Preparation and characterization of soluble macronuclear chromatin from the hypotrich *Euplotes eurystomus**

Carmen L.Cadilla, Joel Harp, John M.Flanagan, Ada L.Olins and Donald E.Olins

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

Received 11 September 1985; Revised and Accepted 18 December 1985

ABSTRACT

Euplotes eurystomus is a hypotrichous ciliate containing a transcriptionally active macronucleus (MAC) and a transcriptionally inactive micronucleus. Soluble MAC chromatin contains a normal complement of inner histones, an H1-like protein which is very sensitive to proteolysis, and a considerable proportion of non-histone proteins. A combination of N-Tosyl lysine chloromethyl ketone (TLCK) and PMSF was found to be most effective in preventing proteolysis. Micrococcal nuclease digestion yielded an average nucleosome repeat length of 187 ± 25 bp for soluble chromatin; and 190 ± 9 bp for isolated macronuclei. Thermal denaturation profiles of MAC chromatin in 0.25 mM EDTA display two main transitions at about 76 and 83°C, resembling the melting of soluble chicken erythrocyte chromatin. Circular dichroic spectra of MAC chromatin were compared to soluble chicken erythrocyte chromatin under the same ionic strength conditions and were found to be very similar. 2D chromatin/DNA agarose gel electrophoresis resulted in a diagonal line of DNA staining, which establishes a strict correlation between DNA and chromatin electrophoretic mobility.

INTRODUCTION

The vegetative cells of hypotrichous ciliated protozoa possess two types of nuclei: a transcriptionally active macronucleus containing gene-sized DNA molecules and a transcriptionally inactive micronucleus composed of chromosome-size DNA. The macronucleus is derived from micronuclear fusion products following conjugation, in a process involving a complex series of events which include the polytenization of micronuclear chromosomes, transectioning of polytene chromosomes at interbands and the subsequent elimination of most (93%) of the nucleotide complexity. The result of this DNA processing is a drastic decrease in the size and in the sequence complexity in the macronucleus. Macronuclear DNA is thus a modified subset of DNA sequences containing 3-7% of the micronuclear sequences. Each macronuclear DNA fragment is a small yet functional genetic unit that must contain sequence information required for its own transcription, for regulation of its own expression and for its own replication (1). Macronuclear DNA can be separated on agarose

gels yielding a typical and reproducible banding pattern with specific genes present in only one or a few bands (1). The chromatin fragments are composed of nucleosomes (2) and, in the case of *Oxytricha nova*, migrate electrophoretically in proportion to their DNA length (3). Thus, the hypotrichs offer an ideal system to study the structure and composition of a eukaryotic gene as chromatin fragments, removed from the complexity present in eukaryotic genomes.

Thermal denaturation and circular dichroism (CD) have been used extensively to characterize chromatin and nucleosomes from other organisms, and recently to study the chromatin from both macronuclei and micronuclei of the hypotrich *Oxytricha* (4,5). These two methods are very sensitive to changes in DNA-protein and protein-protein interactions within chromatin. Recently, these techniques were used to study the effect of binding enzymatically phosphorylated H1 histone on the structure of reconstituted chromatin (6) and also the effect of binding a high mobility group protein (HMG 17) on the stability and conformation of acetylated and control HeLa high molecular weight core chromatin (7). These studies have often been performed at very low ionic strength conditions (0.25 mM EDTA) due to the solubility properties of chromatin.

Whole chicken erythrocyte chromatin melted in 0.25 mM EDTA, pH 7.0, exhibits three transitions (Iw, IIw and IIIw) (5). The 52 base pairs per repeating unit which melted in transition Iw (62°C) at low ionic strength were incorporated into the structural domains represented by transitions IIw and IIIw at higher ionic strength. The 30% of the total which melted in transition IIw at 4.0 mM Na phosphate, 0.25 mM EDTA, pH 7.0 was considered to represent the linker region between core particles, and the remaining 70% which melted in transition IIIw was considered to represent the 140 base pairs of DNA in the core particle (5). An additional small component (transition IIIa) identified in native chicken chromatin at 1 mM Na phosphate, 0.2 mM EDTA, pH 7.4, has been attributed to the linker and/or core DNA stabilized by H1 (6). This small component disappeared when the chromatin was stripped of H1 (6). The optical activity ($[\theta]_{283} = 4000 \pm 300 \text{ deg cm}^2/\text{dmol nucleotide}$) of the DNA in whole chromatin was unaltered by changes in ionic strength from 0-5.0 mM NaPO_4 at 23°C (5).

High molecular weight chromatin devoid of non-histone proteins and the lysine-rich histones (core chromatin) has three melting transitions (Ic, IIc, IIIc), in 0.25 mM EDTA, pH 7.0 (5), with 50% of the total hyperchromicity displayed in transition Ic (T_m 51°C). The remaining 50% of the

DNA melts in transitions IIc and IIIc. Transition Ic, which is thought to represent the melting of linker DNA and some DNA which resides in the core particle (8), varied greatly with ionic strength in the range of 0-5.0 mM NaPO_4 , pH 7.0, and behaved similarly to free DNA (5). The circular dichroism of core chromatin displayed decreasing ellipticities at 283 nm with increasing ionic strengths. These results demonstrated a conformational transition over this range in ionic strength, suggesting that at least part of the DNA had characteristics similar to that of free DNA.

Oxytricha MAC chromatin displays four melting transitions in 0.25 mM EDTA, pH 7.0 and three transitions at higher ionic strength (4). The lowest melting transition exhibits a large ionic dependence. The optical activity of the DNA in this chromatin also displays a substantial ionic strength dependence. These results are consistent with an apparent lack of an H1 protein in this organism's macronucleus.

In the present work, we studied the chromatin of the related protozoan Euplotes eurystomus to compare the MAC chromatin biophysical properties and the protein and DNA composition with Oxytricha and chicken erythrocyte chromatin. There are three main advantages to using Euplotes vs Oxytricha: 1) the Euplotes strain we employed does not senesce; 2) it is commercially available, 3) it can be synchronized by starvation. The use of protease inhibitors other than phenyl methyl sulfonyl fluoride (PMSF), and NaHSO_3 was employed, which enabled us to prevent degradation of an H1-like protein. The properties of Euplotes MAC chromatin are closer to those of whole chicken erythrocyte chromatin than to core erythrocyte chromatin or Oxytricha MAC chromatin.

MATERIALS AND METHODS

The Euplotes eurystomus cell line was obtained from Carolina Biological. Cell stocks were maintained in 100 mm dishes in Carolina Springwater containing 6-7 previously boiled wheat seeds. Large-scale cultures of Euplotes were grown in large photographic trays containing 5 L of Pringsheim salt solution (0.02 g/l Na_2HPO_4 , 0.02 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g/l $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 0.026 g/l KCl, pH 7.2) and were fed live algae, Chlorogonium elongatum. The algae stocks were obtained from Dr. David Prescott, (University of Colorado, Boulder) and were maintained axenically in a yeast extract-sodium acetate medium (2.67 g/l yeast extract and 1.33 g/l sodium acetate, anhydrous). Algae to be used for feeding the Euplotes were pelleted and resuspended in Pringsheim medium.

The Euplotes cells were harvested by filtering through 6 μ m nylon mesh and lysed in a nuclear isolation buffer containing 10 mM Pipes, pH 6.75, 1 mM spermidine phosphate, 5 mM $MgCl_2$, 1 mM PMSF, 1 mM TLCK and 0.5% NP-40. The nuclei were centrifuged and resuspended in the nuclear isolation buffer and further purified by buoyant density centrifugation in a linear 10-60% metrizamide gradient in nuclear isolation buffer for 1 hr at 4°C and 25,000 rpm in a SW41 rotor. The fractions containing nuclei were diluted sevenfold with nuclear isolation buffer and washed three times with the same buffer. Then the nuclei were pelleted and lysed by the addition of 10 mM EDTA, 5 mM TEA.HCl pH 7.0, 1 mM PMSF and 1 mM TLCK for 1 hr in ice. Lysis was completed by pipetting, and insoluble material was removed from the soluble chromatin by centrifugation at 3000 X g in a Sorvall centrifuge for 30 min at 4°C. The soluble chromatin was extensively dialyzed against 0.25 mM EDTA, 5 mM TEA.HCl pH 7.0, 0.1 mM PMSF, 0.1 mM TLCK.

Micrococcal nuclease digestions of nuclei were performed in 10 mM Tris, 1 mM $CaCl_2$ and 30 units/ml of micrococcal nuclease (Boehringer) at 37°C. When soluble chromatin was digested it was dialyzed overnight against 10 mM Tris, 0.1 mM EDTA, 0.1 mM PMSF, 0.1 mM TLCK prior to the digestion. Subsequently 1 mM $CaCl_2$ and 30 units/ml of micrococcal nuclease were added at 37°C. At the desired time points, the reaction was terminated by adding EDTA to a final concentration of 2 mM, and by adding 200 μ g/ml proteinase K and incubating at 37°C, 1 hr.

Chicken erythrocyte soluble chromatin was prepared according to Olins et al. (9) and loaded in linear 10-60% sucrose gradients in 20 mM NaCl, 10 mM Tris.HCl, pH 7.0, 0.2 mM EDTA pH 7.0, 0.1 mM PMSF and centrifuged for 3 hrs, 5°C, 25,000 rpm in a SW41 rotor. Fractions that were similar in DNA size to Euplotes macromolecular DNA were pooled and used in the physical studies.

Perchloric Acid Extraction of Proteins

Soluble chromatin was made 5% in perchloric acid (PCA) by addition of an equal volume of 10% PCA. Sample was kept for 20-30 min at 4°C while shaking. Insoluble material was removed by centrifugation in a microfuge for 5 min. The supernatant was made 20% in trichloroacetic acid (TCA) and shaken at 4°C overnight. Proteins soluble in 5% PCA were collected by centrifugation at 4°C in a microfuge for 3 min. The pellet was washed once with 4% HCl in acetone (v/v) and twice with ice-cold acetone. The sample was then vacuum-dried.

Protease Inhibitors

The chromatin isolation was performed as described above but in addition

to using PMSF and TLCK together, a comparison was made of the protein electrophoresis pattern when TPCK, leupeptin or soybean trypsin inhibitor were used together with PMSF, and when PMSF was used alone. TPCK and PMSF were used at the same molar concentrations of inhibitors throughout the chromatin isolation procedure described above. Leupeptin was tested at a concentration of 0.1 mM and soybean trypsin inhibitor at 5 μ g/mL throughout the chromatin isolation procedure.

DNA and Protein Gel Electrophoresis

Electrophoresis of proteins was performed according to Laemmli (10) in 15% SDS-polyacrylamide gels using "minislab" gels (11) (75 $\times$ 100 $\times$ 0.5 mm), and the modified electrode buffer of Thomas and Kornberg (12). Electrophoresis of DNA fragments in 1.25% agarose in 40 mM Tris (pH 7.9), 5 mM sodium acetate, and 1 mM EDTA was done for 26 hr at 70 volts, using water cooling and buffer exchange.

Two-Dimensional Gel Electrophoresis

The two-dimensional chromatin/DNA gels were adapted from those described by Levinger et al. (13). In the chromatin first dimension, samples were prepared by the addition of one-fifth volume of 135 mM Tris-borate, 3.75 mM EDTA, pH 8.3, 50% glycerol and 0.1% bromophenol blue. The electrophoresis gel consisted of 1.0% agarose containing 3.0 mM EDTA and 2 mM Na acetate (pH 5.5). A 3.0 mm wide strip was excised from the central region of the chromatin-containing lane and soaked for 2 hrs in 1.0% sarkosyl and 0.025% bromophenol blue. The second dimension gel consisted of 1.2% agarose containing 40 mM Tris-HCl (pH 7.9), 5 mM Na acetate, 1 mM EDTA and 1.0% sarkosyl. The chromatin strip was inserted in a slot in the DNA gel and overlayed with 0.3% agarose in DNA electrophoresis buffer. Both dimensions were run 16 hr at 70 volts using water cooling and buffer exchange.

Electron Microscopy Studies

Electron micrographs of Euplotes chromatin were obtained by fixing soluble chromatin in 0.1% glutaraldehyde, 0.2 mM EDTA, 0.5 mM TEA.HCl pH 7.0 for 15 hr at 4°C, then zephiran chloride was added to a final concentration of 0.0002% (14). The sample was then spread onto unglowed carbon film coated grids for 30-60 seconds, rinsed in distilled water, dried using dilute photo-flo, and stained with 0.1% aqueous uranyl acetate (15). Micrographs were taken using a Siemens Elmiskop 102 operated in the darkfield mode (16). The magnification was calibrated using a carbon grating replica with 2160 lines/mm (E. F. Fullam Co.). Chromatin molecules were measured from negatives projected onto a Tektronix 4954 graphics tablet, enlarged 11.5-fold.

Physical Studies

Chromatin samples were prepared for thermal denaturation and circular dichroism by exhaustive dialysis against 0.25 mM Na₂ EDTA, pH 7.0, containing 0.1 mM PMSF and sodium phosphate (as indicated) to achieve the desired ionic strength. Experiments were conducted on samples with an absorbance of 260 nm of 0.9-1.2. Thermal denaturation experiments were performed with a Gilford 2000 spectrophotometer equipped with a digital absorbance meter and interfaced to a PDG 11/20 computer linked to the data acquisition system. The differential melting curves were fit to a sum of gaussians, of the form:

$$\frac{d A_{260}}{d T} = \sum_{n=1}^i A_n e^{-B_n(T_{m,n}-T)}$$

where i is the number of transitions, A_n , $T_{m,n}$ the height and melting temperature of the n th transition, and B_n is inversely proportional to the width of the transition, the values of these constants were determined using LMDER1, the non-linear equation solver of the MINPACK library. This FORTRAN routine uses the Levenberg-Marquardt algorithm (17,18) and requires the calculation of derivatives. The values of A , B , and T_m were constrained in each transition so that A was positive, $B < 0.1$ and $2 < T_m < 90$, peaks violating these constraints were eliminated and the fitting proceeded with $i-1$ transitions. The area of each peak was determined using QUANC8 (19), an adaptive quadrature algorithm. CD spectra were collected with a Jasco J-40 spectropolarimeter and analysed by computer (4). Absorbance data were collected either on a Cary 15 or a Zeiss PMQ II spectrophotometer.

RESULTS

Soluble MAC Chromatin Proteins

Fig. 1a shows an SDS-PAGE gel of Euplotes soluble MAC chromatin proteins when different protease inhibitors were employed in the chromatin isolation procedure. If PMSF was the only inhibitor used (lane 3), a prominent band of about 17 kD was diminished and some low molecular weight bands appeared below the fastest histone, which may be H4. When TLCK was used, in addition to PMSF (lane 2), this protein was not degraded and no low molecular weight bands appear below the presumed H4. When TPCK was used (lane 1), there appeared to be a small amount of protein degradation taking place. Macro-nuclear DNA was not degraded when any of these three protease inhibitors was present. Leupeptin was effective in preventing proteolysis but the DNA was degraded during the chromatin isolation procedure. When soybean trypsin

Fig. 1(a). Effect of Protease Inhibitors. SDS-Polyacrylamide Gel Electrophoresis of *Euplotes* soluble macronuclear chromatin isolated in the presence of, from left to right: Lane 1, TPCK and PMSF; Lane 2, TLCK and PMSF; Lane 3, PMSF. (b) SDS-PAGE of various chromatin samples. From left to right: Lane 1, chicken erythrocyte soluble chromatin; Lane 2, *Euplotes* macronuclear proteins; Lane 3, 5% PCA extract of *Euplotes* macronuclear soluble chromatin.

inhibitor was employed, there was substantial protein degradation but the DNA was not affected (data not shown).

Fig. 1b compares nuclear proteins from chicken erythrocytes (lane 1), and *Euplotes eurytostomus* (lane 2). Lane 3 shows the 5% PCA extracted proteins of *Euplotes* MAC soluble chromatin. The 17 kD band that is sensitive to proteolysis is preferentially extracted in 5% PCA, suggesting that it is an H1 or an HMG protein. The use of TLCK did not alter the pattern of chicken erythrocyte nuclear proteins in SDS-gels.

It has been shown by Krueger (20) that TLCK prevents the solubilization of chromatin from insoluble chromatin fibers by inhibition of a nucleolytic activity. TLCK inhibited proteolysis of the presumptive H1 in this system but PMSF did not. Krueger (19) proposes that TLCK may inhibit an endogenous nuclease or a proteolytic enzyme which, in turn, is responsible for activation of the endogenous nuclease. Both our study and Krueger's published study suggest that TLCK is a good reagent to use as a protease inhibitor, in combination with PMSF, when degradation of lysine-rich histones is a problem that PMSF alone does not solve. Among the MAC non-histone proteins, there is a ~53 kD band (Fig. 1a) that resembles the 50 kD band that Lipps (21) described in *Oxytricha*. We expect micronuclear proteins to have a negligible effect on the MAC protein pattern due to the fact that the DNA and protein contents of the macronucleus is ~200-fold greater than that of the micronucleus (22) and micronuclei are a very minor (<0.1%) contaminant in our macronuclei preparations.

Previous work on macronuclear chromatin of a related hypotrich, *Oxytricha nova* failed to demonstrate the presence of an H1-like protein (4),

Fig. 2. Electrophoresis of DNA Fragments from Micrococcal Nuclease Digestions of *Euplotes* Macronuclei, Macronuclear Chromatin and Chicken Erythrocyte Nuclei. From left to right: Lane 1, 123 bp ladder DNA molecular weight marker; Lane 2, DNA Hind III restriction fragments; Lanes 3-9, DNA from macronuclei digested 0, 0.5, 1, 2, 4, 8, 24 minutes; Lane 10, 123 bp ladder DNA molecular weight marker; Lane 11, DNA Hind III restriction fragments; Lanes 12-19, DNA from macronuclear chromatin digested for 0, 0.5, 1, 2, 4, 8, 16, 32 minutes; Lanes 20-21, 123 bp ladder DNA molecular weight marker; Lanes 23-24, chicken erythrocyte DNA digested 30 min., 1 hr. and 2 hrs.; Lane 25, chicken soluble chromatin digested 1 hr., DNA from pooled sucrose density gradients.

despite the use of several reagents to inhibit proteolysis, such as 1 mM PMSF, 50 mM NaHSO₃, 0.12% spermidine and 5-10 mM MgCl₂. Variations of the nuclear isolation procedure were also tried with no success in detecting an H1-like protein in 2-D protein gels (4). Unfortunately, since the original *Oxytricha* strain has died out, it has not been possible to examine whether TLCK would be effective in preserving an H1-like protein in the macronuclear chromatin of that species.

Nuclease Digestions

Euplotes eurytostomus macronuclei were digested with micrococcal nuclease as described in the Materials and Methods. Time courses of typical diges-

Table I. Analysis of micrococcal nuclease digestions of *Euplotes* macronuclei and macronuclear soluble chromatin

n	Macronuclei		Soluble Chromatin	
	Δbp^0	bp^0/n	Δbp	bp^0/n
1		203.7		218.5
2	163.8	183.8	145.8	182.0
3	184.9	184.2	107.2	157.1
4	205.9	189.6	290.6	190.5
$\bar{X} \pm S.D.$	184.9 ± 21.1	190.3 ± 9.3	181.1 ± 96.7	187.0 ± 25.3
Nominal X:	186			

The nominal X represents the average of the means obtained for the nucleosome DNA repeat lengths of macronuclei and macronuclear soluble chromatin.

tions are shown in Fig. 2. Fragment sizes were determined by calibration with ϕ X 174 and λ DNA restriction fragments in addition to the 123 base pair (bp) ladder DNA marker (available from Bethesda Research Laboratories) electrophoresed on the same slabs as the *Euplotes* samples. Macronuclear DNA contains many discrete fragments at very early digestion time points (lanes 3 and 12); these vary from approximately 500 bp to <23 kilobase pairs (kb). These are the gene-sized fragments originally described by Prescott (1). Digestion with micrococcal nuclease generates a repeating pattern of fragments that reflects the nucleosomal organization macronuclear chromatin. Oligonucleosomes as large as hexamers can be seen (Fig. 2, lanes 6, 7 and 15). The nucleosomal repeat was determined by measuring the DNA size in bp for mononucleosomes to tetranucleosomes, after extrapolating to zero time of digestion, and dividing each DNA fragment bp value by the number of nucleosomes in that fragment. These values were then averaged to obtain the bulk nucleosomal repeat length. For macronuclear digests, this value was found to be 190.3 ± 9.3 bp (standard deviation of 3 experiments); for digestion of MAC soluble chromatin the nucleosome repeat length was 187.0 ± 25.3 bp, (standard deviation for 2 experiments).

Calculation of repeat lengths using the difference (Δ bp) between adjacent oligomers yielded similar results (see Table 1). Within experimental error, the nucleosome repeat lengths of macronuclei and soluble MAC chromatin are the same.

Micrococcal nuclease digestions of chicken erythrocyte nuclei were performed as described in Materials and Methods. Lane 25 of Fig. 2 shows a typical chicken erythrocyte nucleosomal ladder. Lane 24 shows the DNA size of the fragments of chicken erythrocyte chromatin employed in the biophysical

Fig. 3(a). Agarose Gel Electrophoresis of Euplotes Macronuclear Soluble Chromatin. (b). 2-D Agarose Gel Electrophoresis of Soluble Chromatin and DNA. Left lane: Euplotes macronuclear chromatin DNA. Center: Second dimension DNA separation of Euplotes macronuclear chromatin. Right Lane: 123 bp ladder DNA molecular weight marker.

studies (CD and thermal denaturation) after pooling fractions from linear sucrose density gradients.

Two-Dimensional Gel Electrophoresis

The electrophoretic mobility of Euplotes MAC soluble chromatin is shown in Fig. 3a. We excised a narrow strip from the central region of a chromatin lane, deproteinized the chromatin and ran the material in a second dimension DNA gel (Fig. 3b). The results were quite similar to what has already been observed with Oxytricha MAC soluble chromatin (3). A diagonal line of ethidium bromide stained DNA indicates a good correlation between electrophoretic mobility of the chromatin and its corresponding DNA length. Spots corresponding to two of the more prominent bands, such as the rRNA gene band at 7.5 Kb (23) can be seen along the diagonal line. The top part of the diagonal line shows a slight curvature due to the higher concentration of agarose in the second dimension (DNA) separation as compared to the concentration of agarose in the first dimension gel.

Electron Microscopy Studies

The analysis of spreads of Euplotes MAC chromatin revealed that the length of the molecules depends linearly on the number of nucleosomes. Since

Fig. 4. Left: Darkfield electron micrograph of gene-size *Euplotes* macronuclear chromatin, fixed for 15 hrs in 0.1% glutaraldehyde, 0.2 mM EDTA, 0.5 mM TEA.HCl, pH 7.0. Right: Plot of chromatin molecule length (μm) and number of nucleosomes per molecule. The least square linear regression line has a slope of $0.0261 \pm 0.0036 \mu\text{m}$ per nucleosome. Bar = $0.1 \mu\text{m}$.

the nominal nucleosome repeat length is 186 bp, the length of DNA in one nucleosome would be $6.324 \mu\text{m}$. From the slope of the molecule length (in μm) versus number of nucleosomes plot (Fig. 4), we obtain a value of 27.0 nm per nucleosome. Therefore, we estimate a DNA compaction ratio of 2.34/1 in spread chromatin.

Fig. 5. Thermal Denaturation Profiles, dh/dt versus T , as a function of NaPO_4 concentration. a. Chicken erythrocyte soluble chromatin. b. *Euplotes* macronuclear soluble chromatin. —, 0 mM NaPO_4 ; ---, 2 mM NaPO_4 ; ···, 4 mM NaPO_4 . Dots represent data points. Curves were calculated as described in the Materials and Methods.

Table II. Transition midpoints and relative areas of thermal transitions in whole chicken and Euplotes macronuclear chromatin

Buffer	Chicken Soluble Chromatin			<u>Euplotes</u> Macronuclear Chromatin			
	T _m I	T _m II	T _m III	T _m Ia	T _m Ib	T _m II	T _m III
0.25 mM EDTA pH 7.0	63.0 ± 0.2	73.8 ± 0.4	82.6 ± 0.3	32.7 ± 0.7	66.0 ± 2.8	76.7 ± 0.3	83.8 ± 1.2
% area	25.7 ± 0.9	25.7 ± 2.8	48.6 ± 2.6	2.6 ± 0.2	7.2 ± 2.0	18.1 ± 1.4	72.1 ± 1.8
0.25 mM EDTA 2 mM Na Phosphate pH 7.0	65.6 ± 0.3	73.6 ± 0.1	85 ± 0.2	40.1 ± 1.4	59.6 ± 5.6	72.3 ± 0.3	81.6 ± 0.2
% area	10.8 ± 4.0	27.4 ± 1.3	61 ± 4.0	5.2 ± 2.0	12.8 ± 0.8	54.6 ± 1.9	27.4 ± 0.8
0.25 mM EDTA 4 mM Na Phosphate pH 7.0	65.2 ± 1.2	72.3 ± 0.2	85.9 ± 0.2		55.7 ± 3.0	69.5 ± 0.5	81.7 ± 0.2
% area	0.9 ± 0.7	23.4 ± 1.2	75.8 ± 1.2		16.3 ± 3.2	42.7 ± 2.8	41.0 ± 5.2

Thermal Denaturation of Whole Chicken Erythrocyte Soluble Chromatin

First derivative thermal denaturation profiles of chicken erythrocyte soluble chromatin are shown in Fig. 5a. For each melting curve, i , the number of transitions was varied from 2-4, A_n and $T_{m,n}$ were estimated visually and B_n was always initially set to 0.03. The actual number of transitions i , could not be determined unequivocally since all the melting curves could be explained very well with two transitions as judged by χ^2 , though increasing the number of transitions to three always produced substantial reductions in this value. Subsequent increases of i had little effect on the χ^2 value or the visual quality of the fit. The fitted values of A_n , B_n and $T_{m,n}$ for $i=2$ or 4 were very dependent upon the initial values of these parameters, while when $i=3$ they were much less so. This lead us to conclude that there were three major transitions present in the three ionic strengths employed.

In 0.25 mM EDTA there appear to be three transitions (5): transition Iw with $T_m=62.98^\circ\text{C}$; transition IIw, $T_m=73.75^\circ\text{C}$; and transition IIIw, $T_m=82.63^\circ\text{C}$. The melting temperatures and relative areas for each thermal transition also correlate well with the data of Fulmer and Fasman (5) for whole chicken erythrocyte soluble chromatin at different low ionic strengths (see Table II). For example, the relative areas measured by us of the transitions in 0.25 mM EDTA, are $25.7 \pm 0.9\%$, $25.7 \pm 2.8\%$ and $48.6 \pm 2.6\%$ for transitions Iw, IIw and IIIw, respectively. Fulmer and Fasman (5) obtain 26%, 23% and 51% for the three transitions at the same ionic strength. The relative area of transition Iw decreased with increasing ionic strength, as mentioned in the

Introduction. Transitions IIw and IIIw represent 23.4 and 76%, respectively, of the total hyperchromicity at 4.0 mM Na phosphate. The DNA melting in transition IIw is thought to represent the 60 bp of linker DNA per nucleosomal repeating unit, while the DNA melting in transition IIIw is considered to represent the 140 bp of DNA in the core particle (5,24).

Thermal Denaturation of Euplotes Soluble Macronuclear Chromatin

First derivative melting profiles of Euplotes macronuclear chromatin are shown in Fig. 5b. The profiles are also multiphasic, and the transitions were resolved using the same method that was used for the chicken soluble chromatin samples. In 0.25 mM EDTA there appear to be at least four transitions which we will designate as Tm Ia, Tm Ib, Tm II and Tm III. Under these same conditions, purified macronuclear DNA melts at 45°C, with a very small fraction (7%) of the DNA melting at 35°C. The four chromatin transitions occur at 32.7, 66.0, 76.7 and 83.8°C. Tm III, which has been attributed in chromatin of higher eukaryotes to DNA within the core particle is 72.1% of the total hyperchromicity. Tm II however represents 18.1% of the total hyperchromicity. Transition Ia, although it represents only a small portion of the hyperchromicity (~3%), was highly reproducible. Since purified MAC DNA has a small fraction which melts at 35°C, this suggests that Tm Ia involves DNA which is not stabilized by proteins. As the ionic strength is increased, Tm Ia shifts to a higher temperature; in 4 mM Na phosphate Tm Ia and Ib are no longer resolved. Tm II, however, shifts to slightly lower temperatures as the ionic strength is increased from 2-4 mM Na phosphate. Tm III remains unchanged over this range of ionic strengths. These changes in the thermal transition profiles that occur over this small range of ionic strengths are in many ways reminiscent of the behavior of whole chicken erythrocyte chromatin, but there are some differences which may be due to the large amount of non-histone proteins present in Euplotes macronuclear chromatin (see Fig. 1). Overall, the thermal denaturation of Euplotes macronuclear chromatin resembles more that of chicken erythrocyte chromatin containing lysine-rich histones, than it does H1-depleted chromatin (4,5).

Circular Dichroism of Whole Chicken Erythrocyte Chromatin

Another valuable tool for studying the structure of chromatin is circular dichroism, which is an excellent probe to follow or detect conformational changes (25). Fig. 6a shows the CD spectrum of chicken soluble chromatin in 0.25 mM EDTA. There is strong suppression at 280 nm, which is a general feature of chromatin CD spectra, and has been explained as a contribution

Fig. 6(a). CD spectrum of chicken erythrocyte soluble chromatin dialyzed against 0.25 mM EDTA. (b). CD spectra of *Euplotes* macronuclear soluble chromatin (—) and DNA (----) after dialysis into 0.25 mM EDTA. The spectra are normalized according to the concentration of DN phosphate. $\epsilon_L - \epsilon_R$ in 1/cm.mole and wavelength in nanometers (nm).

from DNA ψ -type CD (26) producing a large negative component at 27 nm. In 0.25 mM EDTA, the circular dichroism ($\Delta\epsilon$) at 282.5 nm is 1.02 ± 0.13 l/cm.mole. The reported value for $\Delta\epsilon$ at this ionic strength is 1.21. As the ionic strength is increased from 2–4 mM Na phosphate, the $\Delta\epsilon$ at 282.5 nm remains relatively constant (see Table III). This phenomenon has been observed previously in whole chicken erythrocyte chromatin and is thought to be due to stabilization of a portion of DNA by lysine-rich histones (5).

Table III. CD at 282.5 nm, $\Delta\epsilon_{282.5}$, as a function of Na phosphate concentration for whole chicken erythrocytes and *Euplotes* macronuclear chromatin

Buffer	Chicken Soluble Chromatin $\Delta\epsilon$ (L/cm mole)	<i>Euplotes</i> Soluble Chromatin $\Delta\epsilon$ (L/cm mole)
0.2 mM EDTA pH 7.0	1.02 ± 0.13	1.04 ± 0.08
0.2 mM EDTA 2 mM NaPO ₄ pH 7.0	0.92 ± 0.02	1.07 ± 0.05
0.2 mM EDTA 4 mM NaPO ₄ pH 7.0	0.93 ± 0.10	1.06 ± 0.11

Circular Dichroism of Euplotes Macronuclear Soluble Chromatin

Fig. 6b shows the CD spectrum of Euplotes macronuclear chromatin and purified DNA in 0.25 mM EDTA. The CD spectrum of Euplotes MAC chromatin exhibits some similarities to that of whole chicken chromatin. In 0.25 mM EDTA the circular dichroism, $\Delta\epsilon$ at 282.5 is 1.04 ± 0.08 l/cm.mole. For Oxytricha macronuclear chromatin in that same buffer, $\Delta\epsilon = 1.56 \pm 0.03$ l/cm.mole (4). Thus, the intensity of the CD signal in 0.25 mM EDTA is consistent with the presence of lysine-rich histones in Euplotes MAC chromatin. The observed magnitude of the 282.5 nm CD peak is insensitive to small changes in ionic strength (see Table III). Again, this is reminiscent of the CD behavior of whole chicken erythrocyte chromatin.

DISCUSSION

In the hypotrichous ciliated protozoa, the existence of macronuclei containing transcriptionally-active genes of reduced DNA complexity, which are fragmented into gene-size pieces, affords an excellent and unique opportunity to study the structural properties of defined chromatin molecules (1). The present work compares the properties of bulk macronuclear chromatin with the transcriptionally inactive chromatin of the chicken erythrocyte. We have developed a chromatin isolation procedure that yields soluble MAC chromatin with no detectable nucleolysis or proteolysis, and remains stable for at least one week at 4°C.

Euplotes MAC chromatin appears to have an H1-like protein, which is preferentially extracted in 5% PCA, has a molecular weight of 17 kD (smaller than H1 proteins from higher eukaryotes), and is very sensitive to proteolysis. The use of the protease inhibitor, TLCK, in all the chromatin preparation solutions enabled us to detect and preserve this protein. The migration pattern of Euplotes macronuclear proteins in SDS gels, particularly the histones, is similar to the chromosomal proteins of Entomophthora (a fungus) (27). This fungus also has an H1-like protein which is preferentially extracted in 5% PCA, and has a molecular weight of approx. 18.9 kD (also smaller than most H1 proteins). The nucleosome repeat length of this fungus is 197 bp, while that of Euplotes is 186 bp. Euplotes macronuclei exhibit condensed chromatin, a feature also in common with Entomophthora nuclei. Ralph-Edwards and Silver (27) have proposed that fungal chromatins which exhibit a short (154-170 bp) nucleosomal DNA repeat length and no condensed chromatin appear to either lack histone H1, or contain an H1 protein less basic than that of higher eukaryotes. They stated that nucleo-

some repeat length, at least in fungi, does not appear to be directly determined by the basicity of histone H1, but that a correlation does appear to exist between long nucleosomal DNA repeat length and chromatin condensation. In that respect, Euplotes macronuclear chromatin appears to be similar to the chromatin of this fungus, since its nucleosome repeat length falls in the range of "long" repeat lengths for lower eukaryotes.

Both whole chromatin and lysine-rich histone-depleted chromatin undergo multiphasic thermal denaturation (5,23). A characteristic difference between chromatin with and without lysine-rich histone is the significant dependence with ionic strength of the lowest melting transition when H1 is absent; whole chromatin displays only a decrease in the relative area of this transition with increasing ionic strength. This low melting transition apparently represents linker DNA which, when lysine-rich histones are absent, behaves similarly to free DNA (5). The higher melting transitions are probably due to core particle DNA, T_m III is ascribed to the 90-100 bp central region of core DNA. The thermal denaturation profiles of Euplotes macronuclear chromatin show three main transitions and a minor transition in 0.25 mM Na₂ EDTA and show three transitions at higher ionic strength. The three main transitions occur at around the same temperatures as do the transitions of whole chicken erythrocyte chromatin I, II and IIIw (5), although they are not as clearly resolved. When the ionic strength is increased to 2 and 4 mM NaPO₄, the two highest transitions are better resolved. T_m II shifts to slightly lower temperatures at 4 mM NaPO₄. This decrease in melting temperature may be due to dissociation or destabilization of non-histone proteins. Overall, the thermal denaturation profile of Euplotes MAC chromatin at 4 mM NaPO₄ looks much like that of whole chicken chromatin in that same buffer. T_m Ia could not be observed at that ionic strength, presumably the DNA that melted at 33°C in 0.25 mM EDTA is now more stable, due to reduced electrostatic repulsions. Since RNase treatment was not employed in our chromatin isolation procedure, the possibility that T_m Ia represents the melting of RNA can not be ruled out, and will be subjected to future examination. The small amount of this presumed DNA melting at transition Ia might not be associated or stabilized by typical chromatin proteins since the same transition is observed in purified Euplotes macronuclear DNA. One possibility is that the DNA that melts in transition Ia corresponds to the termini of the linear DNA molecules, all of which have protruding 3' single stranded regions and inverted terminal repeats (28). The double strand to single strand DNA

junction in Oxytricha macronuclear chromatin possesses a preferred micro-coccal nuclease cleavage site, and a "telomeric-complex" has been proposed to exist in the terminal 100 bp (29). Following this line of reasoning, Tm Ia could be explained if one assumed that the protein or proteins in the complex do not stabilize the terminal DNA in the way that the histone octamer does. The percentage of DNA in the telomeric complex is approx. 9%, (averaged over the entire size range of macronuclear DNA), while the amount of DNA melting in transition Ia is 3%. Interestingly, the 50 kD DNA binding protein identified by Lipps (21), which has been suggested as a potential candidate for the protein component of the terminal complex, seems to be present in Euplotes macronuclear chromatin. From the analysis of thermal denaturation data we can conclude that the bulk of Euplotes MAC chromatin appears to be stabilized in a manner consistent with the presence of lysine-rich histones.

The circular dichroism of chromatin has been interpreted as representing a linear combination of two structural domains (25). Optical activity at wavelengths above 250 nm corresponds entirely to DNA, while the region below 250 nm represents a superposition of contributions from both histone peptides and DNA nucleotides. The CD of Euplotes MAC chromatin does not display an ionic strength dependence, nor do we see any significant difference between the CD of whole chicken chromatin and that of Euplotes. The lack of an ionic strength dependent conformational change in Euplotes MAC chromatin is also consistent with the presence of lysine-rich histones in this chromatin.

The results that we have obtained from a variety of techniques lead us to the following conclusions: TLCK prevents proteolysis of a macronuclear H1 like protein. The nucleosome repeat lengths of Euplotes macronuclei and soluble MAC chromatin are essentially identical and are in the long range of repeat lengths for lower eukaryotes, which might be expected if an H1 protein is present. Two dimensional agarose gel electrophoresis (first dimension separation of chromatin, second dimension separation of DNA), established a strict correlation between chromatin and DNA electrophoretic mobility. Electron microscopic analysis of spreads of Euplotes MAC chromatin revealed that the length of the molecules depends linearly on the number of nucleosomes; in other words, the spread chromatin molecules are very uniform in average linker length. Circular dichroism and thermal denaturation studies of chromatin structure revealed that Euplotes MAC chromatin behaves like H1-containing chromatin. The differences in the thermal denaturation profiles of Euplotes MAC chromatin and chicken erythrocyte chromatin are very

likely due to the extensive amount of non-histone proteins present in Euplotes MAC chromatin, compared to their paucity in chicken chromatin. Future studies from this laboratory will describe subfractionation of soluble MAC chromatin, with concomitant enrichment of specific DNA containing fragments.

ACKNOWLEDGEMENTS

We thank James Finch for excellent photographic work. This research was sponsored by the U.S. Department of Energy, under contract DE-AE05-84R2100 with the Martin Marietta Energy Systems, Inc., by grants from NIH to D.E.O. (GM-19334), and to A.L.O. (GM-29429), and by an NIGMS National Research Service Award to C.L.C. (GM-10079-02).

*Research supported by NIH research grants GM-19334 to D.E.O., and to A.L.O. (GM-29429), by an NIGMS National Research Service Award to C.L.C. (GM-10079-02) and by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-84OR21400 with the Martin Marietta Energy Systems, Inc.

REFERENCES

1. Prescott, D. M. (1983) in *Modern Cell Biology*, McIntosh, J. R., Ed., Vol. II, pp 329-352, Alan R. Liss, New York.
2. Lipps, H. J., Nock, A., Riewe, M. and Steinbruck, G. (1978) *Nucl. Acids Res.* 5, 4699-4709.
3. Laughlin, T. J., Hermann, A. L. and Olins, D. E. (1984) *Mol. Cell. Biochem.* 62, 157-163.
4. Butler, A. P., Laughlin, T. J., Cadilla, C. L., Henry, J. M. and Olins, D. E. (1984) *Nucl. Acids Res.* 12, 3201-3217.
5. Fulmer, A. W. and Fasman, G. D. (1979) *Biopolymers* 18, 2875-2891.
6. Kaplan, L. J., Bauer, R., Morrison, E., Langan, T. A. and Fasman, G. D. (1984) *J. Biol. Chem.* 259, 8777-8785.
7. Sasi, R. and Fasman, G. D. (1984) *Biochim. Biophys. Acta* 782, 55-66.
8. Bryan, P. N., Wright, E. B., Hsie, M. H., Olins, A. L. and Olins, D. E. (1978) *Nucl. Acids Res.* 5, 3603-3617.
9. Olins, A. L., Carlson, R. D., Wright, E. B. and Olins, D. E. (1976) *ibid* 3, 3271-3291.
10. Laemmli, U. K. (1970) *Nature (London)* 227, 680-685.
11. Matsudaira, P. T. and Burgess, D. R. (1978) *Anal. Biochem.* 87, 386-396.
12. Thomas, J. O. and Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. USA* 72, 2626-2630.
13. Levinger, L., Barsoum, J. and Varshavsky, A. (1981) *J. Mol. Biol.* 146, 287-304.
14. Ottensmeyer, F. P., Whiting, R. F., Schimdt, E. E. and Clemens, R. S. (1975) *J. Ultrastruct. Res.* 52, 193-201.
15. Thoma, F. and Koller, Th. (1977) *Cell* 12, 101-107.
16. Olins, A. L. (1979) in *Chromatin Structure and Function*, Nicolini, C. A., Ed., NATO Advanced Study Institutes Series: Series A, Vol. 21, pt. A, pp 31-40, Plenum Press, New York.

17. Levenberg, K. (1944) *Quart. Appl. Math.* 2, 164-168.
18. Marquardt, D. (1963) *SIAM J. Appl. Math.* 11, 431-441.
19. Forsythe, G. E., Malcolm, M. A., and Moler, C. B. (1977) in *Computer Methods for Mathematics Computations*, pp. 84-109, Prentice Hall, New Jersey.
20. Krueger, R. C. (1983) *Biochem. Biophys. Res. Commun.* 110, 216-219.
21. Lipps, H. J., Gruissem, W. and Prescott, D. M. (1982) *Proc. Natl. Acad. Sci. USA* 79, 2495-2499.
22. Ammermann, D. and Muenz, A. (1982) *Eur. J. Cell. Biol.* 27, 22-24.
23. Swanton, M. T., McCarroll, R. M., and Spear, B. B. (1982) *Chromosoma* 85, 1-9.
24. Fulmer, A. W. and Fasman, G. D. (1979) *Biochemistry USA* 18, 659-668.
25. Fasman, G. D. (1979) in *Chromatin Structure and Function*, Nicolini, C. A. Ed., NATO Advanced Study Institutes Series: Series A, Vol. 21, pt. A, pp. 67-105, Plenum Press, New York.
26. Shin, Y. A. and Eichhorn, G. L. (1984) *Biopolymers* 23, 325-335.
27. Ralph-Edward, A. and Silver, J. C. (1983) *Exp. Cell Res.* 148, 363-376.
28. Klobutcher, L. A., Swanton, M. T., Pierluigi, D. and Prescott, D. M. (1981) *Proc. Natl. Acad. Sci. USA* 78, 3015-3019.
29. Gottsllinch, D. E. and Cech, T. R. (1984) *Cell* 38, 501-510.