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**Physical structure of gene-sized chromatin from the protozoan *Oxytricha*\***

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A.P.Butler<sup>+</sup>, T.J.Laughlin<sup>§</sup>, C.L.Cadilla, J.M.Henry and D.E.Olins

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University of Tennessee, Oak Ridge Graduate School of Biomedical Sciences and Biology Division,  
Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA

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**ABSTRACT**

*Oxytricha nova* is a hypotrichous ciliate containing a transcriptionally active macronucleus and a transcriptionally inactive micronucleus. Two-dimensional gel electrophoresis shows that macronuclei contain a normal complement of inner histones. However, despite extensive efforts, no classical H1-like protein has been detected. Micrococcal nuclease digestion indicates a nucleosome repeat length of approximately 220 bp for macronuclear chromatin. Thermal denaturation profiles of macronuclear chromatin in 0.2 mM EDTA display four transitions at about 46, 57, 64, and 79°C. The lowest of these shifts to higher temperature as the ionic strength is raised to 3-5 mM Na phosphate. These results are consistent with the absence of H1 and a nucleosome repeat of 220-230 bp. Circular dichroism (CD) results agree with these findings. By contrast, micronuclear chromatin displays a much smaller premelt and a more suppressed DNA CD signal at 285 nm, consistent with a micronuclear chromatin repeat of 165-185 bp as determined by micrococcal nuclease digestion.

**INTRODUCTION**

The hypotrichous ciliate *Oxytricha nova* contains two types of functionally and morphologically distinct nuclei (1), a transcriptionally active macronucleus and a transcriptionally inactive micronucleus. In the vegetative cell, the macronuclei are responsible for virtually all of the cell's RNA synthesis and control the phenotype of the cell, as is the case with other ciliated protozoa (2). Development of a macronucleus from the diploid zygote micronucleus is accompanied by both a great increase in DNA content and a reduction in DNA sequence diversity (3). Thus macronuclear chromatin represents a natural enrichment for transcriptionally active genes. Furthermore, in hypotrichous ciliated protozoa (such as *Oxytricha*), macronuclear DNA is found in discrete fragments of about 0.4-20 Kbp in length, or approximately gene-sized (4). Macronuclear chromatin is organized into correspondingly small fragments (5). These features make *Oxytricha* ideal for study of the structure and composition of specific active genes as chromatin fragments.

Thermal denaturation and circular dichroism (CD) have been extensively used to characterize chromatin and nucleosomes from other organisms. Both of these methods are sensitive to variations in DNA-protein and protein-protein interactions within chromatin. Recent studies on homogeneous nucleosome core particles (6-8), nucleosomes (9,10), and soluble chromatin (11-13) have greatly improved our understanding of the structural features that contribute to the observed thermal denaturation patterns and CD spectra.

In 0.2 mM EDTA, chromatin and H1-depleted chromatin have generally been reported to display three or four resolvable thermal transitions when monitored by DNA hyperchromicity at 260 nm (11-13). The fraction of DNA denaturing in each of these transitions is quite sensitive to ionic strength, as is the CD of H1-depleted chromatin (11). This sensitivity apparently reflects a salt-dependent conformational transition in nucleosomes at about 1-3 mM Na<sup>+</sup> (14,15).

Oxytricha chromatin follows these established patterns. However, there are marked differences between the transcriptionally inactive micronucleus and the macronucleus. In particular, we suggest that the nucleosomal repeat length is  $\approx$ 50 bp longer in macronuclei than in micronuclei. Although the macronuclear chromatin used in these experiments is enriched in active sequences, it is by no means "pure" transcriptionally active chromatin. None the less, these studies should be a useful baseline for ongoing research on the chromatin structure of individual active genes.

### MATERIALS AND METHODS

The Oxytricha nova cell line used was generously provided by David Prescott (University of Colorado, Boulder). Cell stocks were maintained in 50 ml culture tubes in Ochromonas medium (1 g/l each of glucose, liver extract, bactotryptone, yeast extract, and sodium acetate).

Large-scale cultures of Oxytricha were grown in 50 l fermentation flasks as recently described (16). The Oxytricha cells were harvested by filtering through Miracloth to remove filamentous algae and then pelleting the cells in a Sharples continuous-flow centrifuge (5000 rpm, 1 l/min, 4°C). The cells were disrupted in lysis buffer (5% sucrose, 10 mM Tris (pH 7.0), 50 mM sodium bisulfite, 0.12% spermidine, 0.5% Triton X-100 and 1 mM Phenylmethylsulfonyl fluoride [PMSF]). The macronuclei and micronuclei were separated by differential centrifugation through sucrose gradients in lysis buffer (4). The nuclei were further purified by buoyant density centrifugation in a linear 20% to 40% metrizamide gradient in lysis buffer.

Centrifugation was for 1 hr at 4°C and 25,000 rpm in an SW41 rotor. The fractions containing nuclei were diluted sevenfold with lysis buffer and then pelleted at 1000 rpm for 5 min in an International clinical centrifuge to dilute the metrizamide. Spermidine was removed by resuspending nuclei in 10 mM Tris (pH 7.0), 60 mM NaCl, 0.2 mM EDTA, 0.1 mM PMSF followed by overnight dialysis against this same buffer. The nuclei were lysed by dialysis against four changes (3 l each) of 0.2 mM EDTA (pH 7.0), 0.1 mM PMSF over a two-day period. Lysis was completed by vigorous pipetting, and insoluble material was removed from the soluble chromatin by centrifugation for 15 min in an Eppendorf microfuge.

#### Acid Extraction of Histones

Macronuclei were pelleted (1000 rpm for 5 min) and extracted with 0.2 M H<sub>2</sub>SO<sub>4</sub> (100 µl/12 µg DNA) for 12-18 hr at 4°C (17). The precipitate was removed by centrifugation (15,000 x g for 15 min) and the acid-soluble material was precipitated with trichloroacetic acid at a final concentration of 20%. After 30 min at 4°C the precipitate was collected by centrifugation (15,000 x g for 10 min), washed once with cold 0.02 N HCl in acetone and twice with cold acetone, and dried under reduced pressure. The protein was stored at -20°C until examined by gel electrophoresis.

#### Two-dimensional Gel Electrophoresis

Electrophoresis of nuclear proteins was performed using a modification of the triton/acid/urea system (17,18) in the first dimension and sodium dodecyl sulfate (SDS) (19) in the second dimension. Samples were dissolved in acid-urea sample buffer (5% acetic acid, 8 M urea, 5% 2-mercaptoethanol and 0.1% pyronin Y) containing 1% protamine sulfate. "Minislab" gels (75 x 100 x 0.5 mm) were pre-electrophoresed to constant current (3.5-4 mA), and the samples were electrophoresed for 5-6 hr at 3-4 mA (running buffer, 5% acetic acid). For the second-dimension gel, a lane was cut out of the first-dimension gel and equilibrated for 30 min with two changes of H<sub>2</sub>O followed by 30 min with 1 x SDS sample buffer (19), at 37°C. The separating gel (22% acrylamide, 0.15% N, N<sup>1</sup>-methylenebisacrylamide) was poured with the first-dimension lane in place. The stacking gel (4% acrylamide, 0.03% N, N<sup>1</sup>-methylenebisacrylamide) was then poured. The second-dimension was run at 200 V for 4 hrs. Both first- and second-dimensions were stained with Coomassie brilliant blue in 50% methanol, 5% acetic acid. Assignment of histone identity was made by comparison with 2-D gel patterns of other species (Figure 1). Acid-soluble proteins of Tetrahymena macronuclei were a generous gift of Dr. David Allis. Acid-soluble proteins of chicken

erythrocyte and Stylonychia were prepared in this laboratory. The Stylonychia strain was provided by Dr. Dieter Ammerman.

#### Nuclease Digestion and DNA Electrophoresis

Both macro- and micronuclei were purified through the metrizamide gradient step as described above and resuspended in 10 mM Tris+HCl (pH 7.0), 10 mM CaCl<sub>2</sub> at a DNA concentration of 400 µg/ml. The nuclei were digested with 30 units/ml of micrococcal nuclease at 37°C. At the desired time points, the reaction was terminated by adding 2 mM EDTA and 200 µg/ml proteinase K followed by overnight incubation at 37°C. Electrophoresis of the DNA fragments in 1% agarose in 40 mM Tris (pH 8.9), 5 mM sodium acetate, and 1 mM EDTA was done as previously described (20). Marker DNA fragments (Hind III-digested λ DNA and Hae III digested φX 174 RF DNA) were obtained from Bethesda Research Laboratories.

#### Physical Studies

Chromatin samples were prepared for thermal denaturation and circular dichroism by exhaustive dialysis against 0.2 mM Na<sub>2</sub>EDTA (pH 7.0) containing 0.1 mM PMSF and sodium phosphate as indicated to achieve the desired ionic strength. Phosphate stock solutions (0.2 M) were prepared to give a calculated pH 7. The sodium ion concentration of this stock solution was 0.286 M. The measured pH of the final solution was 6.8-7.0, depending on the ionic strength. Experiments were conducted on samples with an absorbance at 260 nm of 0.3-0.8, except for some samples of micronuclear chromatin that had an absorbance of 0.15.

Thermal denaturation experiments were performed with a Gilford 2000 spectrophotometer equipped with a digital absorbance meter and interfaced to a PDP 11-20 computer. Direct and first-derivative melting profiles were calculated from the smoothed data by a PDP 11-70 computer linked to the data acquisition system. CD Spectra were collected with a JASCO J-40 spectropolarimeter and analyzed by computer. Adsorbance spectra were collected on a Cary 15 spectrophotometer.

## RESULTS

### Histone Composition of Macronuclei

Figure 1 shows a typical two-dimensional gel of acid-soluble proteins of Oxytricha macronuclei, along with the acid-soluble proteins of the protozoans Tetrahymena and Stylonychia and of chicken erythrocyte. The assignment of Oxytricha histones to gel spots indicated in Figure 1 is based

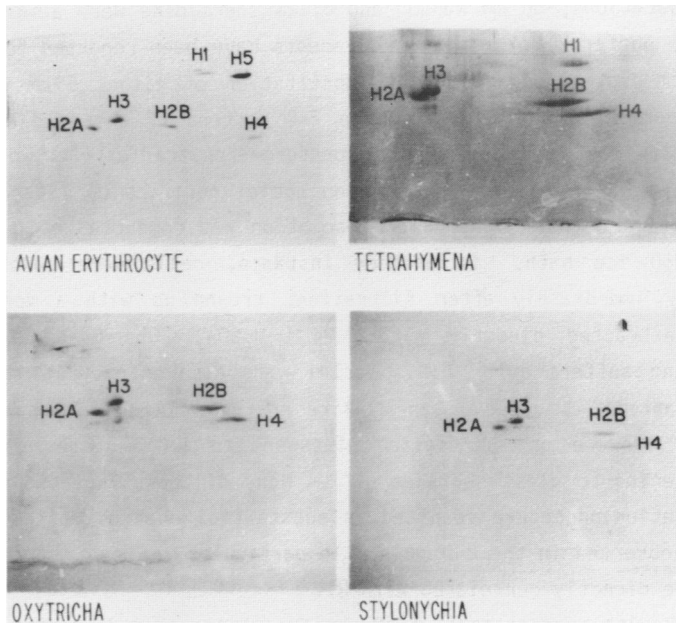


Figure 1. Two-dimensional (acid-urea followed by SDS) gel electrophoresis of acid-extracted histones from A. chicken erythrocyte nuclei, B. *Tetrahymena* macronuclei, C. *Oxytricha* macronuclei, D. *Stylonychia* macronuclei.

on a comparison with the gel patterns of the other species shown. Final identification of these proteins will require detailed study of their amino acid compositions and sequences. However, the mobilities of the major core histones appear to be highly conserved, with the exception that protozoan H2B's have a significantly higher mobility in the triton/acid/urea dimension that does the erythrocyte H2B. It is notable that the *Oxytricha* and *Stylonychia* gels do not show protein spots in the position expected for an H1-like protein. However, *Oxytricha* macronuclei do have a complex group of proteins with low mobility in the first dimension and moderately high molecular weight (20-30,000 daltons) in the second dimension. It is possible that one or more of these polypeptides correspond to the H1-like peptides  $\alpha$ ,  $\beta$ , and  $\gamma$  reported for *Tetrahymena* micronuclei (17).

A number of procedures have been used to ensure that histones have not been lost during isolation of the *Oxytricha* chromatin. All steps of the isolation were conducted in the presence of 1 mM PMSF to inhibit proteolysis.

In most preparations, 50 mM NaHSO<sub>3</sub> and 0.12% spermidine were also included in the lysis buffer. Both of these reagents have been reported to protect histones from proteolysis (21,22). Substitution of either 5 mM or 10 mM MgCl<sub>2</sub> for spermidine did not alter the 2-D pattern of the acid extracts.

Gel analysis of proteins has been performed on acid extracts of nuclei purified on metrizamide gradients and on nuclei purified only through the sucrose gradient steps. One nuclear isolation was conducted at 0°C using an ethanol-H<sub>2</sub>O ice bath. In another instance, cells were quick-frozen in liquid N<sub>2</sub> immediately after filtration, ground up with a mortar and pestle and extracted directly with 0.2 M H<sub>2</sub>SO<sub>4</sub>. One preparation was isolated using buffers at pH 6.0. Nuclei were also extracted with 0.6 M NaCl in an attempt to recover an H1-like protein. All of these methods used plastic labware or siliconized glassware to avoid losses caused by histones adhering to glass surfaces (17). None of these variations of the nuclear isolation procedure resulted in substantial changes in the pattern of proteins observed in the 2-D gels. In particular, no spot was observed that could be directly correlated with H1.

These results suggest that, if Oxytricha macronuclei do contain an H1-like protein, it must differ radically in acid extractability, solubility and/or mobility in triton/acid/urea gels from the H1 of Tetrahymena macronuclei and the H1 and H5 of higher organisms. As shown below, the physical properties of macronuclear chromatin are also consistent with the absence of H1 in these preparations.

#### Nuclease digestions

Macro- and micronuclei were digested with micrococcal nuclease as described in Materials and Methods. Typical digestion time courses are shown in Figure 2. Fragment sizes were determined by calibration of the gels with  $\phi$ X 174 and  $\lambda$  DNA restriction fragments electrophoresed on the same slabs as the Oxytricha samples. Note that the macronuclear DNA contains many discrete fragments at very early digestion time points (Lane 3); these vary in size from ~400 bp to the upper resolution of the gel. These are the "gene-sized" fragments previously described (4). In addition, a prominent band of approximately 500 bp can be seen. Although we have not characterized this band, it is seen in undigested macronuclear chromatin and probably represents the over-amplification phenomenon which has been reported to correlate with the age of a strain (i.e. a clonal population) in the related protozoan, Stylonichia lemna (23). Neither of these features was observed with DNA from micronuclei (Lanes 14-17).

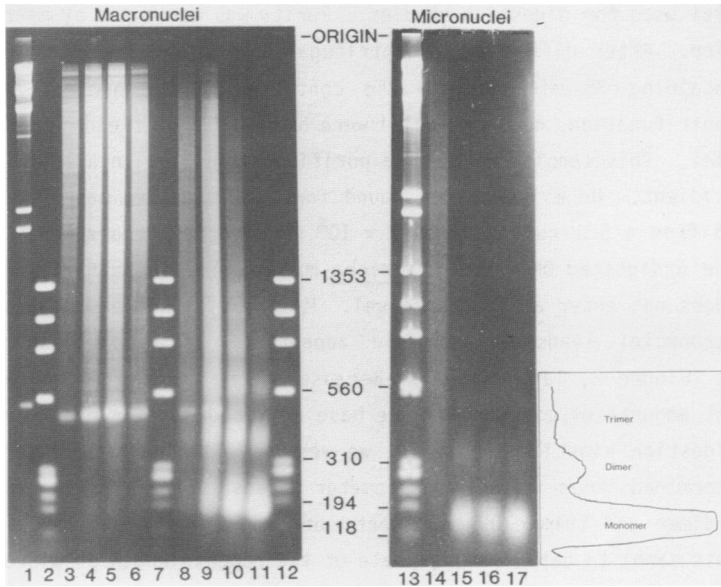


Figure 2. Electrophoresis of DNA fragments isolated from macronuclear and micronuclear chromatin digested with micrococcal nuclease. From left to right: Lane 1, Hind III-digested  $\lambda$  DNA; Lane 2, Hae III-digested  $\phi$ X 174 RF DNA; Lanes 3-6, DNA from macronuclear chromatin digested 0.5, 1, 2, and 4 minutes; Lane 7, Hae III-digested  $\phi$ X 174 DNA; Lanes 8-11, macronuclear DNA from 8, 16, 32, and 64 minutes of digestion. Lane 12, Hae-III digested  $\phi$ X DNA; Lane 13, a mixture of Hae III-digested  $\phi$ X DNA and Hind III-digested  $\lambda$  DNA; Lanes 14-17, DNA from micronuclear chromatin digested 1, 2, 4, and 8 minutes. A laser-densitometer scan of Lane 15, taken from the photographic negative.

Digestion with micrococcal nuclease generates a repeating pattern of fragments that reflects the nucleosomal organization of the macronuclear chromatin. Oligonucleosomes as large as tetramers can easily be seen (Figure 2, Lanes 8-11). The nucleosomal repeat was determined by taking the difference,  $\Delta$ bp, between adjacent oligomers (tetramer-trimer, trimer-dimer, dimer-monomer). These values were then averaged to obtain the bulk nucleosomal repeat length. For macronuclear chromatin, this value was determined to be  $219 \pm 18$  bp (standard deviation of 4 experiments). At longer digestion time ( $> 30$  min), the mononucleosome band appears to be trimmed to a core particle of 140-150 bp.

Because the DNA content of micronuclei is only 1/50 that of macronuclei, special precautions were taken to eliminate any possible contamination of

micronuclei used for digestion studies. Purity was monitored by microscopic observation. After differential centrifugation through sucrose, a typical field containing 35 micronuclei also contained 6 macronuclei. After a second centrifugation, no macronuclei were observed in a field containing 42 micronuclei. This sample was further purified by banding in a 10-70% metrizamide gradient. No evidence was found for contaminating macronuclei, and the yield from a 5 l culture was  $12 \times 10^6$  micronuclei (equivalent to 16  $\mu\text{g}$  DNA). The undigested DNA from *Oxytricha* micronuclei is sufficiently large that it does not enter a 1% agarose gel. Micrococcal nuclease digestion of these micronuclei leads to a rapid appearance of mononucleosome-sized fragments (Figure 2, lanes 14-17). Because of the difficulty of obtaining sufficient amounts of micronuclei, we have not conducted an extensive study of the digestion kinetics. However, we were able to use the difference in size (determined from laser densitometer scans, Figure 2) between the monomer, dimer and trimer DNA fragments of two separate experiments (four time points each) to obtain an estimate of the repeat length of micronuclear chromatin. The value obtained was 176 bp, with a range of 160 to 195 bp.

#### Thermal Denaturation of Macronuclear Chromatin

First derivative thermal denaturation profiles of *Oxytricha* macronuclear chromatin are shown in Figure 3. Several features of these results are informative. First, the profiles are multiphasic. In 0.2 mM EDTA ( $\text{Na}^+ = 0.58$  mM) there appear to be at least four distinct components, which we designate  $T_m^{\text{Ia}}$ ,  $T_m^{\text{Ib}}$ ,  $T_m^{\text{II}}$ , and  $T_m^{\text{III}}$  by analogy to earlier studies

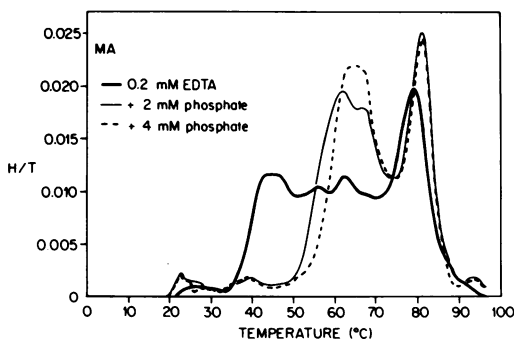


Figure 3. Thermal denaturation profiles,  $dh/dT$  versus  $T$ , of *Oxytricha* macronuclear chromatin as a function of Na phosphate concentration. —, no Na phosphate; — — —, 2 mM Na phosphate; - - - - -, 4 mM Na phosphate. Solutions were dialyzed against the indicated buffers and melted at an initial absorbance of 260 nm at approximately 0.4



(11,12) on the thermal denaturation characteristics of chromatin from higher eukaryotes. Under these same conditions, free macronuclear DNA melts at 40° in a single, relatively sharp transition (data not shown). The four chromatin transitions are at 46, 55-56, 64, and 79°C.  $T_m^{III}$ , which has been attributed to DNA within the core particle (6,7), represents only 41% of the total hyperchromicity. The total hyperchromicity for these samples was 38-42% of the initial  $A_{260}$ . Although transitions Ib and II represent relatively small changes in absorbance, their presence was reproducible between several experiments on each of 3 separate chromatin preparations. At a given ionic strength, the transition temperatures (determined from the first derivative profile of the smoothed data) did not vary more than 1 or 2°C at a given ionic strength. The error in the magnitude of the hyperchromicity represented in transitions I and III, each of which represents 30-40% of the total hyperchromicity at 2 mM EDTA, is approximately 2%.

As the ionic strength is increased (Figure 4),  $T_m^{Ia}$  shifts to a higher temperature. Above 1 mM phosphate,  $T_m^{Ia}$  and  $T_m^{Ib}$  are no longer resolved. The transition temperature of the combined  $T_m^I$  continues to increase until 3 mM phosphate. Above this ionic strength, the thermal transitions are essentially biphasic. Over the range of ionic strengths tested, transition  $T_m^{III}$  increased only from 79°C to 82°C. Transition  $T_m^{II}$  increased from

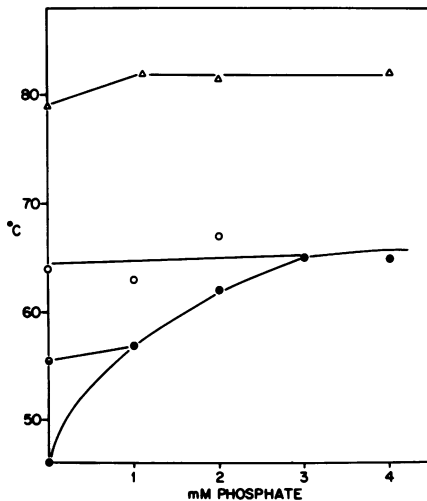


Figure 4. Dependence of *Oxytricha* macronuclear chromatin thermal transition midpoints on Na phosphate concentration. Transition Ia, ●; transition Ib, ○; transition II, ○; transition III, △.

63°-64°C at low ionic strength to 64-66°C above 3 mM phosphate. The major change in transition temperature occurred for  $T_m^I$ , which increased from 46° to 65-66°C. These ionic strength transitions, occurring primarily between 0-2 mM Na phosphate, are similar to ionic strength induced structural transitions reported for H1- and H5-depleted chicken erythrocyte chromatin (11). The sodium ion concentration over which the transition occurs is  $\approx 0.6$  mM to  $\approx 3.5$  mM  $\text{Na}^+$ , a range also similar to that reported for the low ionic strength transitions observed with chicken erythrocyte chromatosomes and nucleosomes depleted in very lysine-rich histones (14,15). Whole chromatin (containing a full complement of very lysine-rich histones) typically does not show an appreciable increase in transition temperature for  $T_m^I$  (11). Furthermore the temperature of the most stable transition of whole chromatin decreases 2°C above 2 mM phosphate (11). Thus, the thermal denaturation of *Oxytricha* macronuclear chromatin much more nearly resembles that of H1-depleted chromatin of higher eukaryotes than that of whole chromatin.

#### Circular Dichroism of Macronuclear Chromatin

Circular dichroism has been widely used as a diagnostic tool in assessing the structural state of chromatin. In Figure 5, the CD spectrum of *Oxytricha* macronuclear chromatin in 0.2 mM EDTA is displayed. The spectrum can be resolved into two regions: for  $250 \text{ nm} < \lambda < 300 \text{ nm}$ , the spectrum reflects predominantly DNA structure; for  $200 \text{ nm} < \lambda < 250 \text{ nm}$ ,

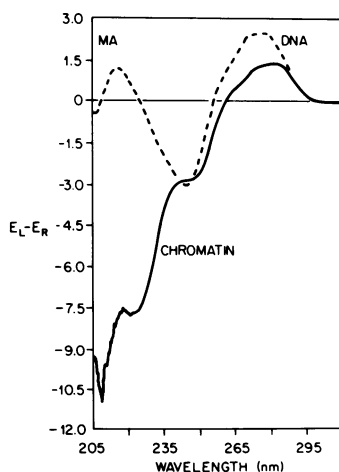


Figure 5. CD spectra of macronuclear chromatin (—) and DNA (-----) after dialysis into 0.2 mM EDTA. The spectra are normalized according to the concentration of DNA phosphates.

the spectrum is a composite of protein secondary structure and DNA contributions. Since it is generally the most diagnostic of chromatin structure and the least ambiguous, we focus our attention on the long wavelength region. Relative to free macronuclear DNA (Figure 5), the spectrum is strongly suppressed at 280 nm. This suppression is a general feature of chromatin CD spectra, and has been tentatively assigned to a contribution from DNA  $\Psi$ -type CD, producing a large negative component at 275 nm (9). Alternatively, the suppression could result from conformational changes within the DNA double helix (24). In 0.2 mM EDTA, the circular dichroism ( $\Delta\epsilon$ ) at 275 nm (per mole nucleoside) is  $1.56 \pm 0.03$ , in good agreement with the value of 1.58 reported for chicken erythrocyte H1-depleted chromatin (11). However, this value is substantially greater than that for whole chromatin from chicken at this ionic strength ( $\Delta\epsilon_{275} = 1.21$ ). Thus the intensity of the CD signal in 0.2 EDTA is consistent with the absence of very lysine-rich histones. It is also apparent, according to the CD criterion, that macronuclear chromatin has a DNA structure at least qualitatively similar to the low ionic strength form of H1-depleted vertebrate chromatin.

The amount of suppression, and the resulting observed magnitude of the 275 nm CD peak, is also sensitive to the low ionic strength structural transition (Table 1). The transition as monitored by CD spans a range of sodium concentration of 0.6-4 mM (0-2 mM  $\text{PO}_4$ ). This again is reminiscent of the transition observed with very lysine-rich histone-depleted chicken erythrocyte chromatin (11). However, the CD spectrum at high ionic strength ( $[\text{PO}_4] > 3 \text{ mM}$ ) is substantially more intense than that of chicken erythrocyte chromatin under similar conditions. This finding suggests that at high ionic strength, macronuclear chromatin DNA may be more "relaxed", i.e.,

TABLE 1: Dependence of Chromatin CD Spectra ( $\Delta\epsilon_{275}$ ) on Ionic Strength\*

$[\text{PO}_4^{-2}]$	Macronuclear	Micronuclear
0	$1.56 \pm 0.03$	1.32 <sup>†</sup>
1	$1.48 \pm 0.04$	N.D.
2	1.40 <sup>†</sup>	N.D.
4	$1.36 \pm 0.03$	N.D.

\*Mean,  $\pm$  S.D. for three independent chromatin isolations

<sup>†</sup>single measurement

more like free B-form DNA, than the DNA in chicken erythrocyte chromatin. Whether this is a reflection of the higher proportion of transcriptionally active sequences in macronuclear chromatin or has another explanation is presently under investigation.

Thermal Denaturation of Micronuclear Chromatin

The DNA content of *Oxytricha* micronuclei is roughly 1/50 that of macronuclei. Furthermore, for physical studies micronuclei must be digested briefly with micrococcal nuclease to solubilize the chromatin, resulting in greater losses during isolation. Because of this limitation of material, we have performed only a few preliminary experiments with micronuclear chromatin and DNA. Figure 6 illustrates the derivative thermal denaturation pattern of micronuclear chromatin after dialysis versus 0.2 mM EDTA. The contrast with macronuclear chromatin under the same conditions is striking. Micronuclear chromatin shows only a very slight premelt, with 55-60% of the hyperchromicity being assignable to a single band centered at 74-77°C (as compared to 79°C for macronuclear chromatin  $T_m^{III}$ ). No hyperchromicity is observed at or near the  $T_m$  (38°C) of free micronuclear DNA. A small amount (<40%) of the chromatin DNA melts as a broad "tail" preceding the main band. This difference between chromatin from the two nuclei may be due in part to the presence of a putative H1 in micronuclei. However, this alone would be insufficient to account for the entire difference. As we show below (Discussion) it is most likely that the nucleosomal repeat length is much shorter in micronuclei than in macronuclei.

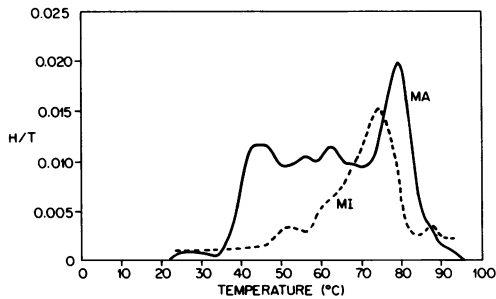


Figure 6. Thermal denaturation of micronuclear chromatin in 0.2 mM EDTA (no added phosphate). The sample was prepared as in Figure 2, but the initial absorbance at 260 nm was 0.15. The profile of macronuclear chromatin denaturation in the same buffer (MA) is included for comparison.

### Circular Dichroism of Micronuclear Chromatin

The CD signal at 275 nm of micronuclear chromatin in 0.2 mM EDTA is more suppressed than that of macronuclear chromatin in the same buffer (Table 1). In fact, the CD intensity of micronuclear chromatin is less than that of macronuclear chromatin under any ionic strength conditions examined. (Due to limitations of available material, we have not yet studied the ionic strength dependence of micronuclear chromatin CD). The magnitude of the CD spectrum suggests that, at low ionic strength, the micronuclear chromatin DNA is much less B-like than that of macronuclei. This is consistent with the absence of a distinct ionic strength transition (if H1 is present), or a smaller contribution from linker DNA (see Discussion), or a combination of these possibilities.

### DISCUSSION

In Oxytricha nova, the existence of macronuclei containing transcriptionally active genes of reduced DNA complexity that are naturally fragmented into gene-sized pieces affords an excellent opportunity for structural studies of active chromatin. Our present results allow us to compare properties of bulk macronuclear chromatin with inactive micronuclear chromatin and with vertebrate chromatin.

Perhaps the most surprising feature that we have discovered is the apparent lack of a very lysine-rich histone class in macronuclear chromatin. We would like to stress that, despite vigorous attempts to inhibit proteolysis or mechanical losses, we can not strictly rule out the possibility that this observation may be an artifact of the isolation procedure. If Oxytricha macronuclei do contain an H1-like protein, it must have highly unusual properties since, in parallel extracts, Tetrahymena macronuclei and chick erythrocyte nuclei displayed H1 while Oxytricha macronuclei did not.

Despite our reservations concerning the protein content of the Oxytricha nuclei, we can combine the results from our nuclease digestions, CD spectra, and thermal denaturation profiles to reach both qualitative and quantitative conclusions concerning the similarities and differences between macro- and micronuclear chromatin and vertebrate chromatin.

A number of analyses of the thermal denaturation profiles of vertebrate chromatin have provided the outlines of a model that can be used to extract information about protein-DNA interactions and nucleosomal structure from such data. Core particles, containing 146 bp of DNA and an octamer of histones, undergo a biphasic thermal denaturation in low ionic strength

buffers (6-11). The initial, reversible phase of this transition involves melting 20-27 bp of DNA from each end of the core particle (6,7). The main hyperchromicity transition occurs simultaneously with the cooperative loss of histone core secondary structure and is irreversible (6). This transition involves the remaining 92 to 106 bp in the central portion of the DNA fragment. This model is based on thermal denaturation of core particles monitored by DNA hyperchromicity (6,7), CD of both DNA and protein components (6) and  $^{31}\text{P}$ -NMR (25) and nuclease digestion at high temperature (26). Comparative studies of nucleosome fragments containing different average DNA lengths (9,27) and of soluble chromatin with and without H1 present (11,13) have extended the basic model to chromatin.

Both chromatin and H1-depleted chromatin undergo multiphasic thermal denaturation (11,13). A characteristic difference between chromatin with and without H1 present is the large variation with ionic strength for the lowest melting transition when H1 is absent, while whole chromatin displays little or no ionic dependence for this transition (11). This low-melting transition apparently represents linker DNA which, in the absence of H1, behaves similarly to free DNA. The higher melting transitions are due to core particle DNA, and in particular, the highest temperature transition corresponds to the 90-100 bp central region of core DNA. In chicken erythrocyte chromatin, this DNA contributes 45% of the total hyperchromicity at 4 or 5 mM Na phosphate. The thermal denaturation profiles we recorded for macronuclear chromatin show four transitions in 0.2 mM Na<sub>2</sub> EDTA and three transitions at higher ionic strength (Figure 3). Of particular interest is the large ionic dependence of the lowest-melting transition (Figure 3 and Figure 4). This is consistent with the apparent lack of an H1 protein (Figure 1). In 4 mM Na phosphate, T<sub>m</sub>III of macronuclear chromatin (the most stable transition) represents approximately 40% of the total hyperchromicity. If this represents a similar region of stable core DNA (90-100bp), then one would estimate a mean nucleosomal repeat of 225-250 bp in Oxytricha macronuclei. Although this is slightly on the high side of the value derived from the nuclease digestion studies (220 bp), the values are sufficiently similar to suggest that the protein-DNA interactions contributing to this region of stability are essentially the same in Oxytricha as in higher eukaryotes.

The circular dichroism of chromatin has similarly been interpreted as representing a linear combination of two structural domains (11,24,28). The first of these has a spectrum similar to free B-form DNA and the second

has an altered structure that produces a large negative ellipticity at 275 nm. This altered DNA is associated with the core histones. Within this framework, the molar ellipticity at a given wavelength,  $\Delta\epsilon_\lambda$  is given by

$$\Delta\epsilon_\lambda = f_C \Delta\epsilon_\lambda^C + f_L \Delta\epsilon_\lambda^L$$

where  $f_C$  and  $f_L$  are the fraction of base pairs in the core and linker domains, respectively, and  $\Delta\epsilon_\lambda^C$  and  $\Delta\epsilon_\lambda^L$  are the molar CD signals characteristic of the core and linker DNA, respectively. Because the data can be adequately described with just these two terms,  $f_C + f_L = 1$ .

In H1-containing mononucleosomes ~140 bp of DNA exist in this altered structure. In H1-depleted mononucleosomes and core particles (at low ionic strength), ~105-110 bp have the altered structure (9,27). However, at higher ionic strength, about 30 additional base pairs are in the altered form in stripped chromatin; the CD of whole chromatin is essentially independent of ionic strength between 0-5 mM Na phosphate.

The CD of Oxytricha macronuclear chromatin also displays a substantial ionic strength dependence. In 4 mM Na phosphate, the CD (at 275 nm) of macronuclear chromatin is  $1.36 \text{ M}^{-1} \text{ cm}^{-1}$ . If we again assume that nucleosomal architecture is similar between Oxytricha and chicken, this ellipticity would be due to  $\approx 140$  bp of core particle DNA of altered structure plus linker DNA in the B-form structure. Using the characteristic ellipticity for chicken core particle DNA,  $0.58 \text{ M}^{-1} \text{ cm}^{-1}$ , and a measured value of  $2.67 \text{ M}^{-1} \text{ cm}^{-1}$  for free Oxytricha DNA (Figure 5), we then calculate a linker size of  $\approx 85$  bp from eqn. 1. Thus, the total nucleosomal repeat is calculated to be 225 bp, consistent with the results of the thermal denaturation analysis. These results are in good agreement with micrococcal nuclease digestion studies, which suggest a nucleosome repeat of approximately 220 bp (Figure 2 and Ref. 29). We note that the closely related hypotrich, Stylonychia mytilus, has also been reported to have a macronuclear repeat of 220 bp (30).

Although our data are more limited for micronuclear chromatin (because of problems associated with isolating the quantities needed for physical studies) we do see significant differences. Micrococcal nuclease digestion gives a nucleosomal repeat of approximately 176 bp (Figure 2). Melting profiles in 0.2 mM EDTA have a much smaller premelt region than those of macronuclear chromatin (Figure 6). We can tentatively assign 55-60% of the total hyperchromicity, representing the main melting band, to the 92-106 bp of highly stable core particle DNA. This agrees with the measured repeat

length for inactive micronuclear chromatin of  $\approx 165$ -185 bp (Figure 2). The CD spectrum at 275 nm is more suppressed than that of macronuclear chromatin, suggesting that a larger fraction of micronuclear DNA exists in an altered (i.e., non B-form) conformation. The CD data are also consistent with a repeat length of 170-180 bp. Thus, we conclude that the linker region of micronuclear chromatin is approximately 40-50 bp shorter than that of Oxytricha macronuclei. Whether the difference we observe reflects the difference in transcriptional activity between micro- and macronuclei, different histone variants, or some other factor, etc., is currently under investigation.

The macronucleus of the hypotrichs affords a unique opportunity to study the physical organization of chromatin containing single active genes. Comparison of these isolated structures with the properties of bulk macronuclear and micronuclear genes should provide considerable insight into the relationship between chromatin structure and gene regulation.

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+ Present address: The University of Texas System Cancer Center, Science Park—Research Division, P.O. Box 389, Smithville, TX 78957, USA

§Present address: Southern Research Institute, 2000 Ninth Avenue S., Birmingham, AL 35255, USA

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