavashisth, T. B., Taylor, A. K., Andalibi, A., Svenson, K. L., & Lusis, A. J. (1989) *Science 245*, 640–643.

Roth, M., Meiying, L., & Prescott, D. M. (1985) *J. Cell Biol. 101*, 79–84.

Shippen-Lentz, D., & Blackburn, E. (1990) *Science 247*, 546–552.

Shore, D., & Nasmyth, K. (1987) *Cell 51*, 721–732.

Sundquist, W. I., & Klug, A. (1989) *Nature 342*, 825–829.

Sussel, L., & Shore, D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7749–7753.

Wang, W., Skopp, R., Scofield, M., & Price, C. (1992) *Nucleic Acids Res.* (submitted for publication).

Williamson, J. R., Raghuraman, M. K., & Cech, T. R. (1989) *Cell 59*, 871–880.

Zakian, V. A. (1989) *Annu. Rev. Genet. 23*, 579–604.