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Telomere Structure in *Euplotes crassus*: Characterization of DNA-Protein Interactions and Isolation of a Telomere-Binding Protein

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The nucleoprotein structure of telomeres from *Euplotes crassus* was studied by using nuclease and chemical footprinting. The macronuclear telomeres were found to exist as DNA-protein complexes that are resistant to micrococcal nuclease digestion. Each complex encompassed 85 to 130 base pairs of macronuclear DNA and appeared to consist of two structural domains that are characterized by dissimilar DNA-protein interactions. Dimethyl sulfate footprinting demonstrated that very sequence-specific and salt-stable interactions occur in the most terminal region of each complex. DNase I footprinting indicated that DNA in the region 30 to 120 base-pairs from the 5′ end lies on a protein surface; the interactions in this region of the complex are unlikely to be sequence specific. A 50-kilodalton telomere-binding protein was isolated. Binding of this protein protected telomeric DNA from BAL 31 digestion and gave rise to many of the sequence-specific DNA-protein interactions that were observed in vivo. The telomeric complexes from *E. crassus* were very similar in overall structure to the complexes found at *Oxytricha* telomeres. However, telomeric complexes from the two ciliates showed significant differences in internal organization. The telomeric DNA, the telomere-binding proteins, and the resultant DNA-protein interactions were all somewhat different. The telomere-binding proteins from the two ciliates were found to be less closely conserved than might have been expected. It appears that the proteins are tailored to match their cognate telomeric DNA.

Telomeres are the natural ends of chromosomes, and as such they consist of the most terminal DNA sequence and the associated chromosomal proteins. The DNA component of telomeres has been studied extensively. Nuclear telomeres from humans, plants, and unicellular eucaryotes all contain short tandemly repeated DNA sequences with a C-rich strand at the 5′ end and a G-rich strand at the 3′ end (1, 5, 25, 32). The telomeric repeats from different organisms are similar but frequently not identical in sequence (5), and in some (if not all) organisms the 3′ strand is longer than the 5′ strand (15).

Although telomeric DNA-binding proteins have been detected in a number of organisms (including yeast, *Physarum*, *Tetrahymena*, *Oxytricha*, and now *Euplotes* species), only a couple of these proteins have been characterized to any extent (3, 4, 6, 8, 12, 28). The best-characterized telomere-binding protein is from the ciliate *Oxytricha nova*. The *Oxytricha* protein is a 98-kilodalton (kDa) heterodimer with subunits of 55 and 43 kDa (29). The purified protein binds in vitro with great specificity to the $(T_4G_4)_2$ extension on the 3′ strand (12, 28, 30), giving rise to many of the DNA-protein interactions that are detected in vivo by chemical footprinting (28).

Oxytricha telomeres are discrete DNA-protein complexes that encompass 100 to 150 base pairs (bp) of DNA (11). The complexes seem to have two structural domains that are characterized by their dissimilar DNA-protein interactions (28). In the most terminal region, binding of the 98-kDa telomere protein dimer results in very sequence-specific and salt-stable interactions (12, 28). In the more internal portion of the complex (45 to 135 bp from the 5′ end), the DNA lies on the surface of protein molecules. The DNA-protein interactions in this region are neither sequence specific nor salt stable; the protein components have not been identified (28).

Although the structure of the *Oxytricha* telomeric complex is now fairly well defined, it is not clear what role the various structural features play in ensuring that the 5′ ends of linear macronuclear DNA molecules are replicated fully. Presumably, binding of the 98-kDa telomere protein is what confers stability to the macronuclear DNA molecules, as degradation and end-to-end joining of the telomeric DNA will be prevented (12, 28). Since telomeric DNA sequences are very conserved, it seems likely that the functionally important features of telomere structure will also be conserved. Thus, structural comparison of *Oxytricha* telomeres with telomeres from other organisms should reveal much about the relative importance of the various features of a telomere. For this reason, I have initiated studies of telomere structure in a second organism, *Euplotes crassus*.

Ciliates such as *Oxytricha* and *Euplotes* species are particularly suited for studies of telomere structure and function because they have an unusually large number of telomeres. In the macronucleus, the DNA occurs as small gene-size molecules (16, 27). The number and size of these molecules vary between hypotrich species, but usually more than 2×10^7 molecules are present per macronucleus. In *O. nova* the number average size is 2,200 bp (39), so over 10% of the total DNA is part of a telomeric complex.

Oxytricha and *Euplotes* telomeric DNAs show both similarities and differences in sequence and organization (17; see Fig. 3E). In both organisms, the telomeric DNA consist of a defined number of copies of the sequence $C_4A_4{\cdot}T_4G_4$. In *E. crassus* there are 28 bp of this double-stranded repeated sequence, whereas in *O. nova* there are only 20 bp. *E. crassus* has a 14-base extension on the 3′ strand, whereas *O. nova* has a 16-base extension. *E. crassus* has a further 5 bp of conserved sequence 17 bp internal to the $C_4A_4{\cdot}T_4G_4$ repeats (17); this 5-bp region may be part of the sequence that directs chromosome fragmentation during macronuclear development (2). An equivalent conserved region has not been found in *O. nova*.

Although *Oxytricha* and *Euplotes* species are both classified as hypotrichous ciliates and show similarities in mor-

phology and macronuclear organization (9, 16), these organisms are now thought to be fairly distantly related. Comparison of the small-subunit rRNAs from *Euplotes aediculatus* and *O. nova* demonstrated considerable divergence in sequence (36). On the basis of this sequence divergence, it appears that the relationship between *Euplotes* and *Oxytricha* species is similar to that between *Paramecium* and *Tetrahymena* species (35). Differences in codon usage are a further indication of the evolutionary divergence between *Euplotes* and *Oxytricha* species. In most ciliates, TGA is the sole stop codon. TAA and TAG are not used as stop codons; instead, they code for glutamine and glutamic acid (21). *Euplotes* species are the only ciliates known to use TAA as a stop codon (14, 24).

In this report, I present information about telomeric chromatin structure and telomere-binding proteins from *E. crassus*. I demonstrate that *Euplotes* telomeres exist as nucleoprotein complexes which are very similar in overall structure to the telomeric complexes found in *O. nova*. However, the telomeric complexes from the two ciliates differ in the component proteins and in the resultant DNA-protein interactions. The difference in the telomere proteins emphasizes the evolutionary distance between *Oxytricha* and *Euplotes* species.

MATERIALS AND METHODS

Culturing of *E. crassus* and *O. nova*. *E. crassus* was grown on *Dunaliella salina*, using a modification of the procedure described by Roth et al. (33). Algae grown in 20-liter carboys were inoculated with *E. crassus* and grown with aeration and illumination until the algae were depleted. The cultures were then fed at 2-day intervals with 7 to 10 g of *Escherichia coli*. The *Euplotes* cells were isolated 1 to 2 days after the second feeding. *O. nova* was grown in nonsterile culture with live *Chlorogonium* sp. as the food source (38).

Isolation of macronuclei. *Euplotes* cells were washed twice with 5 mM Tris (pH 7.5)–5 mM $MgCl_2$, the cells were suspended in the same buffer, and Triton X-100 was added to 0.5%. Protease inhibitors were added to give a final concentrations of 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK), and 1 mM *p*-chloromercuribenzenesulfonic acid. Where necessary, macronuclei were released from the burst cells by homogenization with a Dounce homogenizer. The efficiency of cell lysis varied greatly from culture to culture. The macronuclei were purified by the procedure described by Swanton et al. (38). *Oxytricha* macronuclei were isolated as previously described (28, 38).

Micrococcal nuclease and DNase I digestion. Macronuclei were suspended in TMS (10 mM Tris [pH 7.5], 10 mM $MgCl_2$, 3 mM $CaCl_2$ 0.25 M sucrose) to a final concentration of 3.3×10^6/ml. After addition of micrococcal nuclease, the nuclei were incubated at 37°C for the desired length of time. After addition of DNase I, the nuclei were incubated at 25°C for 10 min. Digestion was stopped by adding EDTA to 25 mM and sodium dodecyl sulfate (SDS) to 0.5%. The DNA was then isolated as previously described (28). Deproteinized DNA (100 μg/ml) was digested by using the conditions described above. The DNA was deproteinized by treatment with SDS and proteinase K, followed by phenol-chloroform extraction.

Gel electrophoresis and Southern blot analysis. The procedures were performed as described previously (11, 18–20, 28).

Footprinting with DMS. Methylation with dimethyl sulfate (DMS) was performed as previously described (28). Live *Euplotes* cells (suspended in seawater), macronuclei, and deproteinized DNA were treated with 10 mM DMS at 25°C. Methylation was stopped by addition of one-fifth volume of 2.5 M 2-mercaptoethanol–2.5% SDS. DNA from the lysed cells or macronuclei was ethanol precipitated, and the DNA was purified. To examine the salt stability of the DMS footprint, macronuclei were suspended in 1 or 2 M NaCl for 2 h at 4°C. Subsequent treatment with DMS was as described above.

DNA was labeled at the 3′ end, using ^{32}P-labeled 3′-deoxyadenosine and terminal deoxynucleotidyltransferase. Labeling of the 5′ strand is described in Results. DNA was cleaved at methylated G residues by heating in 1 M piperidine for 30 min at 90°C (22, 23). To cleave at methylated G and A residues, the DNA was heated in 20 μl of 10 mM sodium phosphate buffer (pH 7.0)–1 mM EDTA for 15 min at 90°C. Then 2 μl of 1 M NaOH was added, and the sample was heated for 30 min at 90°C (37). The cleavage products were analyzed by electrophoresis through either sequencing gels or high-resolution sequence-suppressing gels.

Isolation of the *Oxytricha* and *Euplotes* telomere proteins. Macronuclei were incubated in 2 M NaCl–10 mM Tris (pH 8.0)–0.1 mM PMSF–0.1 mM TPCK for 2 h at 4°C. The insoluble material was removed by centrifugation, and the supernatant was loaded on a Bio-Gel A15M gel filtration column. The DNA-containing fractions were collected, the salt was removed by dialysis, and the DNA was digested with 0.5 U of micrococcal nuclease per ml. The remaining telomere protein was trichloroacetic acid precipitated and examined by electrophoresis on SDS-polyacrylamide gels.

BAL 31 digestion. Column fractions containing DNA and the 50-kDa protein were dialyzed to remove the salt. Then 5× BAL 31 buffer (3 M NaCl, 62 mM $CaCl_2$, 62 mM $MgCl_2$, 5 mM EDTA, 100 mM Tris [pH 8]) and 1 μg of *Hae*III-digested φX DNA were added to a sample of the dialyzed column fractions or to an equivalent sample of deproteinized DNA. After addition of BAL 31, the samples were digested at 30°C. Samples were removed at 2.5- to 40-min intervals, and EDTA and SDS were added to stop the digestion.

RESULTS

Detection of a DNA-protein complex at *Euplotes* macronuclear telomeres. The chromatin structure of *Euplotes* telomeres was examined by nuclease footprinting. Isolated macronuclei or deproteinized macronuclear DNA were digested with micrococcal nuclease. The DNA was isolated, separated by electrophoresis in agarose gels, and visualized with ethidium bromide (Fig. 1A). The DNA was then capillary blotted to nylon membrane, and telomeric DNA fragments were identified by hybridization with ^{32}P-labeled $(C_4A_4)_2$ (Fig. 1B).

Ethidium bromide staining of the micrococcal nuclease-digested macronuclei revealed a canonical nucleosome repeat pattern (Fig. 1A). The average nucleosome size was 160 to 170 bp. This is somewhat smaller than the 190-bp repeat observed for *Euplotes eurystomus* (7). When telomeric DNA fragments were identified by Southern blot, a repeating pattern with 160 to 170 bp per repeat was again observed (Fig. 1B). However, the telomeric DNA fragments were interspersed between the ethidium bromide-stained fragments and had average sizes of approximately 110, 275, 440, 605, etc. bp. Digestion of deproteinized *Euplotes* DNA with micrococcal nuclease resulted in a smear in both the ethidium bromide-stained gels and the blots.

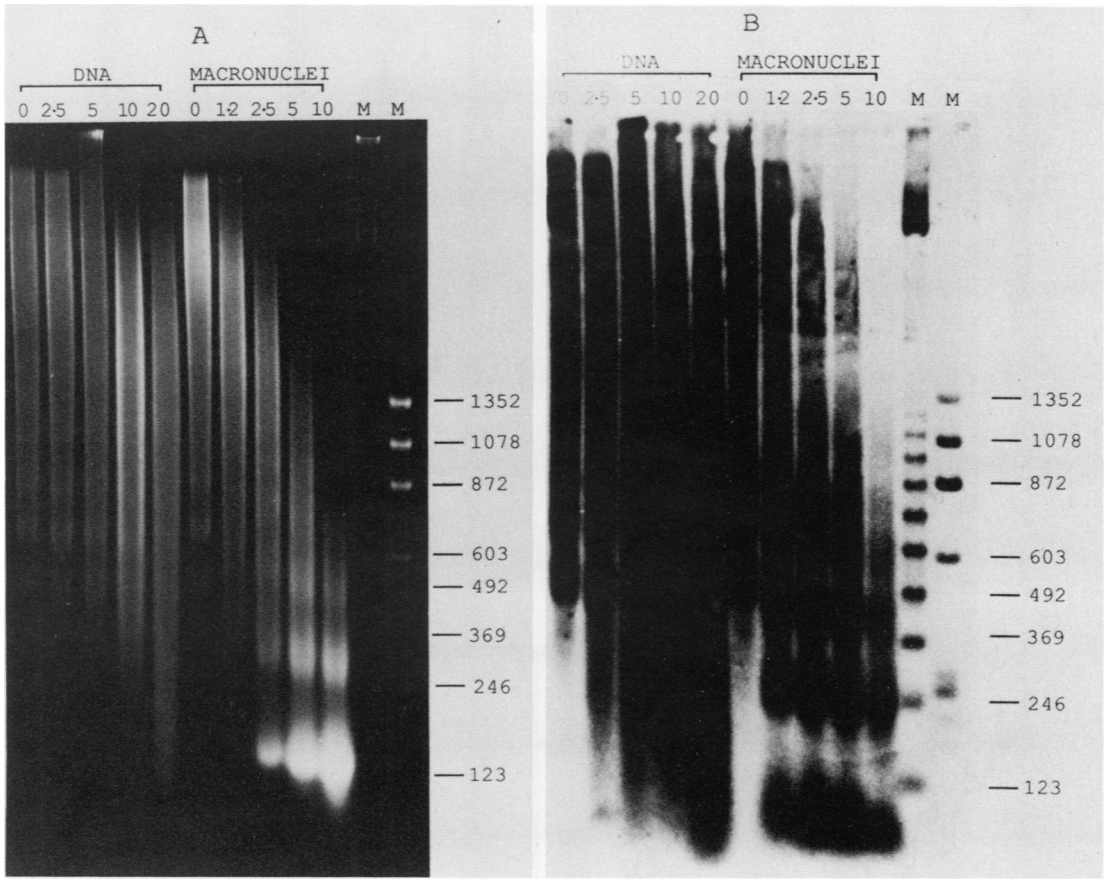

FIG. 1. Protection of *Euplotes* telomeres from micrococcal nuclease digestion. Deproteinized DNA was digested with 0.01 U of micrococcal nuclease per ml; macronuclei were digested with 2 U/ml. The DNA was isolated, separated by electrophoresis in 2% agarose gels, and stained with ethidium bromide. After transfer to nylon membrane, the telomeric DNA was detected by hybridization with ^{32}P-labeled $(C_4A_4)_2$. (A) Ethidium bromide staining of DNA from nuclease-digested DNA and macronuclei. The duration of nuclease digestion (in minutes) is shown above each lane. Lanes M contain size markers. (B) Southern blot of nuclease-digested DNA and macronuclei. The duration of nuclease digestion is shown above each lane. Size (in base pairs) and positions of marker DNA are indicated to the right of each panel.

The nuclease resistance of telomeric DNA within macronuclei suggests that *Euplotes* telomeres are part of a protected complex. This telomeric complex is too small to consist of a complete nucleosome because only 85- to 130 bp of DNA was resistant to the nuclease. The digestion of DNA adjacent to the telomeric complex at intervals of approximately 165 bp suggests that this region of each macronuclear DNA molecule is packaged into phased nucleosomes. Thus, the digestion pattern shown in Fig. 2 reflects cleavage either between nucleosomes or between the telomeric complex and the first nucleosome.

The pattern of digestion shown in Fig. 1 is essentially identical to the pattern observed when equivalent experiments were performed with *O. nova* (11). *Oxytricha* telomeres are known to exist as a nuclease-resistant DNA-protein complex; the adjacent DNA is packaged into phased nucleosomes. Side-by-side comparison of the DNA protected from nuclease digestion in *Euplotes* and *Oxytricha* species showed that the telomeric complexes from the two species are very similar in size (data not shown). In an attempt to determine the exact length of DNA protected by the *Euplotes* complex, the DNA fragments were separated in nondenaturing acrylamide gels and analyzed by Southern blot hybridization. Telomeric fragments of uniform length were not observed; instead, a smear in the size range of 16 to 130 bp was obtained. Apparently, once the DNA adjacent to the telomeric complex is cleaved by micrococcal nuclease, the exonuclease activity of the enzyme gradually degrades the telomeric DNA. This degradation was not apparent in Fig. 1B because DNA of less than 100 bp was not resolved.

Structural analysis of the *Euplotes* telomeric complex by DNase I footprinting. The internal structure of the telomeric complex was examined by nuclease footprinting with DNase I. Isolated macronuclei and deproteinized macronuclear DNA were digested with DNase I, and the DNA was isolated and separated by electrophoresis in denaturing polyacrylamide gels. The DNA was then transferred to nylon membrane, and telomeric DNA fragments from the 5′ strand were detected by hybridization with ^{32}P-labeled $(T_4G_4)_4$. To ensure that a full 28 bp of the probe hybridized to the 28 bp of C_4A_4 at the telomere, hybridization and washing were performed at 49 and 64°C, respectively. Hybridization of short portions of the probe at various places along the C_4A_4 repeats would have obscured the details of the digestion pattern.

Digestion of deproteinized DNA resulted in some sequence- or structure-specific cleavage in the region 30 to 50 bp from the 5′ end; larger digestion products showed no

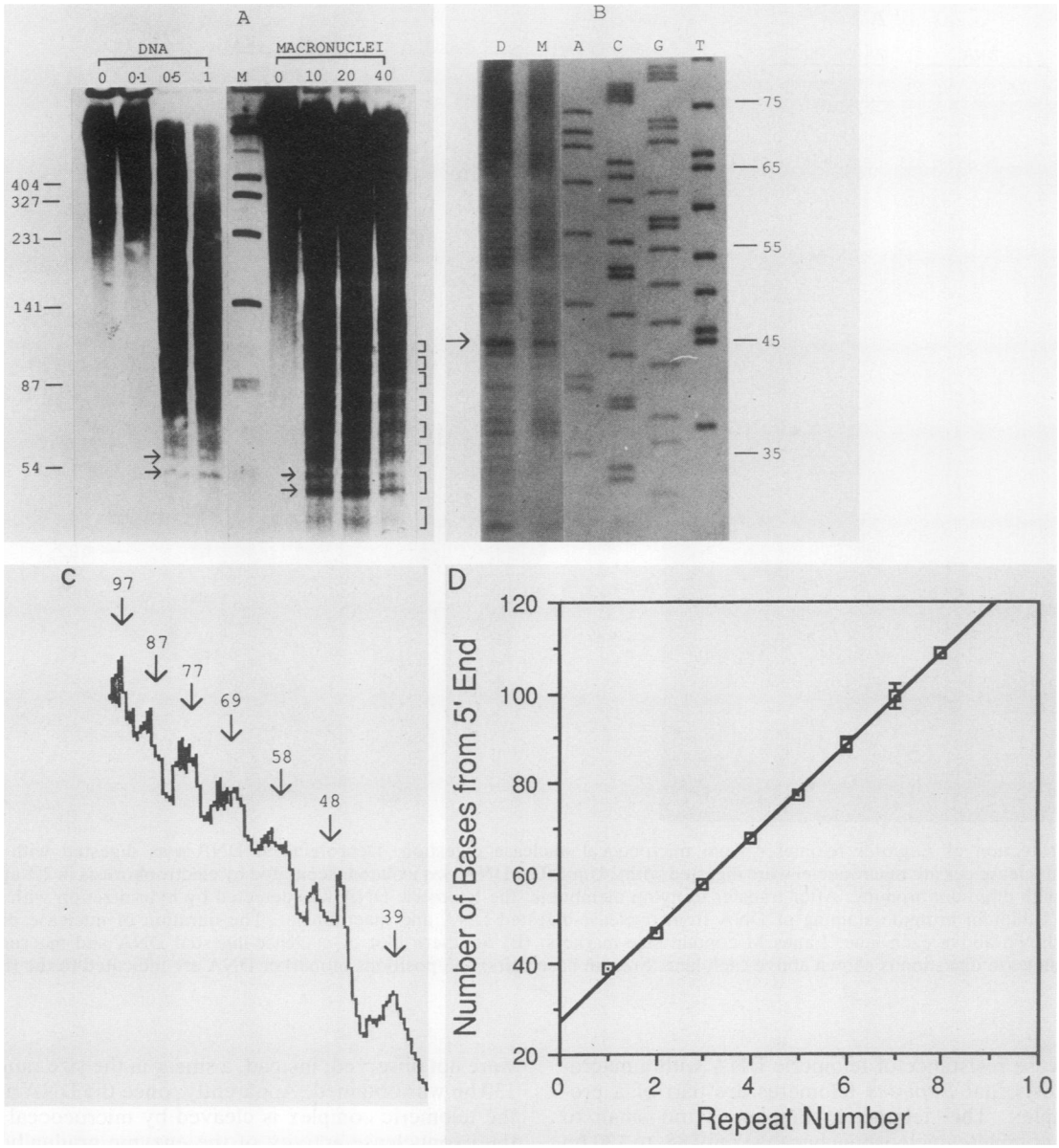

FIG. 2. DNase I digestion of *Euplotes* macronuclei. Macronuclei and deproteinized DNA were digested with DNase I; the DNA was isolated, electrophoresed through denaturing polyacrylamide gels, and transferred to nylon membrane. Telomeric DNA was detected by hybridization with ^{32}P-labeled $(T_4G_4)_4$. (A) Low-resolution 6% polyacrylamide gel. The amount of nuclease used per digestion is shown above each lane (indicated as 10^3 units per milliliter). Lane M contains size markers; the sizes (in base pairs) are shown at the left. Arrows mark the DNase I-hypersensitive sites; brackets mark the cleavage maxima of the repetitive cleavage pattern. (B) 8% sequencing gel. Lanes: D, DNase I-digested DNA; M, DNase I-digested macronuclei; A, C, G, and T, sequence ladders generated with M13 DNA. (C) Densitometric scan of lane 40 of the autoradiogram shown in panel A. (D) Positions of maximum DNase I cleavage were determined from densitometric scans of Southern blots and plotted against the repeat number. The line drawn through the points is the least-squares fit of the data from three separate experiments. The standard deviation of the data is shown by the vertical bar; bars are not visible where the standard deviation is less than the width of the point. The slope has a value of 10.15 bases per repeat.

specific banding pattern (Fig. 2A). The exact positions of the main hypersensitive sites (marked with arrows) were determined by separating the digested DNA on sequencing gels; a sequence ladder of M13 DNA provided markers (Fig. 2B). The most prominent hypersensitive site was 45 bp from the 5′ end; this site corresponds to the first T in the TTGAA conserved sequence that lies 17 bp internal to the C_4A_4 repeats. Additional cleavage was seen 44, 49, and 50 bp from the 5′ end.

Digestion of macronuclei with DNase I resulted in a repetitive cleavage pattern in the region 30 to 120 bp from the 5′ end. The cleavage maxima are marked with brackets in Fig. 2A. Several sites of DNase hypersensitivity are apparent 45 to 50 bp from the 5′ end (marked with arrows). These hypersensitive sites are superimposed on the repetitive cleavage pattern. The hypersensitive sites aligned exactly with the hypersensitive sites seen in the deproteinized DNA digests (Fig. 2B).

To determine the periodicity of the repetitive cleavage pattern, the positions of the cleavage maxima were determined by scanning autoradiograms (such as the ones shown in Fig. 2A and B) with a Hoeffer densitometer (Fig. 2C). The gels, and hence the densitometer scans, do not show good single-base resolution because the macronuclear DNA was of mixed sequence. Consequently, the positions of maximum DNase I cleavage were determined by comparing the densitometer scans of DNase I-digested DNA with scans of marker DNA of known size that was run on the same gel. The positions of maximum DNase I cleavage were then plotted against the repeat number, and the periodicity of cleavage (the slope) was found to be 10.15 bp (Fig. 2D). When DNA lies on a surface (protein or crystalline), DNase I cleavage occurs with a periodicity that reflects the helical repeat of the DNA, i.e., 10 to 10.5 bp (19, 31). Thus, the repetitive digestion pattern shown in Fig. 2 suggests that a portion of the DNA within the *Euplotes* telomeric complex lies on a protein surface.

The pattern of DNase I digestion shown in Fig. 2 is very like the pattern seen when analogous experiments were performed with *Oxytricha* cells (28). In *Oxytricha* cells, DNA in the region 45 to 135 bp from the 5′ end was cleaved at intervals of 10.27 bp. This repetitive digestion pattern was thought to reflect the positioning of the DNA on the outside of a protein surface. Thus, it appears that the overall structures of *Euplotes* and *Oxytricha* telomeric complexes are very similar. The main difference revealed by the DNase I footprinting is that in *Euplotes* cells the repetitive cleavage pattern starts only 2 to 7 bp internal to the C_4A_4 telomeric repeats, whereas in *Oxytricha* cells the distance is 20 to 25 bp.

It is interesting that in *Euplotes* cells, the DNase I hypersensitivity on either side of the conserved TTGAA sequence is retained when the DNA is packaged into the telomeric complex. There must be a small sequence-related change in DNA structure which can be detected by enzymes even when the DNA is packaged into chromatin (10). Maybe the enzymes that act at this site during chromosome fragmentation recognize the slight change in DNA structure (2).

Detection of sequence-specific DNA-protein interactions within the *Euplotes* telomeric complex. The methylating reagent DMS was used to probe for sequence-specific interactions between protein and G or A residues both in the terminal T_4G_4 and C_4A_4 repeats and in the 5-bp conserved TTGAA·AACTT sequence. Living cells, isolated macronuclei, and deproteinized DNA were treated with DMS; the DNA was isolated, 3′ or 5′ end labeled, cleaved at methylated A or G residues, and analyzed by electrophoresis in sequencing gels.

Figure 3A shows the pattern observed when the DNA was 3′ end labeled and cleaved at methylated G residues by using piperidine. The DNA from DMS-treated cells and macronuclei showed strong methylation protection at three G residues in the 3′ $G_2T_4G_4T_4$ tail. Some methylation protection was also apparent at the second G residue in the 3′ tail. However, the amount of protection seen at this residue varied between experiments.

In DMS-treated cells, the overall pattern of methylation at the most 3′ set of G residues in the double-stranded region was different from the pattern observed with DMS-treated control DNA. Although the changes in the methylation level at these G residues were quite small, they were very reproducible. On the basis of densitometer scans of multiple gels, the best interpretation of the data is that G_{17} and G_{18} display methylation protection and G_{15} displays slight methylation enhancement. Examples of the densitometer scans are shown in Fig. 3D. The amount of methylation protection and enhancement seen at G_{15}, G_{17}, and G_{18} is more obvious in cells than in macronuclei, suggesting that some DNA-protein interactions were disrupted during isolation of the nuclei. No methylation protection or enhancement was seen at G residues elsewhere in the double-stranded region of the telomeric DNA.

The control experiment illustrated in lane CON 2 of Fig. 3A showed that the DMS methylation protection (enhancement) of G residues in the 3′ strand was mediated by protein. No methylation protection (enhancement) was apparent when macronuclei were incubated with 0.5% SDS and proteinase K for 30 min before DMS treatment. The methylation protection observed with cells and nuclei could have resulted either from direct interaction of protein with the relevant G residues or from DNA-DNA interactions that are stabilized by protein (e.g., Hoogstein base pairs resulting from folding back of the 3′ tail would cause the N7 of some G residues to be inaccessible to DMS).

Euplotes cells were almost unaffected by a 5-min incubation of DMS. The extremely sturdy pellicle probably retards entry of DMS into the cells. This would explain why cells had to be incubated with DMS for 10 to 2 min in order to see methylation protection, whereas macronuclei required a shorter exposure.

Piperidine treatment of DNA caused a low level of background cleavage at G residues in the absence of DMS (Fig. 3A, lane CON 1). The extent of this cleavage was very much less than the extent of cleavage obtained after DMS treatment. Moreover, the extent of background cleavage was the same for DNA samples from cells, macronuclei, and deproteinized DNA.

The *Euplotes* protein(s) responsible for much of the methylation protection pattern appeared to bind to the 3′ $G_2T_4G_4T_4G_4$ tail in a very salt-stable manner, since it was not dissociated by treatment with 2 M NaCl. Incubation of macronuclei in 0, 1, or 2 M NaCl before DMS treatment resulted in essentially the same pattern of methylation protection (Fig. 3B). The addition of 2 M NaCl slightly altered the DMS reactivity of G residues in the double-stranded $C_4A_4{\cdot}T_4G_4$ repeats of deproteinized DNA. As a result, DMS-treated DNA and macronuclei gave rise to very similar methylation patterns in the double-stranded region. Consequently, it was not possible to tell whether 2 M NaCl affected the interactions between protein and G residues in this region of the *Euplotes* telomeric complex.

DMS footprinting was used to seek sequence-specific interactions between telomere-binding protein(s) and A residues on the 5′ C_4A_4 strand. After treatment of macronuclei with DMS, the macronuclear DNA was isolated and deproteinized. The DNA was then 5′ end labeled and cleaved at G and A residues. Labeling of the 5′ strand was achieved by hybridizing the oligonucleotide $(C_4A_4)_2$ to the 3′ tail of the purified macronuclear DNA and then ligating the oligonucleotide to the 5′ end of the DNA; the resulting protruding 5′ end was labeled with [^{32}P]ATP and T4 polynucleotide kinase. This procedure was used to label the 5′ strand because only a small (possibly unrepresentative) fraction of the natural 5′ ends could be labeled by T4 polynucleotide kinase despite attempts to remove the 3′ tail with T4 DNA polymerase before the labeling step.

The patterns of A methylation were very similar after treatment of cells, macronuclei, or deproteinized DNA with DMS (Fig. 3C). Thus, within the telomeric complex, there

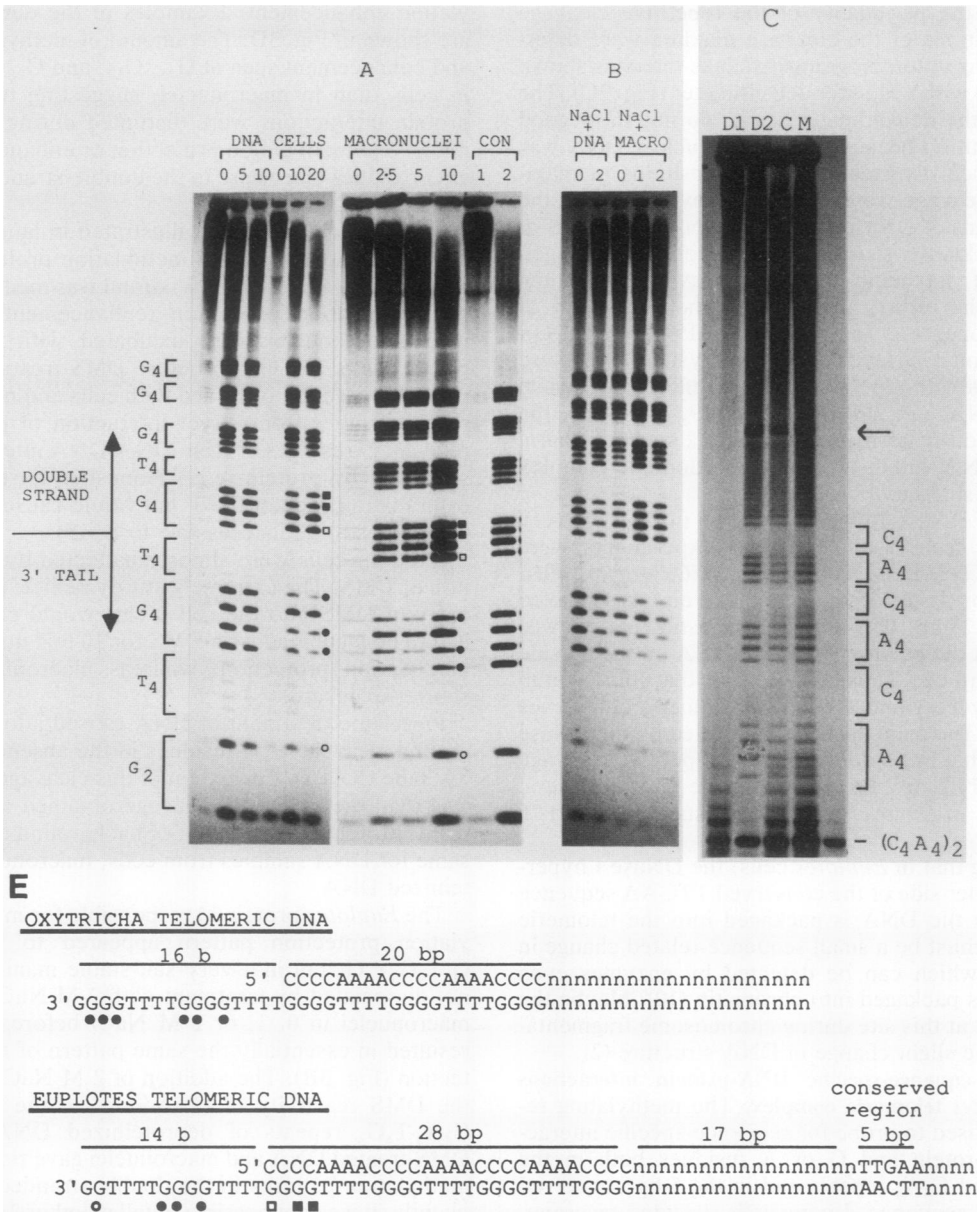

FIG. 3. DNA-protein interactions within the *Euplotes* telomeric complex detected by DMS methylation. (A) Living cells, macronuclei, and deproteinized DNA were treated with DMS. The DNA was isolated, 3′ end labeled, cleaved at G residues, and separated by electrophoresis in sequencing gels. The duration of DMS treatment (in minutes) is shown at the top of each lane. Control lane 1 (CON 1) shows the amount of G cleavage obtained when macronuclei were incubated for 10 min at 25°C in the absence of DMS. Control lane 2 (CON 2) shows the effect of treating macronuclei with proteinase K and 0.5% SDS before DMS treatment. (B) Macronuclei and deproteinized DNA were treated with DMS in the presence of various concentrations of NaCl. The DNA was 3′ end labeled and cleaved at G residues. The molar concentration of NaCl used is shown at the top of each lane. (C) Living cells, macronuclei, and deproteinized DNA were treated with DMS for 10 min. The DNA was isolated, 5′ end labeled, and cleaved at G and A residues. Lanes: D1, deproteinized DNA, no DMS; D2, deproteinized DNA plus DMS; C, cells plus DMS: M, macronuclei plus DMS. (D) Densitometric scans of an autoradiogram showing cleavage at G residues obtained after DMS treatment of macronuclei or deproteinized DNA and 3′ end labeling of the isolated DNA. (E) Comparison of DMS methylation within *Euplotes* and *Oxytricha* telomeric complexes. In panels A and E, G residues that show strong, consistent methylation protection are marked ●, G residues that show variable protection are marked ○, G residues that show slight but consistent methylation protection are marked ■, and G residues that show slight methylation enhancement are marked □.

does not seem to be any interaction between protein and A residues in the minor groove of the telomeric DNA.

In Fig. 3C, there is a position of heavy cleavage (marked with an arrow) that appears to be about 19 bp internal to the telomeric repeats. A corresponding light region is apparent at this position in Fig. 3A and B. The methylation pattern in this region was examined more closely by separating the 5′ end-labeled DNA fragments on sequence-suppressing gels. These gels allow DNA of mixed sequence to be separated with single-nucleotide resolution solely on the basis of size (19). The sequence-suppressing gels confirmed that the position of heavy cleavage is 19 bp internal to the C_4A_4 repeats

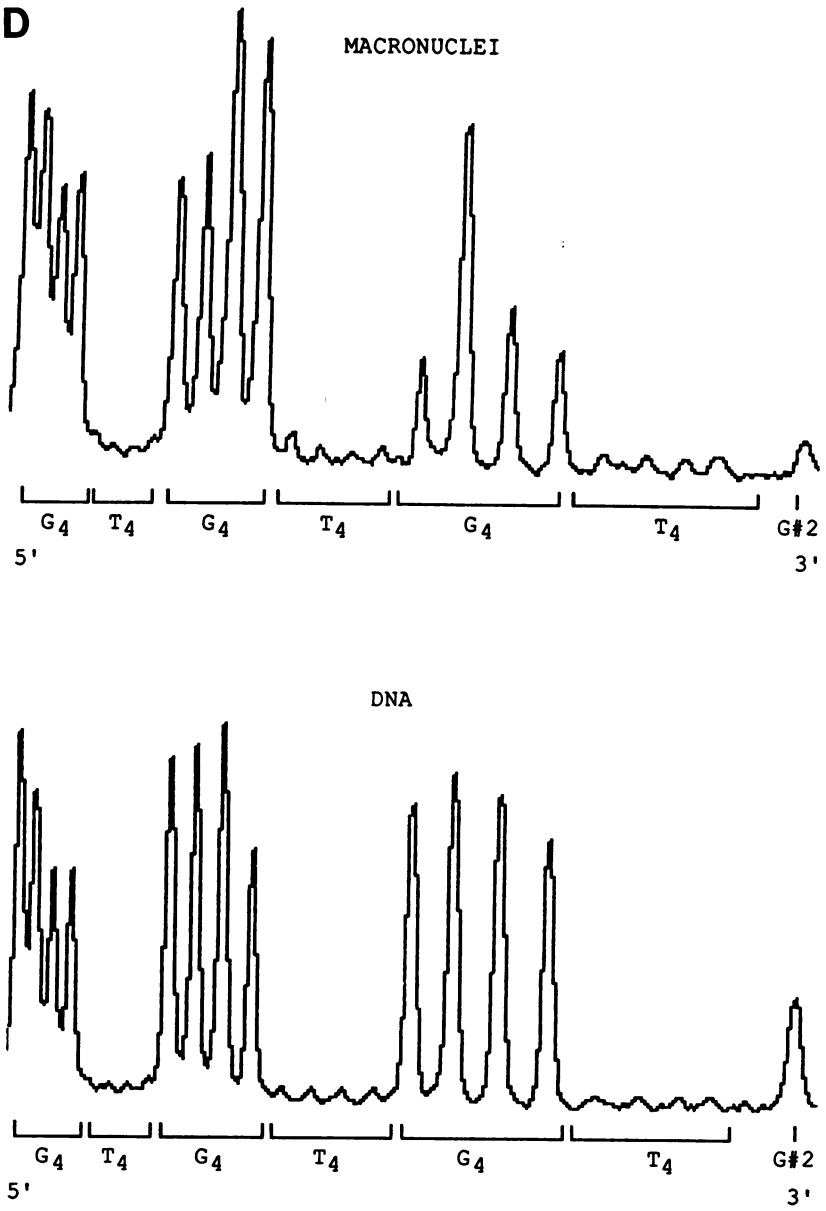

FIG. 3.—*Continued.*

and corresponds to the G residue in the 5-bp conserved TTGAA sequence (Fig. 3D). When the methylation patterns from cells, macronuclei, and deproteinized DNA were compared, no differences were apparent at the conserved G or at the two conserved A residues (data not shown).

Sequence-suppressing gels were also used to separate fragments of 3′ end-labeled DNA that had been cleaved at G and A residues. After DMS treatment, the methylation patterns from cells, macronuclei, and deproteinized DNA were not significantly different (data not shown). This finding suggests that the 3′ strand has no minor groove interactions between protein and A residues in the 5-bp conserved sequence. Sequence-suppressing gels tend to give rather fuzzy bands, so small differences in intensity would not have been detected. It is not particularly surprising that no sequence-specific interactions were detected between protein and the conserved 5 bp, since this sequence is within the region of the telomeric complex where the DNA is lying on a protein surface.

Identification of a telomere-binding protein. The DNA-protein interactions in the most terminal region of the telomeric complex were not destroyed by 2 M NaCl (Fig. 3B), but the salt did dissociate most histones and nonhistone chromosomal proteins from DNA. Consequently, 2 M NaCl treatment was used to purify the salt-resistant telomere-binding protein(s). Macronuclei were incubated in 2 M NaCl plus TE buffer to extract the DNA; the soluble material was then loaded on a Bio-Gel A15M gel filtration column. The DNA-containing fractions were collected and examined for the presence of protein by electrophoresis through SDS-polyacrylamide gels.

After gel filtration, only one protein remained bound to the macronuclear DNA (Fig. 4, lane EUP). This polypeptide had a mass of about 50 kDa and was intermediate in size between

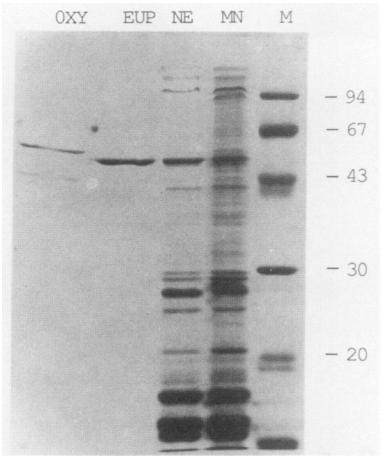

FIG. 4. Coomassie blue-stained SDS-polyacrylamide gel showing that a 50-kDa protein remained bound to *Euplotes* macronuclear DNA in 2 M NaCl. Lanes: OXY, the two subunits of the *Oxytricha* telomere protein; EUP, the one *Euplotes* protein that remained bound to macronuclear DNA after 2 M NaCl treatment; NE, the nuclear extract loaded on the Bio-Gel column; MN, total *Euplotes* macronuclear proteins; M, marker proteins. The molecular weights (in thousands) are shown at the right.

the two subunits (43 and 55 kDa) of the *Oxytricha* telomere-binding protein (lane OXY). No other protein in the >30-kDa size range remained bound to the macronuclear DNA even when the salt concentration was reduced to 1 M (data not shown). In the presence of 1 M NaCl, substantial amounts of histone copurified with the DNA.

A BAL 31 protection experiment was used to determine whether the 50-kDa protein was bound to macronuclear telomeres. Column fractions containing DNA and the 50-kDa protein were dialyzed to remove the salt and treated with BAL 31. Deproteinized macronuclear DNA was treated with the same concentration of BAL 31 for identical periods of time. The DNA was then separated on a 1% agarose gel and capillary blotted to nylon membrane. The telomeric DNA was labeled by hybridization with ^{32}P-labeled $(T_4G_4)_4$. Hybridization and washing were performed at 49 and 64°C, respectively. These conditions prevented hybridization of the probe once BAL 31 had removed 4 to 8 bp of the C_4A_4 repeats.

BAL 31 digestion of the deproteinized DNA gradually removed the telomeric C_4A_4 repeats so that after 10 min of digestion only a small fraction of the DNA was labeled by the $(T_4G_4)_4$ probe (Fig. 5A). In contrast, the macronuclear DNA with bound 50-kDa protein was quite resistant to the BAL 31. Even after 20 to 40 min of digestion, much of the DNA retained sufficient C_4A_4 sequence to be labeled by the $(T_4G_4)_4$ probe.

An internal control was incorporated in the experiment to demonstrate that the BAL 31 was as active in the samples containing DNA plus protein as in the samples containing DNA alone. A 1-μg sample of a *Hae*III restriction digest of phage φX replicative-form DNA was added to both the DNA and DNA-plus-protein samples before the addition of BAL 31. The degree of digestion of the φX DNA by BAL 31 was later visualized on the Southern blot by removing the $(G_4T_4)_4$ probe and rehybridizing the blot with ^{32}P-labeled φX DNA. The BAL 31 showed slightly more activity and digested the φX DNA more in the DNA-plus-protein samples (Fig. 5B).

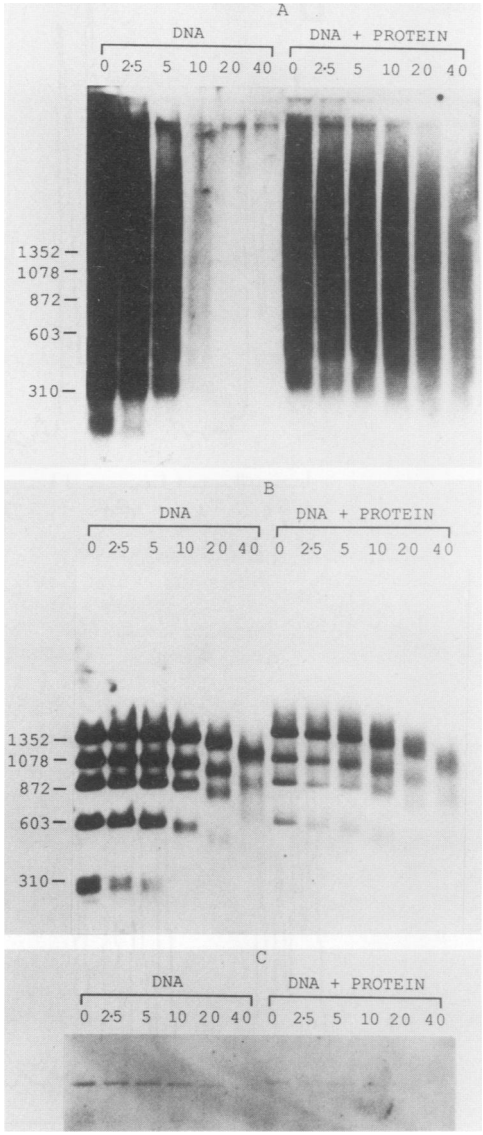

FIG. 5. BAL 31 digestion of the telomeric C_4A_4 repeats in the presence and absence of the 50-kDa protein. Deproteinized DNA or fractions from the Bio-Gel column containing DNA plus 50-kDa protein were treated with BAL 31 for the time (in minutes) indicated above each lane. After separation of the DNA in a 1% agarose gel, the DNA was transferred to nylon membrane and probed with ^{32}P-labeled $(T_4G_4)_4$ (A), ^{32}P-labeled φX *Hae*III DNA (B), or the ^{32}P-labeled 1.9-kbp *Xho-Pst*I fragment of the *Euplotes* V1 gene (C).

To show determine whether roughly the same amount of DNA had been loaded on each lane of the original gel, the blot was stripped of φX DNA and hybridized with a ^{32}P-labeled 1.9 kbp internal fragment of the V1 gene of *E. crassus* (2; kindly supplied by L. Klobutcher). As shown by the intensity of the hybridization signal (Fig. 5C), overall more DNA was present in the DNA samples than in the DNA-plus-protein samples. However, within each set of samples the amount of DNA in each lane was fairly similar, and the results shown in Fig. 5A could not be explained by unequal loading of the gel. In fact, the BAL 31 resistance of the DNA-plus-protein samples is apparent in Fig. 5A even

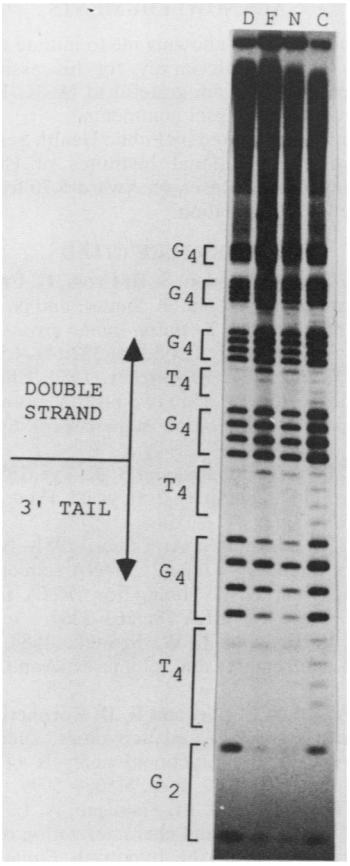

FIG. 6. Binding of the 50-kDa *Euplotes* protein to the 3′ $G_2T_4G_4T_4$ tail of telomeric DNA. Deproteinized DNA (lane D), DNA-containing fractions from the Bio-Gel column (lane F), the nuclear extract loaded on the Bio-Gel column (lane N), and whole cells (lane C), were treated with DMS. The DNA was isolated, 3′ end labeled, cleaved at G residues, and separated by electrophoresis in sequencing gels.

though there was less DNA loaded on this portion of the gel and more BAL 31 activity during digestion of these samples.

The data indicated that the 50-kDa protein was bound at the telomeres of many, if not all, of the macronuclear DNA molecules (Fig. 5). The BAL 31 experiment on its own does not demonstrate that the 50-kDa protein binds specifically to telomeres. The same result would be obtained if the protein coated the length of each macronuclear DNA molecule. However, it is extremely unlikely that the latter event occurred, since there was not sufficient protein present in the samples. Estimations of DNA and protein concentration indicated that there were fewer than five protein molecules per DNA molecule (data not shown). Thus, if the protein bound randomly along the macronuclear DNA molecules, such clear protection of the telomeric DNA from BAL 31 digestion would not have been seen.

Methylation protection of telomeric DNA by the 50-kDa protein. The DMS methylation assay was used to determine whether the 50-kDa protein binds the telomeric T_4G_4 sequence. Column fractions containing DNA and the 50-kDa protein were treated with DMS as soon as they eluted from the Bio-Gel column. The DNA was then isolated, end labeled, and cleaved at methylated G residues. Methylation protection could be observed at G_2, G_7, G_8, and G_{10} (Fig. 6). Since the 50-kDa protein was the only protein present in the column fractions, the data indicate that this protein not only binds the telomeric T_4G_4 repeats but is responsible for at least some of the DMS footprint that is observed in vivo.

The methylation protection displayed by the column eluate always showed the same trend but was sometimes less clear than the methylation protection seen in whole cells or the 2 M NaCl nuclear extract that was loaded on the column. It appears that this degeneration of the methylation protection pattern resulted from gradual denaturation of the 50-kDa protein. Much less protection was observed if the sizing column was run slowly or if the DMS assay was performed after dialysis of the column fractions. Under conditions in which no DMS footprint was observed, the telomeric DNA was still protected from BAL 31 digestion. This finding indicated that the protein was not totally denatured and remained bound to the DNA. It may be that the 2 M NaCl treatment removes additional telomere-binding proteins that are required to stabilize binding of the 50-kDa protein. These proteins may also interact with the telomeric DNA and be responsible for the methylation protection (enhancement) of G_{15}, G_{17}, and G_{18} in the double-stranded region.

DISCUSSION

In recent years there has been a rapid expansion in both our knowledge about the sequence of telomeric DNA from various organisms and our understanding of how this DNA is maintained during DNA replication (13, 26, 41). However, information about the protein component of telomeres has accumulated much more slowly because very few telomere-binding proteins have been isolated. A detailed picture of how telomere-binding proteins interact with telomeric DNA has been restricted to one organism, the ciliate *O. nova*. In this report, I present information about telomeric chromatin structure and telomere-binding proteins from a second organism, *E. crassus*. I show that *Euplotes* telomeres exist as discrete DNA-protein complexes that are structurally very similar to the complexes present at *Oxytricha* telomeres.

In both organisms, the nucleoprotein complexes encompass 100 to 150 bp of DNA and consist of two structural domains that are characterized by their very different DNA-protein interactions (28). In the more internal region, the DNA lies on a protein surface. The DNA in this portion of the complex is of unique sequence, and therefore the DNA-protein interactions must be largely, if not completely, sequence independent. In the most terminal region of each complex, the DNA-protein interactions are sequence specific and also salt stable. In both organisms, treatment with 2 M NaCl leaves most of the DMS methylation protection pattern intact (28).

Comparison of the patterns of DMS methylation from *Euplotes* and *Oxytricha* telomeric complexes (Fig. 3E) shows some distinct similarities and differences. The patterns of methylation protection in the second set of G residues in the 3′ tail are almost identical in the two species. However, *E. crassus* has two fewer G residues in the 3′ tail than does *O. nova*. Consequently, two bases that display methylation protection in *Oxytricha* cells are absent from *Euplotes* cells. It appears that the loss of DNA-protein interactions in the 3′ tail region of *Euplotes* cells is compensated for by interactions between protein and G residues in the double-stranded region. There is no evidence for interaction between protein and G residues in this portion of the *Oxytricha* telomeric complex (28).

Although the telomeres from *Euplotes* and *Oxytricha* cells

are very similar in overall structure, they are by no means identical. Slight variations in the DNA-protein interactions were to be expected, since the telomeric DNAs from the two species are somewhat different. However, it is now apparent that the protein components of the telomeres are also fairly different. Characterization of the *Euplotes* and *Oxytricha* telomere-binding proteins has shown that these proteins differ not only at their DNA-binding sites but also in overall structure. In *Euplotes* cells, binding of a 50-kDa polypeptide to the 3′ $G_2T_4G_4T_4$ tail is responsible for most of the methylation protection pattern seen when living cells are subject to DMS footprinting. In *Oxytricha* cells, the protein that is responsible for methylation protection is a 98-kDa heterodimer with 55- and 43-kDa subunits. Antibodies made against the *Oxytricha* and *Euplotes* telomere proteins show no cross-reactivity (C. M. Price and J. Vermeesch, unpublished data).

It seems probable that the *Euplotes* protein and one subunit of the *Oxytricha* protein are equivalent polypeptides with related DNA-binding domains. However, the proteins are less closely conserved than might have been expected for proteins from ciliated protozoa that bind a similar telomeric DNA sequence. The differences in the telomere proteins from the two ciliates serves to underscore the great evolutionary distance between *Oxytricha* and *Euplotes* species.

The *Oxytricha* and *Euplotes* telomere proteins are rather unusual in that they both remain bound to telomeric DNA in 2 M NaCl (12, 28). Consequently, it will be of great interest to determine exactly how these proteins interact with the DNA (28, 30). However, the two subunits of the *Oxytricha* protein are very tightly associated with each other and have been separated only by using conditions that destroy the DNA-binding activity (29). Consequently, it has not been possible to determine which of the subunits actually recognizes and binds the telomeric DNA. It appears that the *Euplotes* protein may lend itself more readily to this type of analysis. Further studies are needed to determine whether the bound protein consists of one or multiple subunits.

It is notable that the overall structures of *Oxytricha* and *Euplotes* telomeres are so similar although the DNA and protein components show significant differences. It was suggested previously that telomeres from many different organisms might consist of DNA-protein complexes that are structurally conserved but differ significantly in internal composition (28). Clearly this is the case in *Oxytricha* and *Euplotes* cells, in which the protein in the terminal portion of the telomeric complex seems to be tailored to match the cognate telomeric DNA. It will be interesting to determine whether a similar feature is seen in telomeres from more divergent organisms. Many organisms have a larger and more variable number of telomeric repeats (5). Such variation in the telomeric DNA might be accommodated by a more extended internal region of the telomeric complex with variable lengths of DNA lying on protein surface.

It is apparent that various enzymes, such as telomere terminal transferase and the *Oxytricha* telomere DNA primase, must act directly on telomeric DNA during DNA replication (13, 41). Since these enzymes recognize the sequence of the telomeric DNA at the 3′ end, it is hard to envision how they could function when a telomeric complex is present. It seems likely that at least the most terminal portion of each complex dissociates during DNA replication. Re-formation of complexes immediately after replication might then be a way to regulate telomere length as well as ensure chromosome stability by rendering the telomeres resistant to degradation or recombination (28, 30, 34, 40).

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LITERATURE CITED

1. **Allshire, R. C., J. R. Gosden, S. H. Cross, G. Cranston, D. Rout, N. Sugawara, J. W. Szostak, P. Fantes, and N. D. Hastie.** 1988. Telomeric repeat from *T. thermophila* cross hybridized with human telomeres. Nature (London) **332**:656–659.
2. **Baird, S. E., and L. A. Klobutcher.** 1989. Characterization of chromosome fragmentation in two protozoans and identification of a candidate fragmentation sequence in *Euplotes crassus*. Genes Dev. **3**:585–597.
3. **Berman, J., C. Y. Tachibana, and B.-K. Tye.** 1986. Identification of a telomere-binding activity from yeast. Proc. Natl. Acad. Sci. USA **83**:3713–3717.
4. **Blackburn, E. H., and S. A. Chiou.** 1981. Non-nucleosomal packaging of a tandemly repeated DNA sequence at termini of extrachromosomal DNA coding for rRNA in *Tetrahymena*. Proc. Natl. Acad. Sci. USA **78**:2263–2267.
5. **Blackburn, E. H., and J. W. Szostak.** 1984. The molecular structure of centromeres and telomeres. Annu. Rev. Biochem. **53**:163–194.
6. **Buchman, A. R., N. F. Lue, and R. D. Kornberg.** 1988. Connections between transcriptional activators, silencers, and telomeres as revealed by functional analysis of a yeast DNA-binding protein. Mol. Cell. Biol. **8**:5068–5099.
7. **Cadilla, C. L., J. Harp, J. M. Flanagan, A. L. Olins, and D. E. Olins.** 1986. Preparation and characterization of soluble macronuclear chromatin from the hypotrich *Euplotes eurystomus*. Nucleic Acid Res. **14**:823–841.
8. **Cheung, M. K., D. T. Drivas, V. C. Littau, and E. M. Johnson.** 1981. Protein tightly bound near the termini of the *Physarum* extrachromosomal rDNA palindrome. J. Cell Biol. **91**:309–314.
9. **Corliss, J. O.** 1979. The ciliated protozoa: characterization, classification and guide to the literature, 2nd ed. Pergamon Press, Oxford.
10. **Drew, H.** 1984. Structural specificities of five commonly used DNA nucleases. J. Mol. Biol. **176**:535–557.
11. **Gottschling, D. E., and T. R. Cech.** 1984. Chromatin structure of the molecular ends of *Oxytricha* macronuclear DNA: phased nucleosomes and a telomeric complex. Cell **38**:501–510.
12. **Gottschling, D. E., and V. A. Zakian.** 1986. Telomere proteins: specific recognition and protection of the natural termini of *Oxytricha* macronuclear DNA. Cell **47**:195–205.
13. **Greider, C. W., and E. H. Blackburn.** 1989. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. Nature (London) **337**:331–337.
14. **Harper, D. S., and C. L. Jahn.** 1989. Differential use of termination codons in ciliated protozoa. Proc. Natl. Acad. Sci. USA **86**:3252–3256.
15. **Henderson, E. R., and E. H. Blackburn.** 1989. An overhanging 3′ terminus is a conserved feature of telomeres. Mol. Cell. Biol. **9**:345–348.
16. **Klobutcher, L. A., and D. M. Prescott.** 1986. The special case of the hypotrichs, p. 111–154. *In* J. Gall (ed.). The molecular biology of ciliated protozoa. Academic Press, Inc., New York.
17. **Klobutcher, L. A., M. T. Swanton, P. Donini, and D. M. Prescott.** 1981. All gene-sized DNA molecules in four species of hypotrichs have the same terminal sequence and an unusual 3′ terminus. Proc. Natl. Acad. Sci. USA **78**:3015–3019.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lutter, L. C.** 1979. Precise location of DNase I cutting sites in the nucleosome core determined by high resolution gel electrophoresis. Nucleic Acids Res. **6**:41–56.

20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Martindale, D. W.** 1989. Codon usage in *Tetrahymena* and other ciliates. J. Protozool. **36**:29–34.
22. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
23. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing with base specific chemical cleavages. Methods Enzymol. **65**:499–560. Academic Press.
24. **Miceli, C., A. La Terza, and M. Melli.** 1989. Isolation and structural characterization of cDNA clones encoding the mating pheromone Er-1 secreted by the ciliate *Euplotes raikovi*. Proc. Natl. Acad. Sci. USA **86**:3016–3020.
25. **Moyzis, R. K., J. M. Buckingham, L. D. Cram, M. Dani, L. L. Deavan, M. D. Jones, J. Meyne, R. L. Ratliff, and J.-R. Wu.** 1988. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc. Natl. Acad. Sci. USA **85**:6622–6626.
26. **Pluta, A. F., and V. A. Zakian.** 1989. Recombination occurs during telomere formation in yeast. Nature (London) **337**: 429–433.
27. **Prescott, D. M.** 1983. The C-value paradox and genes in ciliated protozoa. Mod. Cell Biol. **2**:329–352.
28. **Price, C. M., and T. R. Cech.** 1987. Telomeric DNA-protein interactions of *Oxytricha* macronuclear DNA. Genes Dev. **1**: 783–793.
29. **Price, C. M., and T. R. Cech.** 1989. Properties of the telomeric DNA-binding protein from *Oxytricha nova*. Biochemistry **28**: 769–774.
30. **Raghuraman, M. K., C. J. Dunn, B. J. Hicke, and T. R. Cech.** 1989. *Oxytricha* telomeric nucleoprotein complexes reconstituted with synthetic DNA. Nucleic Acids Res. **17**:4235–4253.
31. **Rhodes, D., and A. Klug.** 1980. Helical periodicity of DNA determined by enzyme digestion. Nature (London) **286**:573–578.
32. **Richards, E. J., and F. M. Ausubel.** 1988. Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. Cell **53**:127–136.
33. **Roth, M., L. Meiying, and D. M. Prescott.** 1985. Large scale synchronous mating and the study of macronuclear development in *Euplotes crassus*. J. Cell Biol. **101**:79–84.
34. **Runge, K. W., and V. A. Zakian.** 1989. Introduction of extra telomeric DNA sequences into *Saccharomyces cerevisiae* results in telomere elongation. Mol. Cell. Biol. **9**:1488–1497.
35. **Sogin, M. L., and H. J. Elwood.** 1986. Primary structure of the *Paramecium tetraurelia* small-subunit rRNA coding region: phylogenetic relationships within the ciliophora. J. Mol. Evol. **23**:53–60.
36. **Sogin, M. L., M. T. Swanton, J. H. Gunderson, and H. J. Elwood.** 1986. Sequence of the small subunit ribosomal RNA gene from the hypotrichous ciliate *Euplotes aediculatus*. J. Protozool. **33**:26–29.
37. **Solomon, A. J., F. Strauss, and A. Varshavsky.** 1986. A mammalian high mobility group protein recognizes any stretch of six A · T base pairs in duplex DNA. Proc. Natl. Acad. Sci. USA **83**:1276–1280.
38. **Swanton, M. T., A. F. Greslin, and D. M. Prescott.** 1980. Arrangement of coding and non-coding sequences in the DNA molecules coding for rRNAs in *Oxytricha* sp. Chromosoma **77**:203–215.
39. **Swanton, M. T., J. M. Heumann, and D. M. Prescott.** 1980. Gene-sized DNA molecules of the macronuclei in three species of hypotrichs; size distribution and absence of nicks. Chromosoma **77**:217–227.
40. **Zahler, A. M., and D. M. Prescott.** 1988. Telomere terminal transferase activity in the hypotrichous ciliate *Oxytricha nova* and a model for replication of the ends of linear DNA molecules. Nucleic Acids Res. **16**:6953–6972.
41. **Zahler, A. M., and D. M. Prescott.** 1989. DNA primase and the replication of the telomeres in *Oxytricha nova*. Nucleic Acids Res. **17**:6299–6317.