Inaccessibility of theEuplotes telomere binding protein

ARTICLE *in* CHROMOSOMA · JANUARY 1993

Impact Factor: 4.6 · DOI: 10.1007/BF00650896 · Source: PubMed

5 AUTHORS, INCLUDING:

Madhu Dhar
University of Tennessee
60 PUBLICATIONS **742** CITATIONS

SEE PROFILE

Chromosoma (1993) 102:700–711

CHROMOSOMA

Inaccessibility of the *Euplotes* telomere binding protein

Ada L. Olins, Lucia H. Cacheiro, Adria L. Herrmann, Madhu S. Dhar, Donald E. Olins

The University of Tennessee Graduate School of Biomedical Sciences and Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-8077, USA

Received: 4 June 1993; in revised form: 23 August 1993 / Accepted: 10 September 1993

Abstract. The telomere binding protein (TP) from the macronucleus of the ciliate *Euplotes eurystomus* was purified by removal of tenaciously bound DNA with hydroxylapatite, and the purified TP partially sequenced. Rabbit antiserum was generated against a synthetic peptide of 14 amino acids at the amino-terminus of the TP. This antiserum was employed to examine the accessibility of TP antigenic determinants in nuclei and chromatin. Immunofluorescent staining of isolated macronuclei revealed only weak reactivity with specific antiserum. Reactivity within replication bands was demonstrated, and could be augumented by preparation of nuclear scaffolds. Employing a dot immunoblot analysis, the amino-terminal antigenic determinants of TP were revealed after extraction of histone H1 (and some nonhistones). A different aspect of TP inaccessibility was demonstrated by immunoblot analysis of trypsin-treated macronuclei and chromatin; TP was considerably less susceptible to digestion by trypsin than were histones H1 and H3. The relative inaccessibility of TP was not a consequence of chromatin higher-order structure, since soluble macronuclear chromatin in low salt exhibited the same burying of antigenic determinants by dot blot analysis, and the same decreased susceptibility to trypsin, as did isolated nuclei. Electron microscopy of soluble macronuclear chromatin spread in low salt revealed that most telomeres appear unfolded, without stable higher-order structure. The mechanisms for the relative inaccessibility of TP are not yet known, but probably arise as a consequence of the strong interactions of TP with the telomere nucleotide sequence and additional interactions of TP with various chromatin proteins, perhaps including histone H1.

Introduction

Telomeres, the structures at the ends of chromosomes, and their functional roles have been reviewed by Zakian (1989), Blackburn (1991) and Price (1992). Telomeres lend stability to chromosomes by protecting the ends from fusion and recombination, often observed with broken chromosome ends. They may participate in the determination of nuclear architecture, and probably prevent loss of chromosome ends during replication. Each of these functions is probably determined by their unusual terminal DNA sequence, specific telomere binding proteins (TPs) and possibly by other chromatin proteins.

One of the systems favored for the study of telomeres is the macronucleus (MAC) of hypotrichous ciliates, because each nucleus contains a large number of gene-size linear DNA fragments (e.g., $\sim 10^8$ in *Euplotes eurystomus*), each having two telomeric ends. It is not only the number of telomeres that make this a useful biologic model, but also the simplicity of its DNA structure and the probable reduction of telomere functions compared with other eukaryotic nuclei. The structure of these small chromatin gene-size fragments is quite interesting. Each contains one structural gene, a compact transcriptional control region and short constant-length telomeres (Prescott 1992). Each gene-size DNA molecule possesses at least one replication origin, although these have not yet been identified (Murti and Prescott 1983; Allen et al. 1985). Replication in the MAC is confined to a migrating structure called the replication band (RB; see Olins et al. 1988; Olins and Olins 1993).

Telomeres in the hypotrichous ciliates contain G_4T_4 repeats at the distal 3′ ends, including 14 to 16 base single-strand extensions of the same sequence (Klobutcher et al. 1981; Henderson and Blackburn 1989). In *Euplotes* there are 28 bp of the double-stranded region and 14 bases of the single-strand, ending with GG at the 3′ end. The length of these telomere repeat sequences is usually constant in hypotrichous ciliates; however, it is variable (sometimes hundreds or thousands of base pairs long) in higher organisms. The telomeric repeat sequences of other eukaryotes (e.g., G_4T_2 in *Tetrahymena;* $G_{1-3}T$ in yeast; and T_2AG_3 in more than 100 vertebrate species, Moyzis et al. 1988) also have the G-rich repeats on the 3′ end. It should be noted that

Communicated by: P.B. Moens

Correspondence to: A.L. Olins

3′ single-stranded tails have not been demonstrated in most organisms.

Proteins associated with the telomeres may be divided into two categories (Price et al. 1992): those that bind to the subterminal regions and those that show specificity only for the extreme ends. Internal binding proteins from *Physarum,* PPT (Coren et al. 1991; Coren and Vogt 1992), and from yeast, TBFα (Liu and Tye 1991) and RAP1 (Berman et al. 1986; Longtine et al. 1989; Conrad et al. 1990; Lustig et al. 1990) have been identified. Several proteins binding to the extreme ends, and showing specificity for the single-stranded overhang, have been characterized in *Oxytricha* (Gottschling and Zakian 1986; Price and Cech 1987, 1989; Fang et al. 1993), *Stylonychia* (Steinhilber and Lipps 1986; Fang and Cech 1991) and *Euplotes* (Wang et al. 1992; Price et al. 1992). Those proteins that bind to the extreme ends of telomeres bind with great tenacity, even in very high salt. They show considerable specificity for accurate homologous telomere sequences, especially for the single-stranded ends. They protect these ends from Bal31 digestion (Gottschling and Zakian 1986; Hauser et al. 1991) and from chemical methylation (Price and Cech 1989; Price 1990). In *Oxytricha,* the TP is a heterodimer, with subunits of Mr 56,000 and 41,000. Both of these subunits have been expressed in *Escherichia coli* (Hicke et al. 1990; Gray et al. 1991; Fang et al. 1993). In *Euplotes crassus* an Mr 51,000 protein has been isolated and cloned (Wang et al. 1992). Its sequence, binding specificity and protection of DNA are homologous to the proteins seen in *Oxytricha* (Price et al. 1992). These TPs do not bind to telomeres under in vitro conditions that favor the formation of G-quartets (Zahler et al. 1991), consistent with their preference for single-stranded DNA.

In the present study, we investigated the *E. eurystomus* TP. We show that it has a similar amino-terminal peptide sequence to the *E. crassus* protein, characterized by Wang et al. (1992) and by Price et al. (1992). We generated polyclonal antibodies directed against the 14 amino acids located at the amino-terminus of the *E. eurystomus* TP. This antibody has been used to demonstrate that the amino-terminus of TP is inaccessible in chromatin and in nuclei; however, conditions that extract histone H1 from chromatin reveal the TP determinants. Comparing relative sensitivity with trypsin digestion, we demonstrate that TP is less accessible than histones H1 and H3. The relative inaccessibility of the *Euplotes* TP does not appear to be a consequence of chromatin higher-order structure, since it can be observed with soluble MAC chromatin at low ionic strength.

Materials and methods

Cultivation of hypotrichous ciliates. Two ciliate species were maintained in the laboratory: (1) *E. eurystomus* (Carolina Biological Supply Co., Burlington, N.C.), and (2) *Oxytricha nova* (generously provided by Dr. D.M. Prescott, Univ. Colorado, Boulder, Colo.). Both species were grown under non-sterile conditions in Pringsheim medium on a diet of the alga *Chlorogonium elongatum* as previously described (Allen and Olins 1984). For large scale preparations of *Euplotes,* cells were grown in 8–10 l glass jars, aerated with an aquarium pump connected to a fine-tip 5 ml pipet. We routinely obtained cultures of *Euplotes* of greater than 5×10^3 cells/ml.

Preparation and analysis of TP. Euplotes and *Oxytricha* were collected by filter concentration on a 6 μm nylon mesh (Cadilla et al. 1986). The methods for cell lysis, MAC isolation by centrifugation in metrizamide gradients and preparation of soluble MAC chromatin have been previously described (Cadilla et al. 1986). Cell lysis buffer consists of 10 mM Pipes, pH 6.75, 1 mM spermidine phosphate, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM tosyl-lysine chloromethylketone (TLCK) and 0.5% NP-40. Nuclear lysis buffer is 5 mM triethanolamine, pH 7.0 and 10 mM EDTA. Yields of soluble chromatin from the large jars of *Euplotes* routinely contained ca. 3–5 mg of MAC DNA.

The TP-DNA complex was usually obtained from soluble MAC chromatin, employing a modification of previously published procedures (Gottschling and Zakian 1986; Price and Cech 1987). Occasionally, TP-DNA was prepared from purified MACs, which were dissociated in 2 M NaCl/TE for 2 h on ice, centrifuged to remove the 2 M NaCl insoluble residue and the supernatant loaded on a Bio-Gel A15 m (Bio-Rad Laboratories, Richmond, Calif.) column in 2 M NaCl/TE at room temperature. The void volume from the Bio-Gel column contained the TP-DNA.

Euplotes TP was purified away from MAC DNA by binding a dissociated TP-DNA mixture to hydroxylapatite (HAP, Bio-Gel HTP) in a buffer containing 8 M urea, 0.8% SDS, 0.24 M sodium phosphate buffer, pH 7 by the following procedure. The TP-DNA complex in 2 M NaCl, pooled from the Bio-Gel A15m void volume, containing 500–700 μg DNA, was diluted in half with TE, precipitated with ethanol and dried. TP-DNA was dissolved in 200 μl TE. Subsequently, 2 ml of warm (42° C) freshly prepared 9 M urea, 0.9% SDS, 1.1 mM dithiothreitol (DTT), 0.27 M sodium phosphate, pH 7 was added to the dissolved TP-DNA. After mixing, the solution was incubated at 42° C for 30 min. One gram dry HAP was added and incubation at 42° C was continued an additional 30 min. The suspension was centrifuged at low speed in a clinical centrifuge at room temperature. The supernatant containing eluted TP was saved and the HAP washed twice with 1 ml 8 M urea, 0.8% SDS, 1.0 mM DTT, 0.24 M sodium phosphate, pH 7, pooling the three supernatants. MAC DNA remained bound to the HAP matrix and was recovered by washing with 0.5 M sodium phosphate, 1.0 mM DTT, pH 7. As determined from measurements of A_{260}, more than 90% of the DNA was recovered in the high phosphate wash. TP was precipitated from the pooled low phosphate supernatants with 20% TCA, washed with HCl/acetone, acetone and dried. Precipitated TP and released MAC DNA were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The DNA containing fraction was almost devoid of TP; the extracted TP fraction consisted primarily of TP with a variety of minor bands, apparent at the higher loads. Comparing TP electrophoretic band densities with BSA standards, we estimated a final yield of ca. 10 μg TP per 1.0 mg MAC DNA.

For amino-terminal peptide sequence analysis, approximately 5 μg (i.e., ca. 100 pmol of the Mr 50,000 protein) of TP was subjected to sequential Edman degradation using an Applied Biosystems 477A protein sequencer (analysis performed by C. Murphy, Univ. Tenn. Medical Center, Knoxville, Tenn.).

Preparation and characterization of antisera. Approximately 100 μg of TP amino-terminal peptide was synthesized by Dr. R. Cook (Protein Chemistry Facility, Baylor College of Med., Houston, Tex.), consisting of the 14 sequenced amino acids plus a carboxy-terminal GGC to facilitate coupling to carrier protein. The peptide synthesis procedures and the technique of coupling the TP peptide to keyhole limpet hemocyanin (KLH) were essentially as previously described (Lin et al. 1989). Twenty milligrams KLH was reacted with M-maleimidobenzoyl-N-hydroxysuccinimide (MBS) in PBS. Excess MBS was removed with a Sephadex G-25 column. After adjusting the MBS-KLH complex to pH 7.5, approximately 6 mg purified synthetic TP peptide was added, purged with N_2 and incu-

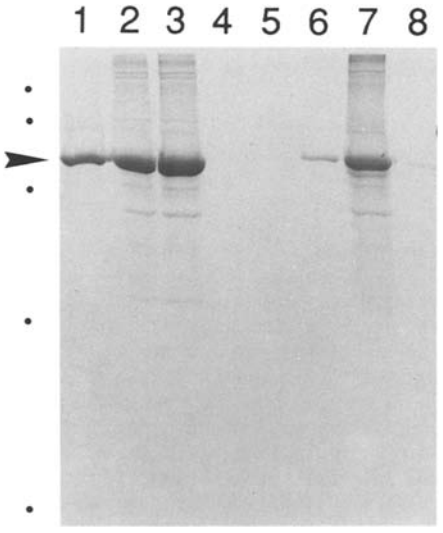

Fig. 1. Coomassie blue stained SDS-polyacrylamide gel of *Euplotes* telomere binding protein (TP)-DNA and the separated TP and DNA fractions, obtained from batchwise hydroxylapatite (HAP) elutions in 8 M urea, 0.8% SDS. Lane *1*, starting TP-DNA; lanes *2* and *3*, increasing loads of the unbound TP; lanes *4* and *5*, increasing amounts of DNA, eluted with 0.5 M sodium phosphate; lane *6*, starting TP-DNA; lane *7*, unbound TP; lane *8*, eluted DNA. Fractions *1–5* and *6–8* are two separate preparations. Lanes *4*, *5*, and *8* are estimated to contain ca. 70, 100 and 90 µg DNA, respectively. *Arrowhead* indicates position of migration of the *Euplotes* TP (Mr ca. 50,000). Mol. wt. markers indicated by *dots* along the gel, in descending order: 97.4, 68, 43, 29, and 18.4 kDa

bated with mixing for 3 h at room temperature. An additional 6 mg TP peptide was added, involving another 3 h incubation. The resulting TP-KLH complex was dialyzed against PBS, split into aliquots each containing ca. 2.5 mg protein and lyophilized.

For immunization, one aliquot of TP-KLH was dispersed in H_2O, emulsified with an equal volume of complete Freund's adjuvant and injected into multiple subcutaneous locations in the back of one adult New Zealand White Rabbit. Pre-immune serum was collected before immunization. Two weeks after the initial immunization, a test bleeding was performed and the animal was boosted with TP-KLH in incomplete Freund's adjuvant. About 2 weeks later the animal was tested and subsequently exsanguinated. All the studies described here have used this final bleeding. Aliquots of centrifuged sera were stored at −20° C. The antibody and pre-immune serum reactivity with total MAC proteins and TP-DNA on immunoblots (Fig. 2B) demonstrate the specificity of anti-TP-KLH. In order to examine the specificity more fully, we prepared a complex of synthetic TP peptide with bovine gamma globulin (TP-BGG), starting with 20 mg BGG and following a procedure essentially identical to that described above for preparation of TP-KLH. Figure 2C verifies the specificity of anti-TP-KLH, showing clear reactions on an immunoblot with dissociated TP-conjugated H and L chains. Preimmune serum showed no reaction with TP-BGG; BGG that was not coupled to the TP peptide did not react with anti-TP-KLH (data not shown).

The rabbit antiserum against soluble MAC chromatin has been described earlier (Herrmann et al. 1987). This potent serum contains antibodies to the inner histones, H1 and numerous nonhistone proteins (Fig. 2A).

Preparation of nuclear and chromatin fractions, and of Euplotes cell extracts. A variety of nuclear and chromatin extracts were tested by immunoblotting procedures. Their designation and preparation are described here. As mentioned earlier, soluble MAC chromatin was prepared following the description of Cadilla et al. (1986). Isolated MACs were lysed for 1 h at 4° C in 10 mM EDTA, 5 mM triethanolamine, pH 7.0, and dialyzed overnight in 0.25 mM EDTA, 5 mM triethanolamine. A low-salt insoluble residue was obtained by centrifugation for 30 min at 3,000 rpm. Both the low-

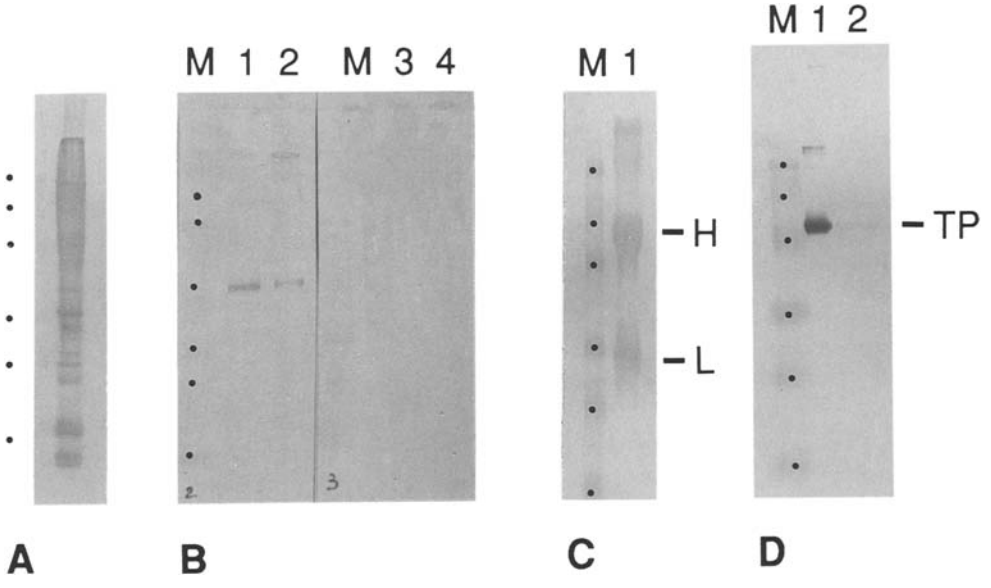

Fig. 2A–D. Immunoblots from SDS-polyacrylamide gels with rabbit anti-*Euplotes* soluble macronucleus (MAC) chromatin and rabbit anti-TP-KLH serum. **A** Immunoblot of anti-soluble MAC chromatin with total *Euplotes* MAC proteins. **B** Comparison of total MAC proteins (lanes *1* and *3*) with TP-DNA (lanes *2* and *4*). *Left panel* anti-TP-KLH; *right panel,* pre-immune serum. The apparent upper band in lanes *1* and *2* is at the origin of the separation gel. **C** Immune reaction of anti-TP-KLH with TP-BGG showing antibody staining of dissociated TP-BGG H and L chains (lane *1*). **D** Immunoblot of anti-TP-KLH with *Euplotes* TP-DNA (lane *1*) and *Oxytricha* TP-DNA (lane *2*). *M*, mol. wt. markers in descending order: 106, 80, 49.5, 32.5, 27.5 and 18.5 kDa

salt insoluble residue and the soluble chromatin supernatant were examined. Dissociation of isolated MACs in 2 M NaCl, TE, also described earlier, yielded two fractions that were saved: (1) TP-DNA; (2) high-salt insoluble residue. The preparation of H1-depleted chromatin was performed as a modification of Herrmann et al. (1987). Soluble MAC chromatin in 5 mM triethanolamine, 0.25 mM EDTA (ca. 1 mg DNA/ml) was made 0.4 M NaCl and incubated with the buffer-equilibrated resin AG50W-X2 (Bio-Rad) at 4° C for 1 h in batchwise treatment. Following centrifugation, the supernatant (H1-depleted chromatin) was dialyzed against 5 mM triethanolamine, 0.25 mM EDTA before use. Isolated *Euplotes* MACs were also extracted with 10 mM lithium – 3′,5′ – diiodosalicylate, (LIS, Sigma Chemical Corp.) in 0.1% digitonin, leading to a LIS extract and a LIS-insoluble residue, essentially as described by Cardenas et al. (1990) for the production of yeast nuclear scaffolds. As described by these authors, exposure of nuclei to 1.0 mM $CuSO_4$ at 37° C for 10 min prior to LIS extraction appeared to be essential, judged from morphological data (see text), and these steps were included in all experiments. The LIS extract was precipitated with 20% TCA, to allow examination by SDS-PAGE.

Immunoblotting techniques. Immunoblot analysis of SDS-PAGE was essentially as described by Herrmann et al. (1987), with the following modifications. Proteins were transferred to Immobilon-P, pore size 0.45 μm (Millipore Corp., Bedford, Mass.). The blocking solution consisted of 5% powdered milk and 10% fetal calf serum in PBS. Primary antisera and secondary antibodies were diluted with the blocking solution. Blots were washed with 1% NP-40 and 1% Tween-20 in PBS. Primary antibody concentrations were usually the following: rabbit anti-TP-KLH, 1:3,500; rabbit anti-*Euplotes* soluble chromatin 1:8,000. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Bio-Rad) and was routinely employed at 1:3,000 or higher dilution. Dot immunoblots were prepared by sucking 100 μl of the appropriate chromatin fraction containing ca. 3 μg DNA onto Immobilon-P sheets clamped in a minifold filtration manifold SRC 96/0 (Schleicher and Schuell, Keene, N.H.). Subsequent blocking, antibody reactions and washing steps were as described for transfers from SDS-PAGE.

Immunofluorescent staining. Liberated *Euplotes* MACs were fixed with 3.7% HCHO, before or after extraction with LIS, and centrifuged on chromalum-gelatin subbed slides, essentially as described previously (Olins et al. 1989, 1991). Photographs were collected on a Zeiss photomicroscope III equipped with epifluorescence and filters for fluorescein isothiocyanate (FITC) and 4′,6-diamidino-2-phenylindole (DAPI), using Kodak TMAX 400 film.

Trypsin digestion of nuclei and soluble chromatin. Bovine pancreas trypsin (2× crystallized, Gibco BRL Laboratory, Grand Island, NY) was dissolved in a stock solution (1 mg/ml in distilled H_2O). Soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO.) was a stock solution (200 μg/ml in PBS). For digestion of isolated MACs, nuclei recovered from a metrizamide ultracentrifugation gradient were washed and pelleted from cell lysis buffer without detergent, then resuspended in the washing buffer to an estimated DNA concentration of ca. 1 mg/100 μl (based upon cell counts, prior to cell lysis). A zero-time sample of MACs was collected and added to trypsin inhibitor. Trypsin was added to yield ca. 1 μg trypsin/mg MAC DNA. Incubation was at room temperature. Samples were collected at times indicated in the text and figure legends, by removing aliquots and adding them to tubes containing trypsin inhibitor at a tenfold weight excess to trypsin. Control and digested MACs were centrifuged and dissolved in SDS sample buffer for SDS-PAGE. Trypsin digestion of soluble MAC chromatin was performed in 5 mM triethanolamine-HCl, pH 7.0. DNA concentrations were measured by absorbance at 260 nm, followed by concentration by centrifugation to ca. 1 mg DNA/100 μl, prior to addition of trypsin. Following termination of digestion with trypsin inhibitor, total proteins were precipitated with 20% TCA, followed by HCl/acetone, acetone and air-drying, and dissolving in SDS sample buffer.

Electron microscopy. Preparation of *E. eurystomus* soluble MAC chromatin for visualization by dark-field electron microscopy has been described (Cadilla et al. 1986). Briefly, chromatin was fixed in 0.1% glutaraldehyde, 0.2 mM EDTA, 0.5 mM triethanolamine-HCl, pH 7.0 for 15 h at 4° C. The sample was put on carbon film in the presence of 0.0002% Zephiran chloride for 30–60 s, rinsed in water and then dried in Photo-flo. Finally, the grids were stained with 0.1% aqueous uranyl acetate.

Results

Amino-terminal sequence of E. eurystomus TP

The N-terminal 14 amino acids from HAP-purified *E. eurystomus* MAC TP were obtained by sequential Edman degradation. In Table 1 (adapted from Wang et al. 1992) this sequence is compared with homologous regions from cloned and sequenced hypotrich TP genes. It is obvious that there are peptide sequence homologies in this amino-terminal region between the *E. eurystomus* MAC TP and other hypotrich TPs. Indeed, a weak cross-reaction was detected when immunoblots of *O. nova* TP-DNA were probed with our rabbit antisera directed against *E. eurystomus* TP (Fig. 2D). However, consider-

Table 1. Comparison of N-terminal amino acid sequences from hypotrich TPs

Hypotrich species	TP name	Homologous region sequence
Euplotes eurystomus		M K T K A A K K D H – – – – – Y E Y T . .
Euplotes crassus	51 kd	M P K Q K A A K K D H – – – – – Y Q Y S . .
Oxytricha nova [a]	56 kd	. . . A P K E G A A K K S D K G H – K Y E Y V . .
Stylonychia mytilus [a]	56V	. . . P A K D G A P K K R E Q S T – R Y K Y V . .
Euplotes crassus [b]	hom	M K R R T D L D T K S S R K V Y K K Y E Y T . .

[a] The sequences shown for *Oxytricha* and *Stylonychia* are internal from the amino-terminal ends
[b] The *Euplotes crassus* TP homolog gene, sequenced by Wang et al. (1992), has no known function

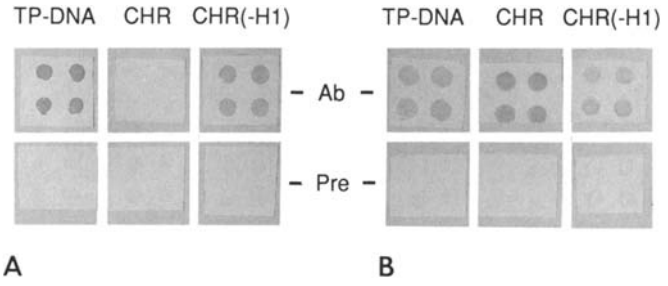

Fig. 3A, B. Dot immunoblots of: 2 M NaCl extracted chromatin, TP-DNA; soluble *Euplotes* MAC chromatin, CHR; H1-depleted soluble chromatin, CHR (-H1). **A** Reacted with rabbit anti-TP-KLH (*Ab; top row*) and the preimmune serum (*Pre, bottom row*). **B** Reacted with rabbit anti-soluble MAC chromatin (*top row*) and the preimmune serum (*bottom row*). Four identical dots are shown, each spotted with 100 µl of the appropriate chromatin fraction

able non-homology may also exist between the TP genes of *E. eurystomus* and *E. crassus*. Southern blots of *E. eurystomus* DNA probes with *E. crassus* probes for TP and its homolog (generous gifts of C. Price) did not show hybridization under a variety of conditions, including those used by Wang et al. (1992).

Inaccessibility of TP amino-terminal antigenic determinants

Attempts to localize TP antigenic determinants by immunofluorescent microscopy with rabbit anti-TP-KLH on isolated MACs (data not shown) proved only marginally successful. MACs revealed only slightly more staining with anti-TP-KLH than with preimmune sera, at a variety of dilutions. On the other hand, the rabbit antiserum against soluble *Euplotes* MAC chromatin routinely revealed intense immunostaining over the entire MAC (data not shown).

The TP antigenic determinants might be rendered inaccessible to specific antibodies at a variety of structural levels within the nucleus (e.g., higher-order chromatin condensation; TP conformation; steric masking by chromatin-associated proteins, or formation of the TP-DNA complex). Figure 2B clearly demonstrates an immunoblot reaction of anti-TP-KLH with SDS-denatured TP dissociated from the DNA. Consequently, the failure to achieve strong localized immunostaining of the MACs was not due to an inability of the antibody to recognize the extracted TP. In order to explore the mechanism of inaccessibility, we developed a dot immunoblotting technique that would not involve denaturation and dissociation of TP. Figure 3 illustrates the results obtained with this method, demonstrating the reactions of three antigens (i.e., soluble MAC chromatin, H1-depleted chromatin and TP-DNA) with the two rabbit antisera (i.e., anti-TP-KLH and anti-soluble chromatin) and their respective preimmune sera. Rabbit anti-soluble chromatin (Fig. 3B) exhibited strong reactions with soluble MAC chromatin, TP-DNA and H1-depleted MAC chromatin; preimmune serum from the same rabbit was uniformly negative. In contrast, rabbit anti-TP-KLH showed no significant reaction with soluble chromatin and gave strong reactions with both TP-DNA and H1-depleted chromatin. The appropriate preimmune serum was uniformly negative. Since all of these chromatin samples were dissolved in the same low ionic strength buffer (i.e., 5 mM triethanolamine, 0.25 mM EDTA, pH 7.0), none would be expected to possess significant higher-order structure or chromatin condensation. The lack of reactivity of the TP antigenic determinants to soluble chromatin, combined with their availability after extraction of H1 (and some nonhistones), or after removal of virtually all histones and nonhistones, suggests two possible structural mechanisms. Either the TP amino-terminal antigenic determinants are sterically masked by proteins such as histone H1, or removal of H1 (and/

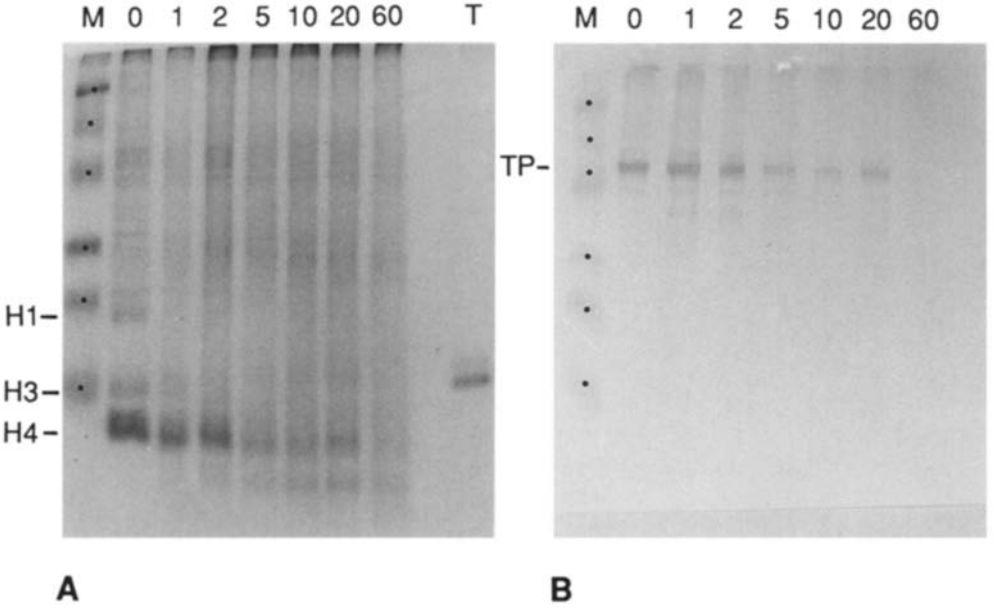

Fig. 4A, B. Trypsin digestion of isolated *Euplotes* MACs examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie blue staining (**A**) and immunoblot analysis with rabbit anti-TP-KLH (**B**). *M*, mol. wt. markers, in descending order: 106, 80, 49.5, 32.5, 27.5 and 18.5 kDa. Digestion times: 0, 1, 2, 5, 10, 20, and 60 min. Lane T, trypsin. *H1, H3, H4* and *TP* indicate the migration positions of the respective proteins

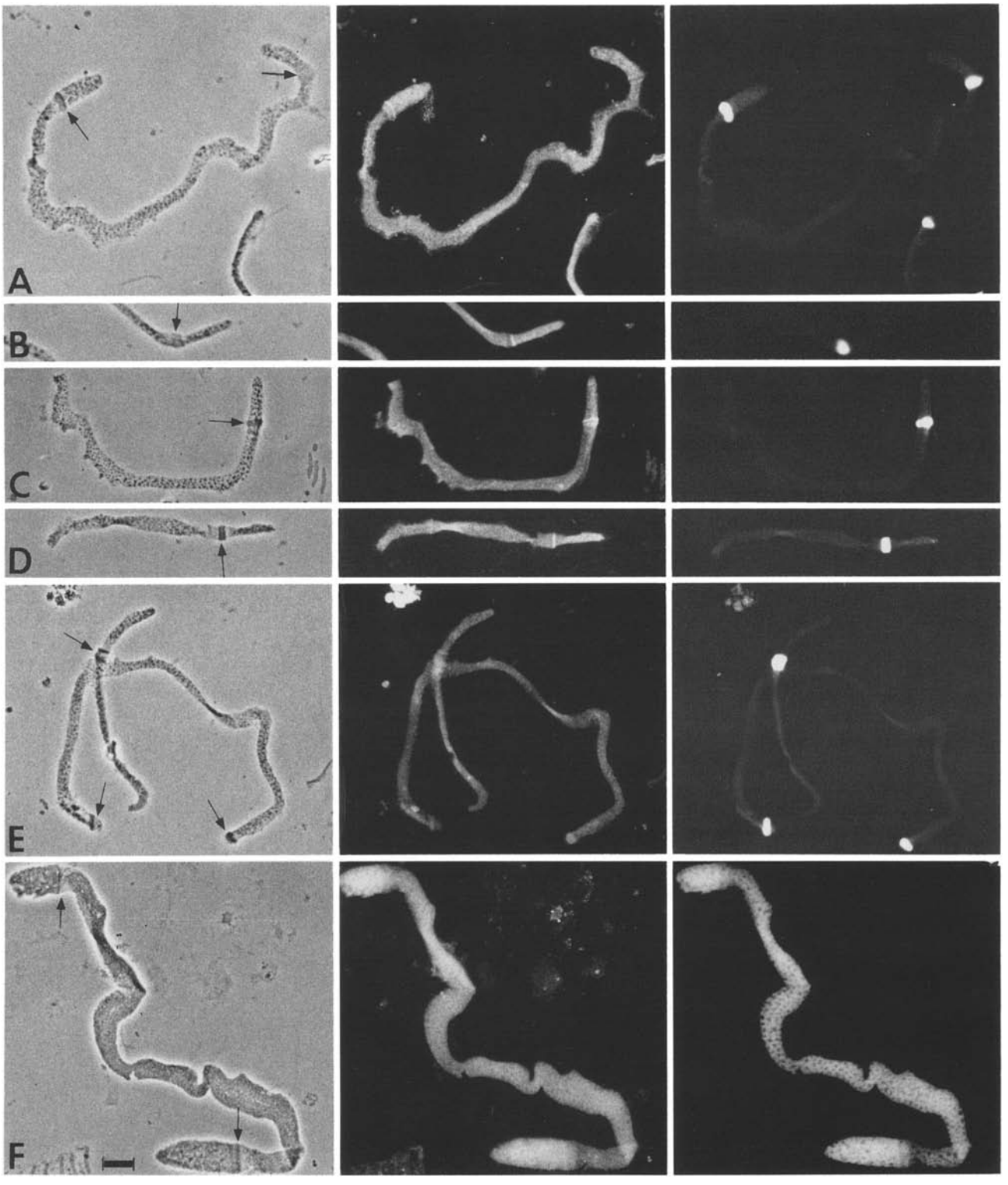

Fig. 5A–F. Immunofluorescent staining of *Euplotes* MACs, with or without extraction by lithium-3′,5′-diiodosalicylate (LIS). *Columns: left,* phase contrast images; *middle,* fluorescein isothiocyanate (FITC) images of antibody staining; *right,* 4′,6-diamidino-2-phenylindole (DAPI) fluorescence of DNA. *Rows:* **A–E** MACs extracted with LIS: **F** MAC treated with digitonin in buffer not containing LIS; **A–D** and **F** MACs immunostained with rabbit anti-TP-KLH; **E** MACs reacted with preimmune serum. *Arrows* indicate the position of replication bands (RBs). Bar represents 10 μm

or nonhistones) permits conformational changes in TP to reveal these determinants.

Trypsin digestion of isolated MACs, soluble chromatin and TP-DNA

Numerous investigators have utilized trypsin digestion of isolated nuclei and chromatin as a probe for the accessibility of histones (for a summary of results employing soluble trypsin, see Bohm and Crane-Robinson 1984; van Holde 1989). Trypsin specifically cleaves peptide bonds adjacent to K and R residues. When isolated nuclei or soluble chromatin from a variety of cell types are treated with trypsin, histones H1 (and H5) are very rapidly degraded, H3 is generally the next most sensitive and H4 is the slowest to be attacked. Cleavage of the inner histones is primarily located on the basic N- and C-terminal tails, which are regarded as more mobile and less structured. Although histone H1 is richer in K+R than the inner histones (i.e., ca. 30 mol% versus 20–25 mol% fraction K+R), the enhanced susceptibility of H1 is primarily attributable to its position external to the nucleosome core. The *Euplotes* TP is also quite rich in K+R (i.e., *E. crassus* Mr 51,000 is 17 mol% K+R, Wang et al. 1992). *E. eurystomus* MAC H1 has been cloned and sequenced in our laboratory (Hauser and Olins 1993). It contains 33 mol% K+R.

The accessibility of TP in MACs to digestion by soluble trypsin, relative to the accessibility of H1 and inner histones, was examined by comparing Coomassie-stained SDS-polyacrylamide gels with immunoblots probed with anti-TP-KLH (Fig. 4). Under the digestion conditions employed (i.e., isolated MACs in cell lysis buffer) H1 is degraded very rapidly (i.e., complete digestion within 1 min), H3 is digested almost as rapidly, and H4 is significantly reduced between 2–5 min. As estimated from the immunoblot (Fig. 4B), TP persists up to 20 min. Soluble chromatin dialyzed into 5 mM triethanolamine buffer, pH 7.0 exhibited a similar relative resistance of TP to tryptic digestion (compared with histones H1 and H3), being digested by ca. 30 min (data not shown).

Price et al. (1992) have examined the products of trypsin digestion of isolated *E. crassus* TP-DNA, rather than MACs or chromatin. They concluded that the principal sites of attack remove the C-terminal ca. Mr 15,000 region, resulting in a ca. Mr 35,000 N-terminal fragment minus the very N-terminal 5 amino acids. These authors reported that the Mr 35,000 tryptic core remained tightly bound to the telomeric nucleotide sequence. We do not observe an Mr 35,000 tryptic peptide detectable by anti-TP-KLH following digestion of MACs or chromatin, but do observe disappearance of the ca. Mr 50,000 TP band. This observation implies that, under our digestion conditions, destruction of the antigenic determinant at the N-terminus precedes degradation of the C-terminal region.

Macronuclear scaffolds reveal TP antigenic determinants

RAP1, a repressor/activator protein of yeast, which binds near chromosomal telomere repeat sequences (Conrad et al. 1990), has been reported to be enriched in yeast LIS nuclear scaffolds (Hofmann et al. 1989). We have adapted their procedure for generating nuclear scaffolds (Cardenas et al. 1990) to isolated *Euplotes* MACs, in suspension and on microscope slides, and examined the distribution of *Euplotes* TP antigenic determinants.

The most striking observation was with MACs extracted in 10 mM LIS, 0.1% digitonin for 5–10 min, while on microscope slides (Fig. 5). MACs remained largely intact and recognizable by this procedure. However, two major differences were observed compared with control slides (i.e., exposed to 0.1% digitonin without LIS): (1) DAPI staining revealed that virtually all MAC DNA was extracted, except that contained within the RB; and (2) the generally low-level immunostaining by rabbit anti-TP-KLH was specifically enhanced at leading and trailing edges of RBs. These specific effects of LIS extraction were entirely dependent upon prior "stabilization" of MACs with 1.0 mM $CuSO_4$ and heat treatment (i.e., 37° C for 10 min); neither treatment alone was effective. Clearly, these results indicate that chromatin structure and TP antigenic accessibility in the

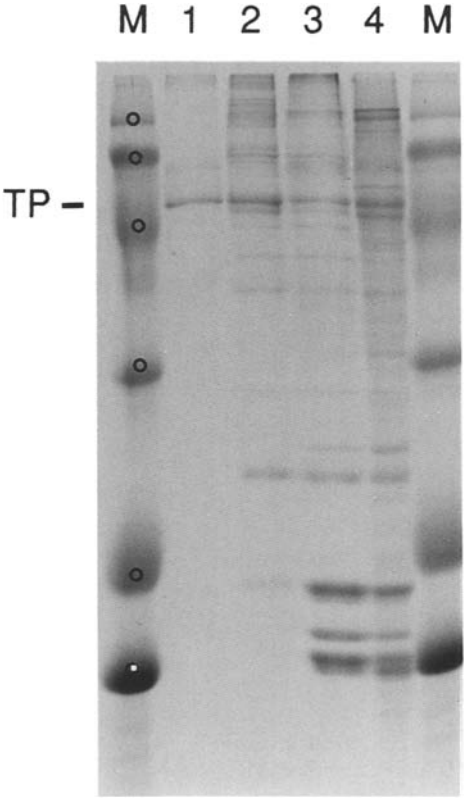

Fig. 6. SDS-PAGE analysis of LIS scaffold and extract from isolated *Euplotes* MACs. Lane *1*, TP-DNA; *2*, LIS scaffold; *3*, LIS extract; *4*, total MAC proteins. *M*, mol. wt. markers in descending order: 97.4, 68, 43, 29, 18.4 and 14.3 kDa. *TP*, telomere binding protein

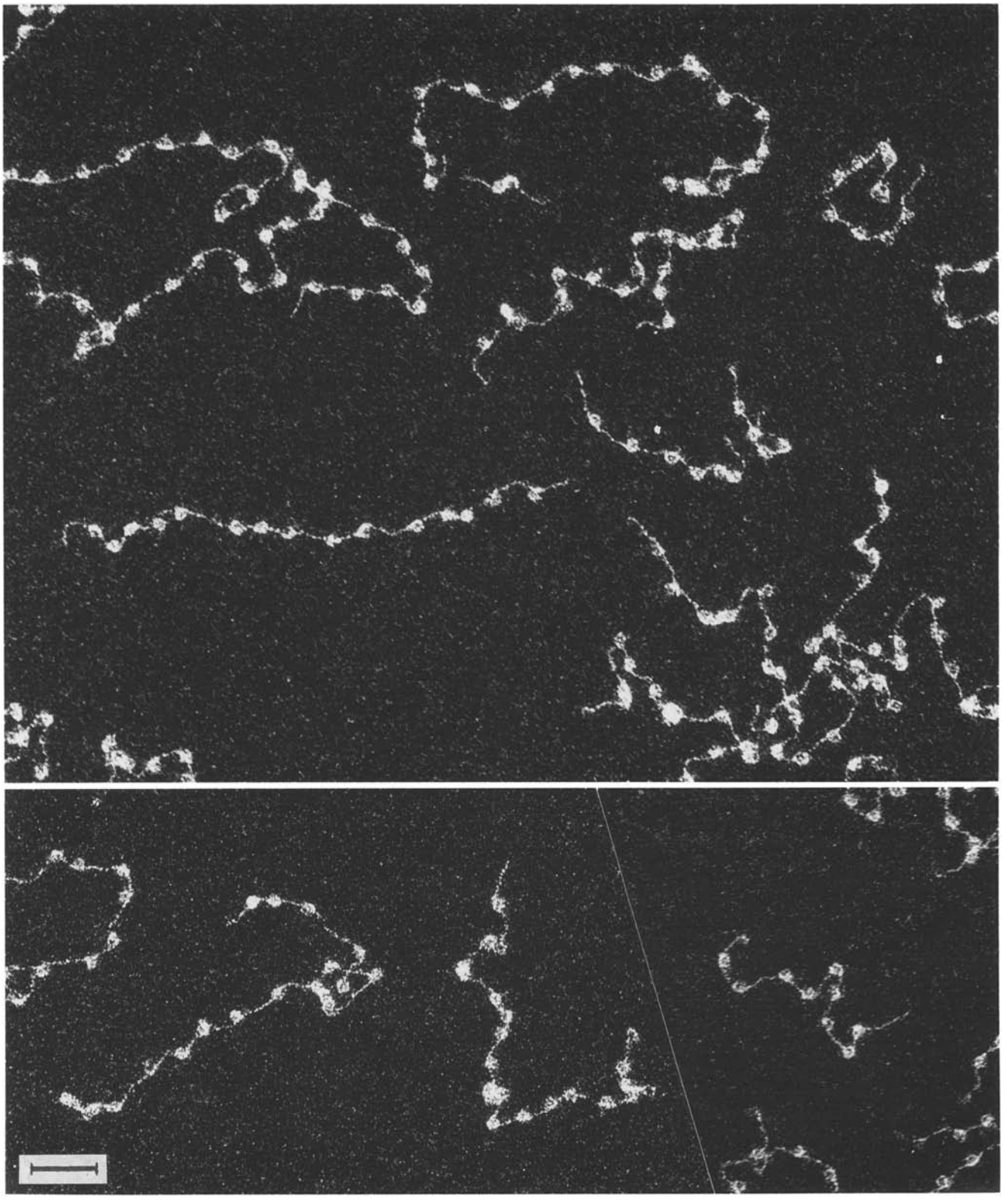

Fig. 7. *Euplotes eurystomus* soluble chromatin spread on a thin carbon film, stained with 0.1% aqueous uranyl acetate and observed in dark field. The stain bound to DNA appears white. Nucleosomes are spaced along individual chromatin molecules; the telomere structures vary, ranging from nucleosome-like to straight. Bar represents 50 nm

RB region differ from their condition in bulk chromatin. LIS extraction of typical nuclei and chromosomes does not usually remove most DNA; the short length of hypotrich MAC DNA may predispose it to loss during the detergent treatment. In an effort to explore whether TP was specifically enriched in LIS preparations of isolated *Euplotes* MACs, biochemical quantities (i.e., microgram amounts) of LIS extracts and LIS scaffolds were prepared and examined by SDS-PAGE (Fig. 6). Electrophoretic analyses of the extracts and scaffolds from numerous experiments yielded results that were as follows: (1) extraction of histones from the scaffolds met with varying success, ranging from complete removal to minimal removal; and (2) TP appeared in both LIS extracts (possibly associated with extracted MAC DNA) and in scaffolds. This was confirmed with immunoblotting experiments, employing anti-TP-KLH (data not shown). It is difficult to make an exact comparison between the microscopic and biochemical experiments. RB chromatin constitutes only a small percentage of total MAC chromatin. Microscopic studies permitted us to focus on changes in the RB structure, whereas, biochemical preparations were weighted toward changes in bulk chromatin. Since it is possible to prepare enriched preparations of RBs (Allen et al. 1985), LIS extractions can be conducted on biochemical preparations more comparable to the microscopy experiments.

An alternative method for preparing nuclear matrices is the extraction of isolated nuclei with high NaCl (for reviews and discussions of different techniques, see Berezney 1991; Jack and Eggert 1992; Roberge and Gasser 1992). Such experiments are not strictly comparable when performed with isolated MACs from *Euplotes*. Treatment of MACs with high NaCl (e.g., 2 M) dissociates the nucleus and, in fact, constitutes the first stage in isolation of TP-DNA complexes (Gottschling and Zakian 1986; Price and Cech 1987). Under the microscope, extraction with 2 M NaCl results in extensive disruption of isolated MACs. At the biochemical scale, a small amount of high salt-insoluble residue can be obtained following treatment of MACs with 2 M NaCl, TE. Although not a procedure for preparing nuclear matrices, isolated MACs similarly dissolve quickly and almost completely when treated with very low ionic strength buffers (e.g., 5 mM triethanolamine plus 10 mM EDTA, pH 7.0). After centrifugation, a low salt-insoluble residue can be separated from the solubilized MAC chromatin. All of these fractions were prepared from isolated *Euplotes* MACs and compared by SDS-PAGE with Coomassie blue and anti-TP-KLH staining (data not shown). Despite considerable differences among the various samples, assayed by Coomassie blue staining, TP antigenic determinants were detected in all four fractions (i.e., high salt-soluble, high salt-insoluble, low salt-soluble and low salt-insoluble) and in total MACs. The TP antigenic determinants were clearly enriched in the high salt-soluble fraction (i.e., first stage in preparation of TP-DNA). Exact quantitative estimates of TP distribution can be performed, but the yields of MACs and the various chromatin fractions would make such experiments a technical tour-de-force that would not extend our understanding of telomere structure.

The scaffold (matrix) experiments generate two principal conclusions relating TP to MAC structure: (1) most TP does not remain bound to the generated scaffold; and (2) the presence and persistence of TP with RB scaffolds is documented. Clearly, the conditions and characteristics of such MAC residual structures require more detailed exploration.

Electron microscopic observations of soluble chromatin telomeres

In *Euplotes*, where the DNA is naturally cut into gene-size fragments, chromatin is solubilized in low salt without the use of nucleases. Combining the methodologies of Miller and Beatty (1969) and Thoma and Koller (1977) we have spread chromatin on carbon film and have analyzed the structure of the ends of the chromatin molecules (Fig. 7). The stain we have used, 0.1% aqueous uranyl acetate behaves as a positive stain for the DNA, even though it is applied as a negative stain (i.e., not washed off). We observe that the proteins are not contrasted in these preparations, but the path of the DNA strand is obvious and nucleosomes are easily recognized by the circular path of DNA. The structure of the telomeres is more difficult to discern: 62% of the ends appear as straight or undulating DNA, having a mean length = 33.2 nm (SD = 10.1; n = 109) or = 98 bp (SD = 30); 38% of the ends are folded and their length cannot be measured. Biochemical data (i.e., Bal31 digestion, Gottschling and Zakian 1986; Hauser et al. 1991), nucleosome positioning studies (Roberson et al. 1989; Hauser et al. 1991; Gottschling and Cech 1984; Budarf and Blackburn 1986) and partial melting studies analyzed in the electron microscope (unpublished observations) clearly demonstrate that the chromatin ends are not naked. Figure 7 demonstrates the variation observed in a typical low ionic strength chromatin spread preparation. Note that some ends look nucleosomal, some have more open bends, and some are almost straight.

Discussion

In most cases studied so far (e.g., Budarf and Blackburn 1986; Gottschling and Cech 1984; Wright et al. 1992; Roberson et al. 1989; Hauser et al. 1991), the telomeric nucleotide sequences are non-nucleosomal, and associated with non-histone proteins called TPs. In the present study we have examined the structural stability of the TP in *E. eurystomus* and its accessibility to antibodies and to trypsin digestion. The TP of *E. eurystomus* resembles that in other hypotrichs and can be isolated by the same procedures developed by Gottschling and Cech (1984) and by Price (1990). In addition, we describe conditions, using HAP, that permitted separation of TP from DNA in the tenacious TP-DNA complex.

Amino acid sequence determination of the isolated TP identified 14 amino acids at the amino-terminal end.

A corresponding synthetic 14-mer was synthesized and covalently bound to KLH, a carrier immunogen, to produce polyclonal rabbit antibodies that were capable of specific TP recognition. We observed a strong reaction of the antibodies by immunoblotting with TP in both total MAC proteins and TP-DNA complexes.

These antibodies have been useful probes for the accessibility of the amino-terminal antigenic determinants of TP in situ and in solution. In contrast to the strong reactions on immunoblots from SDS-PAGE, where TP is denatured and separated from DNA, attempts to localize TP within intact MACs by immunofluorescence were less rewarding. A generalized localization over the entire MAC was seen; specific fluorescence was marginally greater than that observed with pre-immune serum, employed under the same conditions. The dot immunoblots showed that soluble chromatin at low ionic strength does not react with the TP-KLH antibodies, establishing that a higher-order structure, such as a 20–30 nm fiber, is definitely not responsible for the TP protection. The dot immunoblot also established that treatments that remove H1 expose the TP determinants. However, cautious consideration of the data suggests two other explanations: (1) that other factors beside H1 are removed by the mild extraction procedure and are responsible for TP protection; or (2) that the extraction procedure alters TP conformation directly.

Attempts to maintain nuclear structure and reveal the TP determinants led us to investigate LIS-extracted scaffolds. This extraction method removes most of the chromatin from *Euplotes* MACs, probably due to the small size of the DNA, whereas in other eukaryotes, most of the DNA stripped of histones remains attached to a scaffold. In yeast, LIS scaffolds retain the TP RAP1 (Hofmann et al. 1989). In *Euplotes,* the only place where DNA remains in the scaffold is the RB; TP staining can be detected weakly throughout the scaffold, with highest concentrations around the RB. The biochemistry of RB chromatin is dramatically different from the rest of the MAC, including the presence of high concentrations of protein sulfhydryl groups in RBs (Olins et al. 1988). It is possible that these sulfhydryl groups may be oxidized by the $CuSO_4$ and heat treatment, prior to the LIS extraction, forming the basis for TP and DNA retention. It is also possible that TP plays a special role during chromatin replication, which leads to a strong binding to the replication machinery.

Under the conditions employed, trypsin digestion of isolated *Euplotes* MACs and of soluble MAC chromatin revealed that the entire TP is protected, persisting for about 20 min, even though H1 and H3 were digested by approximately 1 min of digestion. It is important to caution that despite the rapid H1 digestion, fragments of H1 may remain bound to and protective of TP. Since we do not observe any change in TP size during trypsin digestion, it appears that not only is the amino-terminus protected, but that the entire TP molecule is relatively resistant to trypsin in intact chromatin.

To date, the proteins that bind to the single-strand 3′ overhang of telomeres have only been identified in *O. nova, S. mytilis* and *E. crassus*. The *Oxytricha* and *Stylonychia* heterodimer proteins are most similar, exhibiting 80% homology of both α- and β-subunits (Fang and Cech 1991). The *E. crassus* protein has a single Mr 51,000 subunit, having only 36% amino acid sequence identity with the *Oxytricha* α-subunit Mr 56,000 peptide. In a recent paper, Fang et al. (1993) demonstrated that telomere-binding specificity resides in the amino-terminal two-thirds of the α-subunit; the α/β-subunit interaction occurs in the carboxy-terminal third of the α-subunit and the amino-terminal two-thirds of the β-subunit. In addition, the carboxy-terminus of the β-subunit resembles histone H1, but its function is unknown. It is interesting that although the DNA-binding specificity resides in the α-subunit, the binding strength is enhanced more than 6,000-fold when a ternary complex is formed by α-β-DNA interaction. In *E. crassus*, a single subunit forms a tenacious complex with the telomere DNA. Specificity resides in the amino-terminal Mr 35,000 fragment, and the function of the carboxy-terminal Mr 15,000 fragment is not known. We propose that homology between the *E. crassus* and *E. eurystomus* proteins will be found in the amino-terminal region, as already shown for the first 14 amino acids in Table 1. However, the absence of hybridization on a Southern blot in which probes from *E. crassus* TP or its homolog are tested against *E. eurystomus* MAC DNA, indicates that the TPs can tolerate much greater sequence variation than the highly conserved telomere DNA sequences of the hypotrichous ciliates.

Price et al. (1992) have noted that lysine-5 at the amino-terminal end of the *E. crassus* protein is cleaved by trypsin. We do not have information addressing the question of whether or not the homologous lysine-4 of *E. eurystomus* TP is cleaved by trypsin. However our data, in which trypsin digestion conditions are somewhat different, indicate that sufficient sequence of the 14 amino acid antigenic determinants remains after trypsin digestion to react with anti-TP-KLH.

Among the multiplicity of functions attributed to telomeres (Zakian 1989; Blackburn 1991; Price 1992) only a few will be mentioned in this discussion. It is likely that, since the hypotrich gene-size DNA fragments and short telomeres are more simple than in classical chromosomes, some of the telomere functions may be absent or reduced in scope in hypotrich MACs. The telomeres of larger chromosomes influence the time of replication of adjacent genes (McCarroll and Fangman 1988; Ferguson and Fangman 1992) and the transcription of genes (Gottschling et al. 1990; Aparicio et al. 1991; Gottschling 1992). The telomere position effects in yeast are primarily inhibitory and can extend for considerable distances (e.g., greater than 20 kb from the telomere; see Ferguson and Fangman 1992). Electron microscopic studies of presumptive replicating DNA molecules from *Euplotes* MACs (Murti and Prescott 1983; Allen et al. 1985) indicate that replication origins are near to the ends of the short gene-size DNA molecules (molecular lengths range from 0.5 to ca. 20 kb, average ca. 2 kb). Regulation of transcription is not well understood in hypotrich MAC genes. However, in those genes that have been sequenced, the initiation sites and presumptive

control regions are close to the telomeres (e.g., Hauser et al. 1991).

Acknowledgements. The authors thank P. Mazur and E. Michaud for critical reading of the manuscript, and J. Harp for the electron micrograph. This material is based on work supported by the National Science Foundation under grant no. MCB 8915715 to A.L.O., no. MCB 9117123 to D.E.O., and by the Department of Energy under contract DE-AC05-84OR21400 with Martin Marietta Energy systems, to D.E.O.

References

Allen RL, Olins DE (1984) Cytochemistry of the replication band in hypotrichous ciliated protozoa staining with silver and thiol-specific coumarin maleimide. Chromosoma 91:82–86

Allen RL, Olins AL, Harp JM, Olins DE (1985) Isolation and characterization of chromatin replication bands and macronuclei from *Euplotes eurystomus*. Eur J Cell Biol 39:217–223

Aparicio OM, Billington BL, Gottschling DE (1991) Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. Cell 66:1279–1287

Berezney R (1991) The nuclear matrix: a heuristic model for investigating genomic organization and function in the cell nucleus. J Cell Biochem 47:109–123

Berman J, Tachibana CY, Tye B-K (1986) Identification of a telomere-binding activity from yeast. Proc Natl Acad Sci USA 83:3713–3717

Blackburn EH (1991) Structure and function of telomeres. Nature 350:569–573

Blackburn EH (1992) Telomerases. Annu Rev Biochem 61:113–129

Bohm L, Crane-Robinson C (1984) Proteases as structural probes for chromatin: The domain structure of histones. Biosci Rep 4:365–386

Budarf ML, Blackburn EH (1986) Chromatin structure of the telomeric region and 3′-nontranscribed spacer of *Tetrahymena* ribosomal RNA genes. J Biol Chem 261:363–369

Cadilla CL, Harp J, Flanagan JM, Olins AL, Olins DE (1986) Preparation and characterization of soluble macronuclear chromatin from the hypotrich *Euplotes eurystomus*. Nucleic Acids Res 14:823–841

Cardenas ME, Laroche T, Gasser SM (1990) The composition and morphology of yeast nuclear scaffolds. J Cell Sci 96:439–450

Conrad MN, Wright JH, Wolf AJ, Zakian VA (1990) RAP1 protein interacts with yeast telomeres in vivo: overproduction alters telomere structure and decreases chromosome stability. Cell 63:739–750

Coren JS, Vogt VM (1992) Purification of a telomere-binding protein from *Physarum polycephalum*. Biochim Biophys Acta 1171:162–166

Coren JS, Epstein EM, Vogt VM (1991) Characterization of a telomere-binding protein from *Physarum polycephalum*. Mol Cell Biol 11:2282–2290

Fang G, Cech TR (1991) Molecular cloning of telomere-binding protein genes from *Stylonychia mytilis*. Nucleic Acids Res 19:5515–5518

Fang G, Gray JT, Cech TR (1993) *Oxytricha* telomere-binding protein: separable DNA-binding and dimerization domains of the α-subunit. Genes Dev 7:870–882

Ferguson BM, Fangman WL (1992) A position effect on the time of replication origin activation in yeast. Cell 68:333–339

Gottschling DE (1992) Telomere-proximal DNA in *Saccharomyces cerevisiae* is refractory to methyltransferase activity in vivo. Proc Natl Acad Sci USA 89:4062–4065

Gottschling DE, Cech TR (1984) Chromatin structure of the molecular ends of *Oxytricha* macronuclear DNA: phased nucleosomes and a telomeric complex. Cell 38:501–510

Gottschling DE, Zakian VA (1986) Telomere proteins: Specific recognition and protection of the natural termini of *Oxytricha* macronuclear DNA. Cell 47:195–205

Gottschling DE, Aparicio OM, Billington BL, Zakian VA (1990) Position effect at *S. cerevisiae* telomeres: reversible repression of pol II transcription. Cell 63:751–762

Gray JT, Celander DW, Price CM, Cech TR (1991) Cloning and expression of genes for the *Oxytricha* telomere-binding protein: specific subunit interactions in the telomeric complex. Cell 67:807–814

Hauser LJ, Olins DE (1993) Cloning and analysis of the macronuclear gene for histone H1 from *Euplotes eurystomus*. Nucleic Acids Res 15:3586

Hauser LJ, Roberson AE, Olins DE (1991) Structure of the macronuclear polyubiquitin gene in *Euplotes*. Chromosoma 100:386–394

Henderson ER, Blackburn EH (1989) An overhanging 3′ terminus is a conserved feature of telomeres. Mol Cell Biol 9:345–348

Herrmann AL, Cadilla CL, Cacheiro LH, Carne AF, Olins DE (1987) An H1-like protein from the macronucleus of *Euplotes eurystomus*. Eur J Cell Biol 43:155–162

Hicke BJ, Celander DW, MacDonald GH, Price CM, Cech TR (1990) Two versions of the gene encoding the 41-kilodalton subunit of the telomere binding protein of *Oxytricha nova*. Proc Natl Acad Sci USA 87:1481–1485

Hofmann JF-X, Laroche T, Brand AH, Gasser SM (1989) RAP-1 factor is necessary for DNA loop formation in vitro at the silent mating type locus HML. Cell 57:725–737

Jack RS, Eggert H (1992) The elusive nuclear matrix. Eur J Biochem 209:503–509

Klobutcher LA, Swanton MT, Donini P, Prescott DM (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

Lin R, Leone JW, Cook RG, Allis CD (1989) Antibodies specific to acetylated histones document the existence of deposition- and transcription-related histone acetylation in *Tetrahymena*. J Cell Biol 108:1577–1588

Liu Z, Tye B-K (1991) A yeast protein that binds to vertebrate telomeres and conserved yeast telomeric junctions. Genes Dev 5:49–59

Longtine MS, Wilson NM, Petracek ME, Berman J (1989) A yeast telomere binding activity binds to two related telomere sequence motifs and is indistinguishable from RAP1. Curr Genet 16:225–239

Lustig AJ, Kurtz S, Shore D (1990) Involvement of the silencer and UAS binding protein RAP1 in regulation of telomere length. Science 250:549–554

McCarroll RM, Fangman WL (1988) Time of replication of yeast centromeres and telomeres. Cell 54:505–513

Miller OL, Beatty BR (1969) Visualization of nucleolar genes. Science 164:955–957

Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu J-R (1988) A highly conserved repetitive DNA sequence, $(TTAGGG)_n$, present at the telomeres of human chromosomes. Proc Natl Acad Sci USA 85:6622–6626

Murti KG, Prescott DM (1983) Replication forms of the gene-sized DNA molecules of hypotrichous ciliates. Mol Cell Biol 3:1562–1566

Olins AL, Olins DE, Derenzini M, Hernandez-Verdun D, Gounon P, Robert-Nicoud M, Jovin TM (1988) Replication bands and nucleoli in the macronucleus of *Euplotes eurystomus:* an ultrastructural and cytochemical study. Biol Cell 62:83–93

Olins DE, Olins AL (1993) Inhibition of DNA synthesis in the macronuclear replication band of *Euplotes eurystomus*. J Eukaryotic Microbiol 40:459–466

Olins DE, Olins AL, Cacheiro LH, Tan EM (1989) PCNA/cyclin in the ciliate *Euplotes eurystomus:* localization in the replication band and in micronuclei. J Cell Biol 109:1399–1410

Olins DE, Olins AL, Herrmann A, Lin R, Allis CD, Robert-Nicoud M (1991) Localization of acetylated histone H4 in the macronucleus of *Euplotes*. Chromosoma 100:377–385

Prescott DM (1992) Cutting, splicing, reordering, and elimination of DNA sequences in hypotrichous ciliates. BioEssays 14:317–324

Price CM (1990) Telomere structure in *Euplotes crassus:* characterization of DNA-protein interactions and isolation of a telomere-binding protein. Mol Cell Biol 10:3421–3431

Price CM (1992) Centromeres and telomeres. Curr Opin Cell Biol 4:379–384

Price CM, Cech TR (1987) Telomeric DNA-protein interactions of *Oxytricha* macronuclear DNA. Genes Dev 1:783–793

Price CM, Cech TR (1989) Properties of the telomeric DNA-binding protein from *Oxytricha nova*. Biochemistry 28:769–774

Price CM, Skopp R, Krueger J, Williams D (1992) DNA recognition and binding by the *Euplotes* telomere protein. Biochemistry 31:10835–10843

Roberge M, Gasser SM (1992) DNA loops: structural and functional properties of scaffold-attached regions. Mol Microbiol 6:419–423

Roberson AE, Wolffe AP, Hauser LJ, Olins DE (1989) The 5S RNA gene minichromosome of *Euplotes*. Nucleic Acids Res 17:4699–4712

Steinhilber W, Lipps HJ (1986) A telomeric binding protein in the macronuclei of the hypotrichous ciliate *Stylonychia lemnae*. FEBS Lett 206:25–28

Thoma F, Koller Th (1977) Influence of histone H1 on chromatin structure. Cell 12:101–107

van Holde KE (1989) Chromatin. Springer, Berlin Heidelberg New York

Wang W, Skopp R, Scofield M, Price C (1992) *Euplotes crassus* has genes encoding telomere-binding proteins and telomere-binding protein homologs. Nucleic Acids Res 20:6621–6629

Wright JH, Gottschling DE, Zakian VA (1992) *Saccharomyces* telomeres assume a non-nucleosomal chromatin structure. Genes Dev 6:197–210

Zahler AM, Williamson JR, Cech TR, Prescott DM (1991) Inhibition of telomerase by G-quartet DNA structures. Nature 350:718–720

Zakian VA (1989) Structure and function of telomeres. Annu Rev Genet 23:579–604