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Subunit structure of a naturally occurring chromatin lacking histones F1 and F3

(*Tetrahymena*/macronuclei/micronuclei/staphylococcal nuclease)

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ABSTRACT **Macronuclei of the ciliated protozoan *Tetrahymena pyriformis* contain at least five classes of histones, including two with properties like those of histones F3 and F1 of higher eukaryotes. Micronuclei isolated under identical conditions contain little or no detectable F3 or F1. Digestion of both macronuclei and micronuclei with staphylococcal nuclease results in DNA fragments of discrete sizes. The electrophoretic mobilities of the larger fragments suggest that they are oligomers of the smallest ones. These results indicate that the periodic subunit structure observed in the chromatin of higher organisms also occurs in protozoans, and that this structure does not depend on the presence of either histone F1 or F3, even in an organism which has the genetic information for synthesizing these proteins.**

Recent studies indicate that the chromatin of higher organisms has an underlying subunit structure which can be visualized directly by electron microscopic techniques (1–5). The existence of this repeating structure can also be inferred by digesting isolated nuclei or chromatin with relatively nonspecific (endogenous or exogenous) nucleases. When used under conditions which completely degrade free DNA, these nucleases digest only about 50% of the DNA in chromatin to acid-soluble fragments (6). The majority of the undigested DNA is found in a nucleoprotein particle (7), and can be isolated as fragments containing approximately 50–200 base pairs (7–11). If nuclei or chromatin are only partially digested under conditions in which 10–30% of their DNA content becomes acid-soluble, the remaining DNA is found in an oligomeric series of nucleoprotein particles (10, 12). As first reported by Hewish and Burgoyne (13), the DNA molecules of such partial digests have discrete sizes. They also form an oligomeric series, the size of the larger fragments being integral multiples of the smallest fragment, which contains 100–200 base pairs (10, 12, 13).

Based on results of the type described above (10) and many of the physical, chemical and evolutionary properties of histones, Kornberg has proposed a model in which approximately 200 base pairs of DNA and eight histone molecules (two each of F3, F2A1, F2B, and F2A2) form basic repeating units which are joined in a flexible chain to form a chromatin fiber (14). He also suggested that a tetramer consisting of two molecules of F2A1 and two molecules of F3 formed the "core" of this repeating subunit. The extreme evolutionary conservatism, stoichiometry in chromatin, and dissociation properties of F2A1 and F3, coupled with the fact that these two histones form a 2:2 tetrameric complex in solution (15–17), were taken as support for the central role of an F3-F2A1 complex in chromatin structure (14). One possible test of this postulated role for F3 and F2A1 in chromatin would be to examine the structure of chromatin lacking one (or both) of these histones.

The macro- and micronuclei of the ciliated protozoan *Tetrahymena pyriformis* offer an opportunity to assess the role played by histone F3 in the structure of a naturally occurring chromatin. In ciliates, macro- and micronuclei are formed from daughter products of a mitotic division during the sexual process of conjugation and, in *Tetrahymena*, 80–90% of the DNA sequences in the two nuclei are the same (18). In this report, we present evidence that macronuclei isolated from *Tetrahymena pyriformis* contain histone F3, but that micronuclei do not. Nonetheless, when micronuclei are digested with staphylococcal nuclease, a series of DNA molecules of discrete sizes is obtained which is similar to that produced when macronuclei or calf thymus nuclei are digested under identical conditions.

MATERIALS AND METHODS

Cell Culture and Isolation of Nuclei. *Tetrahymena pyriformis* (strains HSM, WH-6, or B-1868-VII) were cultured in enriched proteose peptone and macro- and micronuclei were isolated by techniques which are described in detail elsewhere (19). Calf thymus nuclei were isolated as described by Shaw *et al.* (12).

Extraction and Electrophoretic Analysis of Histones. Histones were extracted from isolated macro- and micronuclei as described previously (20). Electrophoresis in long 2.5 M urea–15% polyacrylamide gels was performed by the method of Panyim and Chalkley (21). Gels were stained in fast green and were scanned in a Gilford spectrophotometer (22).

Nuclease Digestion and DNA Isolation. Digestion with staphylococcal nuclease (Worthington Biochemical Corp.) was performed at 37° either in 0.3 M sucrose, 1 mM $CaCl_2$, 0.5 mM Tris adjusted to pH 7.3 with solid cacodylic acid (digestion buffer of ref. 12) or in 0.3 M sucrose, 25 μM $CaCl_2$, 5 mM phosphate buffer, pH 6.7. The reaction was stopped by the addition of 3–4 volumes of buffer containing 1% sodium dodecyl sulfate, 0.5 M ethylenediaminetetraacetate, 18 mM Tris, pH 9.5 (23), and samples were incubated at 65° for at least 20 min. Pronase was added to give a concentration of 1 mg/ml and digestion was carried out for 4 hr at 50°. The digests were then diluted with an equal volume of water, extracted with water-saturated phenol, and precipitated overnight at −20° with 2 volumes of 95% ethanol. The precipitated DNA was collected by centrifugation, washed three times in 70% ethanol, dried briefly under vacuum, and resuspended in electrophoresis buffer (see below) containing 0.1 or 0.2 M sucrose and a small amount of bromophenol blue.

Agarose Gel Electrophoresis. Electrophoresis was carried

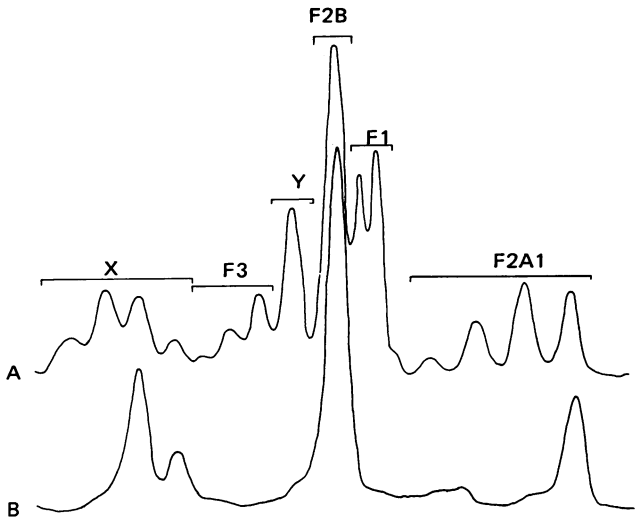

FIG. 1. Densitometer tracing of long polyacrylamide gels containing (A) macronuclear and (B) micronuclear histones of strain HSM. The identification of the electrophoretic fractions, except for X and Y which remain incompletely uncharacterized, is the subject of other communications (refs. 20 and 25; Johmann and Gorovsky, manuscript in preparation). Electrophoresis was at 250 V for 25 hr.

out in gels containing 1.5% agarose as described by Helling *et al.* (24). Fragments of DNA from phage P22 which had been digested by restriction endonuclease *Eco*RI (kindly provided by Dr. Meng-Chao Yao) were used as molecular weight markers. After electrophoresis gels were stained with 1–2 μg/ml of ethidium bromide in buffer and photographed on Polaroid 55 P/N film using a short-wavelength ultraviolet lamp for illumination.

Isotope Labeling and Counting Procedures. Macro- and micronuclei were labeled with [^{14}C]- and [^{3}H]thymidine as described previously (25). To determine the extent of digestion of labeled nuclei, appropriate-sized aliquots of the digests after Pronase digestion were pipeted onto 2.3 cm Whatman filter discs. Three filters from each sample were washed extensively in 5% trichloroacetic acid to remove acid-soluble radioactivity, while a duplicate set of filters was left unwashed to determine total radioactivity. Both sets of filters were digested with 0.5 ml of NCS (Amersham-Searle) at 50° for 3–12 hr and counted in Spectrafluor (Amersham-Searle). Disintegrations per minute were calculated as described previously (25). Agarose gels were soaked in 10% (v/v) glycerol for at least 1 hr, frozen, sliced into 1.0 or 1.5 mm segments, digested in NCS, and counted (25). Due to low levels of radioactivity, in some experiments, the disintegrations per minute for two slices were summed prior to plotting the data.

RESULTS

Histone F3 is present in macronuclei

Histone fraction F3 can be clearly identified in *Tetrahymena* macronuclei (C. A. Johmann and M. A. Gorovsky, manuscript in preparation). The bands labeled F3 in Fig. 1 have the same solubility and chromatographic properties as histone F3 of calf thymus when analyzed by the methods of Johns (26) or Sommer and Chalkley (27). These three bands are the only *Tetrahymena* histones which can be oxidized by atmospheric oxygen to give a single, slow-moving, band on acrylamide gels; this slow-moving band can, in turn, be reduced by mercaptoethanol to again yield the three molecular species seen in Fig. 1A. These bands are also the only

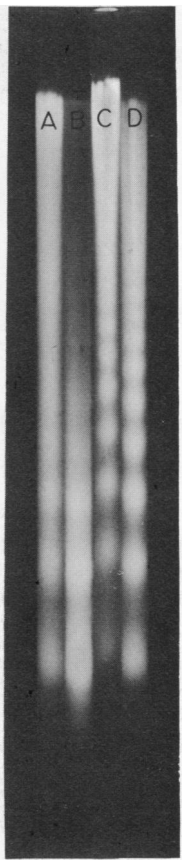

FIG. 2. Photograph of agarose gels containing nuclease digestion fragments of DNA from macronuclei (A, B) and calf thymus nuclei (C, D). Digestion was carried out at 37° in digestion medium containing 56 units/ml of enzyme and approximately 10^8 nuclei per ml. The percentage of trichloroacetic-acid-soluble material was not monitored in this experiment. Electrophoresis was at 22°, 100 V for 180 min in 150 mm × 6.0 mm gels. (A) macronuclei, 5 min digestion; (B) macronuclei, 15 min digestion; (C) calf thymus nuclei, 5 min digestion; (D) calf thymus nuclei, 15 min digestion.

fractions which become labeled when whole *Tetrahymena* histones are reacted with *N*-[^{14}C]ethylmaleimide. Thus, this fraction not only has solubility and chromatographic properties like calf thymus histone fraction F3, but, as is the case in many other organisms (28), is the only *Tetrahymena* histone which contains cysteine. The microheterogeneity of this fraction is probably due to acetylation (M. A. Gorovsky and J. B. Keevert, unpublished observations). The mobilities of the F3 subfractions are the same in all strains of *Tetrahymena* which have been examined, but differ slightly from those of calf thymus F3 (C. A. Johmann and M. A. Gorovsky, manuscript in preparation).

Histone F3 is absent in micronuclei

We have previously reported that histones extracted from isolated micronuclei contain little detectable histone F1 (25) or acetylated subspecies of F2A1 (20). In Fig. 1B, it is also apparent that histones extracted from micronuclei contain little or no histone F3. Histones extracted from micronuclei of strains HSM, WH-6, and B-1868-VII routinely contain only small amounts of F3, which can be accounted for by the low levels of macronuclear contamination which are usually found in isolated micronuclear preparations (19). F3 cannot be extracted from isolated micronuclei even if the entire nucleus isolation procedure is carried out in the presence of 0.5 M 2-mercaptoethanol to prevent possible losses

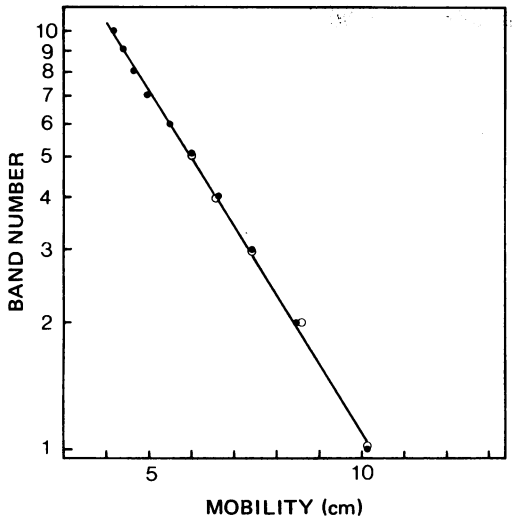

FIG. 3. Plot of the logarithm of the band number versus mobility on agarose gels. Electrophoresis was in 150 mm × 0.6 mm gel at 100 V for 150 min. ●, calf thymus digest; ○, macronuclear digest.

of F3 due to the formation of disulfide bonds with other proteins. We have previously described mixing experiments (25) which argue strongly against the possibilities that there are artifactual losses of histones from micronuclei during our isolation procedures. Also, micronuclear chromatin does not appear to be histone deficient when examined by a number of physical and chemical criteria (25). Rather, it appears that histone F1 and F3 (and Y) are not present in micronuclei and that their absence in micronuclear chromatin is compensated by increased amounts of the other *Tetrahymena* histones (F2A1, F2B, and X).

Staphylococcal nuclease digestion of macronuclei

There is some controversy regarding the extent of digestion and the significance of the DNA fragments which remain after prolonged digestion of chromatin with deoxyribonucleases (8, 29, 30). We have also found some variability in the extent of digestion, depending on the particular conditions employed. Therefore, we have chosen to examine the DNA fragments produced only after brief digestion with staphylococcal nuclease.

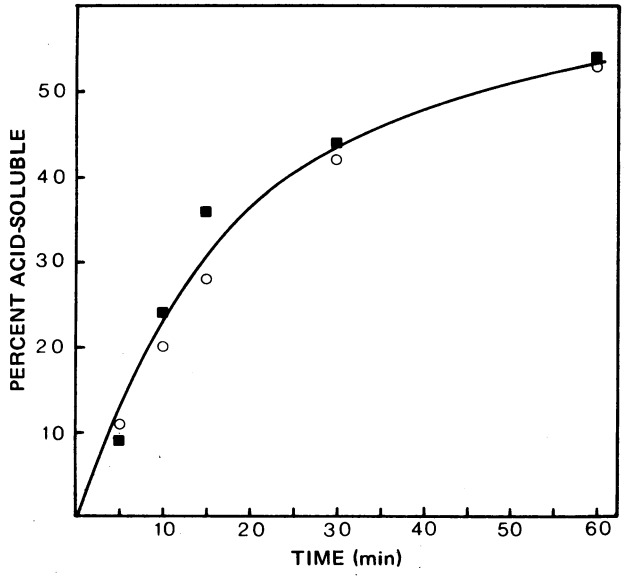

FIG. 4. Kinetics of digestion of [^{14}C]thymidine-labeled macro- and [^{3}H]thymidine-labeled micronuclear DNA. Digestion medium contained 1 mM $CaCl_2$, 10^8 calf thymus nuclei per ml as carrier, 10^7 macronuclei per ml, trace amounts of micronuclei, and 56 units/ml of nuclease. ○, macronuclear DNA; ■, micronuclear DNA. Digestion was at 37°. *Acid-soluble* refers to trichloroacetic acid.

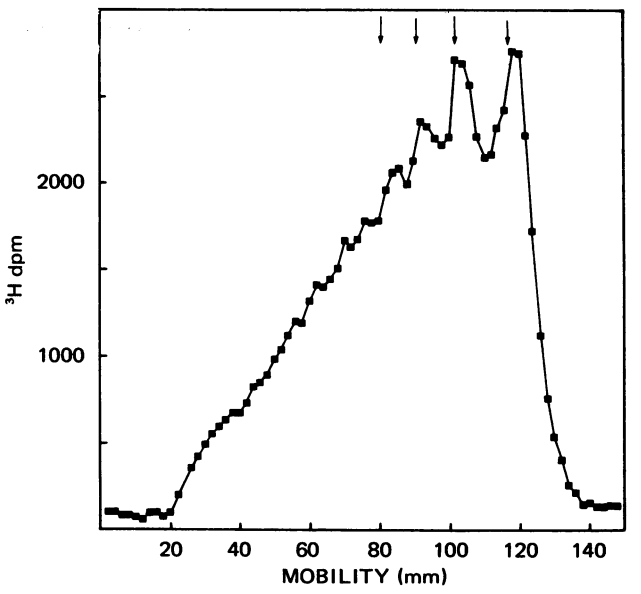

FIG. 5. Electrophoretic analysis of [^{3}H]thymidine-labeled micronuclear DNA after 5 min of digestion under the conditions described in Fig. 4. The radioactivity in the ^{14}C-labeled macronuclear DNA was too low to allow resolution of distinct peaks in this experiment. Electrophoresis was at 100 V for 150 min in 6.0 × 150 mm gels. The arrows mark the positions of the stained bands produced by digestion products of the calf thymus nuclei.

If, after limited digestion, macronuclear DNA is isolated and examined by electrophoresis on agarose gels, a series of bands is seen (Fig. 2) whose relative electrophoretic mobilities are indistinguishable from those of similarly digested calf thymus nuclei (Figs. 2 and 3). When the logarithms of

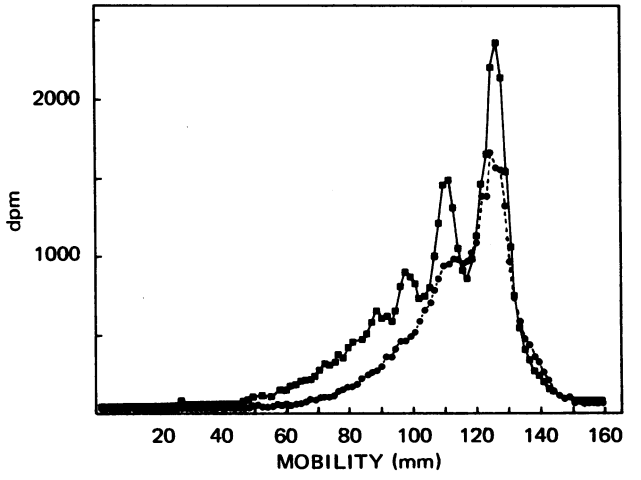

FIG. 6. Electrophoretic analysis of partially digested [^{3}H]thymidine-labeled micronuclear DNA (6% soluble in trichloroacetic acid) and [^{14}C]thymidine-labeled macronuclear DNA (10% soluble in acid). The digestion medium contained 25 μM $CaCl_2$ and 48 units/ml of nuclease. The number of nuclei was not determined because of partial lysis during washing in digestion medium. Digestion was for 10 min at 37°. Electrophoresis was at 100 V for 180 min in 6.0 × 150 mm gels. The 160 mm length of the gel probably represents expansion upon freezing and error in the nominal size (1.5 mm) of the slices. ■, micronuclei; ●, macronuclei.

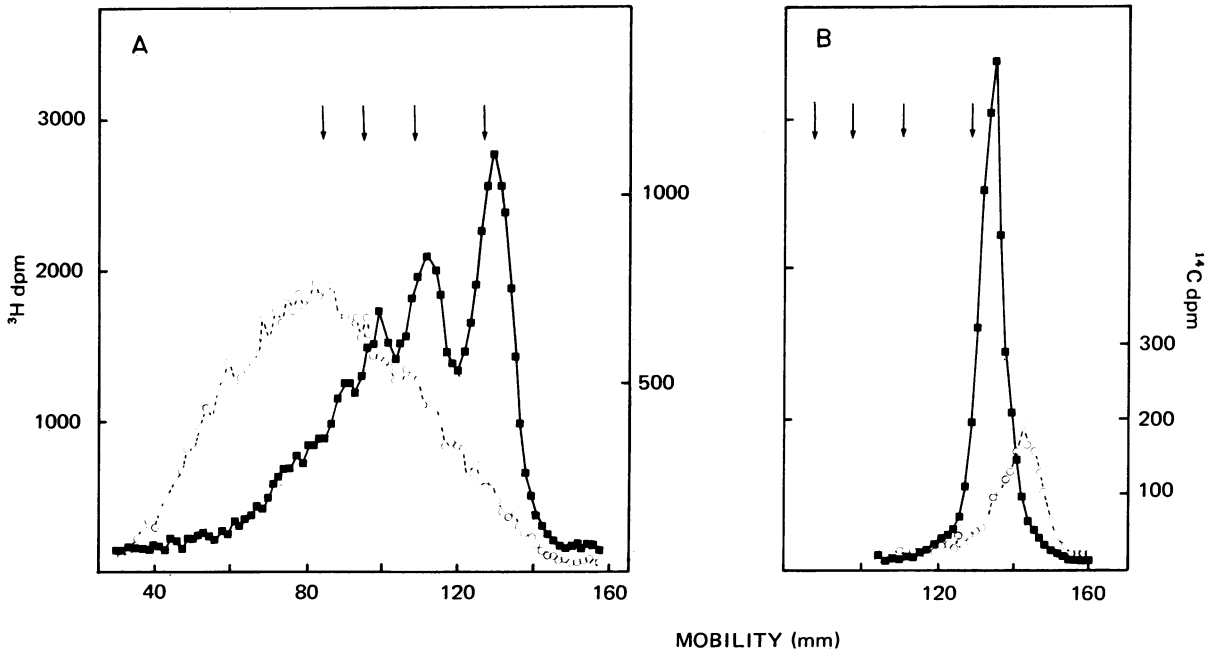

FIG. 7. Electrophoretic analysis of [^{3}H]thymidine-labeled micronuclear DNA and [^{14}C]thymidine-labeled macronuclear DNA. The digestion mixture contained 1 mM $CaCl_2$, 50 units/ml of nuclease, 2.0 mg/ml of *free* [^{14}C]thymidine-labeled macronuclear DNA and trace amounts of [^{3}H]thymidine-labeled micronuclei. Electrophoresis was as in Fig. 6. (A) 5 min digestion. About 5% of the micronuclear DNA and 10% of the free macronuclear DNA were acid-soluble. (B) 30 min digestion. About 25% of the micronuclear DNA and 50% of the macronuclear DNA were acid-soluble. ■, micronuclear DNA; ○, free macronuclear DNA. The arrows indicate the positions of stained bands produced by adding carrier calf thymus digestion products to each gel immediately prior to electrophoresis.

the band numbers are plotted against electrophoretic mobility, the data can be approximated by a straight line (Fig. 3), suggesting that the sizes of the larger DNA fragments are integral multiples of that of the smallest fragment. When coelectrophoresed with fragments from an *Eco*RI digest of phage P22 DNA, the mass of the basic repeating unit of both calf thymus and *Tetrahymena* macronuclei is found to be approximately 0.95–1.05 × 10^5 daltons or about 150 base pairs. Thus, the macronuclear chromatin of *Tetrahymena pyriformis* has a subunit structure which is remarkably similar to that of higher eukaryotic organisms.

Staphylococcal nuclease digestion of micronuclei

When isolated micronuclei are digested with staphylococcal nuclease, the kinetics of digestion are similar to those of macronuclei (Fig. 4). After partial digestion, the micronuclear DNA fragments can be clearly resolved into a series of oligomeric subunits with electrophoretic mobilities which are similar to (but may be slightly greater than) those of calf thymus digestion products run in the same gel (Fig. 5). Similar results are obtained (Fig. 6) when nuclei are digested under conditions (25 μM $CaCl_2$) reported to prevent the migration of histones from one DNA molecule to another (6). If micronuclei are digested in the presence of a large excess of free macronuclear DNA (Fig. 7), a similar pattern is obtained. The digestion pattern of the free DNA in this experiment indicates that production of an oligomeric series of DNA molecules is not a function of restricted site specificity of the action of staphylococcal nuclease on *Tetrahymena* DNA. Thus, the products of digestion of micronuclei are very similar to those of macronuclei or calf thymus nuclei, even when digestion is carried out under conditions in which migration of histones is prevented (Fig. 6) and in which there is no source of exogenous F3 to associate with micronuclei (Fig. 7).

DISCUSSION

We have presented evidence that macro- and micronuclear chromatin of the ciliated protozoan *Tetrahymena pyriformis* has an underlying particulate subunit structure which can be demonstrated by digestion with staphylococcal nuclease. Therefore, the particulate structure of chromatin which has been demonstrated for the chromatin of higher organisms (1–7, 10–13), occurs in protozoans also. Coupled with the recent demonstration of subunit structure in yeast (31), these results make it likely that this structure is a basic feature of eukaryotic chromatin. Since macronuclei divide amitotically (see ref. 32 for a description of the properties of *Tetrahymena* macro- and micronuclei), it is also clear that this structural organization is not restricted to nuclei capable of mitotic division.

We have also presented evidence that micronuclei do not contain histones F3 (this paper) or F1 (25). The absence of F3 in micronuclei which divide mitotically, coupled with the presence of this histone in macronuclei (which divide amitotically) makes it unlikely that this histone plays an indispensible role in mitotic chromosome condensation as has been suggested (33, 34). Evidence has been presented which indicates that micronuclear chromatin is organized in a particulate fashion similar to that of macronuclei and of calf thymus. These results make it unlikely that histone F3 is essential for the maintenance of the particulate subunit structure of chromatin as has been suggested (14). A similar conclusion has been reached by Lohr and Van Holde (31) based on nuclease digestion studies of yeast chromatin, which is also reported to be missing histones F3 and F1 (35). It could have been argued that the existence of particulate subunit structure of yeast chromatin without F3 was a case of convergent evolution in which one of the (as yet) uncharacterized yeast histones played a structural role homologous to that of F3 in higher organisms. This explanation seems un-

likely in the case of *Tetrahymena*, in which a typical histone F3 is found in the macronucleus. This conclusion, i.e., that the presence of F3 is not essential for the particulate structure of chromatin, is quite independent of whether F3 is actually present in micronuclear (or yeast) chromatin *in vivo*. It should be noted that these findings do not rule out the possibility that in chromatin which contains F3, this histone is complexed with F2A1 to form a tetramer which is important in chromatin structure. However, to our knowledge, there is no direct evidence for the existence of such a tetramer *in vivo*, and recent studies of the effects of aldehyde fixation of histones in chromatin did not reveal any preferential association between histones F3 and F2A1 (36).

Finally, it should now be possible to study the microheterogeneity of nuclease digestion products in *Tetrahymena* macro- and micronuclei and to compare these after extraction of other histones by gentle techniques. In this way, it may be possible to elucidate the role of particular histones in the subunit structure of chromatin.

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1. Olins, A. L. & Olins, D. E. (1974) *Science* **183**, 330–332.
2. Woodcock, C. L. F. (1973) *J. Cell Biol.* **59**, 368a.
3. Langmore, J. P. & Wooley, J. C. (1974) *J. Cell Biol.* **63**, 185a.
4. Griffith, J. D. (1975) *Science* **187**, 1202–1203.
5. Oudet, P., Gross-Bellard, M. & Chambon, P. (1975) *Cell* **4**, 281–300.
6. Clark, R. J. & Felsenfeld, G. (1971) *Nature New Biol.* **229**, 101–106.
7. Sahasrabuddhe, C. G. & Van Holde, K. E. (1974) *J. Biol. Chem.* **249**, 152–156.
8. Clark, R. J. & Felsenfeld, G. (1974) *Biochemistry* **13**, 3622–3628.
9. Axel, R., Melchior, W., Jr., Sollner-Webb, B. & Felsenfeld, G. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 4101–4105.
10. Noll, M. (1974) *Nature* **251**, 249–251.
11. Oosterhof, D. K., Hozier, J. & Rill, R. L. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 633–637.
12. Shaw, B. R., Corden, J. L., Sahasrabuddhe, C. G. & Van Holde, K. E. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1193–1198.
13. Hewish, D. R. & Burgoyne, L. A. (1973) *Biochem. Biophys. Res. Commun.* **52**, 504–510.
14. Kornberg, R. D. (1974) *Science* **184**, 868–871.
15. Kornberg, R. D. & Thomas, J. O. (1974) *Science* **184**, 865–868.
16. Roark, D. E., Geohegan, T. E. & Keller, G. H. (1974) *Biochem. Biophys. Res. Commun.* **59**, 542–547.
17. D'Anna, J. & Isenberg, I. (1974) *Biochemistry* **13**, 4992–4997.
18. Yao, M. C. & Gorovsky, M. A. (1974) *Chromosoma* **48**, 1–18.
19. Gorovsky, M. A., Yao, M. C., Keevert, J. B. & Pleger, G. L. (1975) *Methods in Cell Biol.*, ed. Prescott, D. (Academic Press, New York) Vol. IX, 311–327.
20. Gorovsky, M. A., Pleger, G. L., Keevert, J. B. & Johmann, C. A. (1973) *J. Cell Biol.* **57**, 773–781.
21. Panyim, S. & Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337–346.
22. Gorovsky, M. A., Carlson, K. & Rosenbaum, J. L. (1970) *Anal. Biochem.* **35**, 359–370.
23. Kavenoff, R. & Zimm, B. (1973) *Chromosoma* **41**, 1–27.
24. Helling, R. B., Goodman, H. M. & Boyer, H. W. (1974) *J. Virol.* **14**, 1235–1244.
25. Gorovsky, M. A. & Keevert, J. B. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 2672–2676.
26. Johns, E. W. (1964) *Biochem. J.* **92**, 55–59.
27. Sommer, K. R. & Chalkley, R. (1974) *Biochemistry* **13**, 1022–1027.
28. Panyim, S., Chalkley, R., Spiker, S. & Oliver, D. (1970) *Biochim. Biophys. Acta* **214**, 216–221.
29. Mirsky, A. E. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2945–2948.
30. Oliver, D. & Chalkley, R. (1974) *Biochemistry* **13**, 5093–5098.
31. Lohr, D. & Van Holde, K. E. (1975) *Science* **188**, 165–166.
32. Gorovsky, M. A. (1973) *J. Protozool.* **20**, 19–25.
33. Sadgopal, A. & Bonner, J. (1970) *Biochim. Biophys. Acta* **207**, 227–239.
34. Bonner, J. & Garrard, W. T. (1974) *Life Sci.* **14**, 209–221.
35. Franco, L., Johns, E. W. & Navlet, J. M. (1974) *Eur. J. Biochem.* **45**, 83–89.
36. Chalkley, R. & Hunter, C. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 1304–1308.