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Absence of histone F1 in a mitotically dividing, genetically inactive nucleus

(chromatin/phosphorylation/*Tetrahymena*/macronucleus/micronucleus)

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ABSTRACT **Histones were extracted from macro- and micronuclear chromatin of the ciliated protozoan *Tetrahymena pyriformis*. Conditions that resulted in macronuclear chromatin containing large amounts of histone F1 yielded micronuclear chromatin in which this histone was absent. Evidence is presented indicating that the absence of F1 from micronuclei is not a preparative artifact and that histone F1 is replaced by other histone fractions. Since micronuclei divide mitotically, while macronuclei divide amitotically, these results suggest that histone F1 and its phosphorylation do not play an indispensable role in the process of mitotic chromosome condensation, in chromosome replication, or in the separation of newly synthesized chromatids.**

Like most ciliated protozoans, the vegetative cells of *Tetrahymena pyriformis* contain a macro- and a micronucleus. These two nuclei are formed from the daughter products of a single mitotic division during the sexual process of conjugation. It is likely, therefore, that they contain the same, or closely related, genetic information. In spite of their common origin and the fact that they reside in the same cytoplasmic millieu, macro- and micronuclei differ considerably in their structure and function (see ref. 1 for review). For example, macronuclei divide amitotically, with no sign of mitotic chromosomes; micronuclei divide mitotically (2–7). In addition, the two nuclei differ greatly in their genetic activity. Macronuclei are sites of intense RNA synthesis while micronuclei synthesize little, if any, detectable RNA (8, 9).

We have recently reported that the macronucleus of *Tetrahymena* contains a histone with properties similar to those of histone F1 of higher organisms (10). Macronuclear F1 isolated from rapidly growing cells was found to be highly phosphorylated, while histones isolated from slowly growing cells contained only small amounts of phosphorylated F1. Thus, the relationship between the rate of cell replication and the extent of phosphorylation of histone F1 observed in a variety of other cell types (11–16) was also observed in *Tetrahymena*. Recent studies of histone phosphorylation in different cells have led to the various suggestions that phosphorylation of histone F1 may be involved in the alteration of chromatin structure during interphase (17), in chromosome replication (18, 19), in the sorting out of newly synthesized chromatids during S-phase (20), in the process of mitotic chromosome condensation (21–23) or in the control of genetic activity (24–26). Since macronuclei divide amitotically, we concluded from these studies that phosphorylation of histone F1 probably does not play a role exclusively in the process of mitotic chromosome condensation.

Due to the striking functional and structural differences between macro- and micronuclei, it seemed that further analysis of histone F1 and its phosphorylation in *Tetrahymena* micronuclei might shed additional light on the function of this histone in eukaryotic nuclei. In this report, we describe the surprising finding that isolated micronuclei of *Tetrahymena* do not contain any detectable histone F1.

MATERIALS AND METHODS

Cell Culture. *Tetrahymena pyriformis*, strains WH-6 (Syngen 1, mating type I; a micronucleate strain) and GL (an amicronucleate strain), were cultured as described (27).

Isolation of Nuclei and Chromatin. Nuclei were isolated as described (27, 28). Chromatin was isolated by a modification of the method of Panyim *et al.* (29). Chromatin deficient in histone fraction F1 was prepared by washing isolated nuclei repeatedly in 0.5 M NaCl. This treatment was found to remove most of histone F1 from macronuclei while extracting little of the other histone fractions.

Isolation of Histones. Histones were isolated from whole nuclei or from chromatin by extraction with either 2.4 M urea/0.2 M H_2SO_4 or with 0.2 M H_2SO_4 (10, 27).

Thermal Denaturation of Isolated Chromatin. Solubilized macro- or micronuclear chromatin was fixed in 0.91% formaldehyde for at least 15 hr and then dialyzed for at least 5 hr against three changes (100 volumes each) of 0.2 mM EDTA. DNA isolated from *Tetrahymena* macronuclei was similarly treated and served as a control. Increase in absorbance at 260 nm with heating was measured in a Gilford 2400 spectrophotometer. The heating rate was approximately 0.3° per min. Turbidity was monitored at 320 nm and was found to be negligible.

Buoyant Density of Isolated Chromatin in CsCl. Solubilized chromatin was fixed for at least 24 hr in 0.91% formaldehyde and was then centrifuged to equilibrium in gradients containing 5.3 ml of 38.5% CsCl (ρ = 1.40) in 0.2 mM EDTA. Centrifugation was performed in a Beckman SW-50 rotor at 32,000 rpm at 20° for at least 60 hr. Fractions were collected directly into scintillation vials, and radioactivity was determined as described below.

Acrylamide Gel Electrophoresis. Electrophoresis in long (25 cm) 2.5 M urea/15% polyacrylamide gels was performed by the method of Panyim and Chalkley (30). Gels were stained in fast green and subjected to densitometric analysis as described (10, 31).

Isotopic Labeling and Counting Procedures. For labeling histones, cells of strain WH-6 were grown for at least 3 generations in medium containing 1 μCi/ml of [^{3}H]lysine (L-[G-^{3}H]lysine, 3.2 Ci/mmol, 1 mCi/ml, New England Nuclear Corp.). To measure the amount of macronuclear contamination in the micronuclear preparation, we similarly labeled a culture of the amicronucleate strain GL with 0.5

μCi/ml of [^{14}C]lysine (L-[*U*-^{14}C]lysine, 312 mCi/mmol, 0.1 mCi/ml, New England Nuclear). The two cell types were mixed immediately before isolation of nuclei. Any ^{14}C appearing in micronuclear histones must be due to contamination (the ^{14}C-labeled GL cells have no micronuclei), and the degree of contamination could be estimated by comparison with the amount of ^{14}C in the macronuclear histones.

The DNA of chromatin was labeled by growing cells in enriched proteose peptone (from which yeast extract had been omitted) containing either 0.2 μCi/ml of [^{14}C]thymidine ([*methyl*-^{14}C]thymidine, 52.7 mCi/mmol, 100 μCi/ml, New England Nuclear) or 1.0 μCi/ml of [^{3}H]thymidine ([*methyl*-^{3}H]thymidine, 50.7 Ci/mmol, 0.5 mCi/ml, New England Nuclear).

Polyacrylamide gels were sliced into 1-mm segments and counted in either 5 or 10 ml of fluor (Spectrafluor, Amersham-Searle, Arlington Heights, Ill.), containing 0.25 ml of NCS (Amersham-Searle). Fractions from CsCl gradients were counted in PCS (Amersham-Searle). All dual isotope experiments were corrected for spillover of ^{14}C into the ^{3}H channel by use of the automatic external standard on a Nuclear Chicago Isocap 300 scintillation counter. In later experiments, efficiency corrections were performed with a Nova 2 computer (Data General Corp.).

RESULTS

Histone Fraction F1 Is Absent in Micronuclei. We have previously reported (10) that F1 isolated from *Tetrahymena* macronuclei is similar to the F1s of other organisms in most of its chemical and physiological properties (perchloric acid solubility, molecular weight, amino-acid composition, phosphorylation associated with cell division, etc.). However, *Tetrahymena* F1 differs from other F1s in having a unique electrophoretic mobility in urea/acrylamide gels of low pH, migrating between fraction F2B and the unacetylated form of fraction F2A1 (10). In macronuclei (Fig. 1A) this fraction is intensely labeled when cells are grown in radioactive lysine. However, there is little or no radioactivity in this region of a gel containing histones extracted from micronuclear chromatin (Fig. 1B). The absence in this gel of any detectable ^{14}C-labeled histones from the macronuclei of the amicronucleate cells attests to the purity of the micronuclear histones. While the experiment in Fig. 1 illustrates one of our best efforts in preparing micronuclear histones completely free of macronuclear contamination, histone preparations isolated from micronuclear chromatin routinely contain little or no F1. The variable amounts of F1 found in some preparations of micronuclear histones can probably be accounted for by low levels (0–20%) of macronuclear contamination frequently found in micronuclear preparations.

Absence of Histone F1 from Micronuclei Is Not a Preparative Artifact. To demonstrate that the isolation of micronuclei does not lead to an artifactual loss of F1, we performed a mixing experiment. Cells of strain WH-6 were labeled with [^{14}C]lysine as described for strain GL. Macronuclei were isolated and fragmented by blending in a Virtis blender. The ^{14}C-labeled macronuclear fragments were then added to [^{3}H]lysine-labeled cells (strain WH-6) and macro- and micronuclei were isolated. Carrier (unlabeled) macronuclei were then added to the micronuclear fraction, chromatin was isolated, and the histones were extracted and analyzed. Fig. 2 shows that ^{14}C-labeled F1 derived from the added macronuclear fragments was present in normal amounts (relative to the other macronuclear histones) in the micronuclear fraction. The histones extracted from [^{3}H]lysine-labeled micronuclei, on the other hand, were deficient in F1. Since the ^{3}H-labeled micronuclei and the ^{14}C-labeled macronuclear fragments should have been subjected to identical conditions during isolation, it is unlikely that the absence of histone F1 from micronuclei is due to loss during isolation.

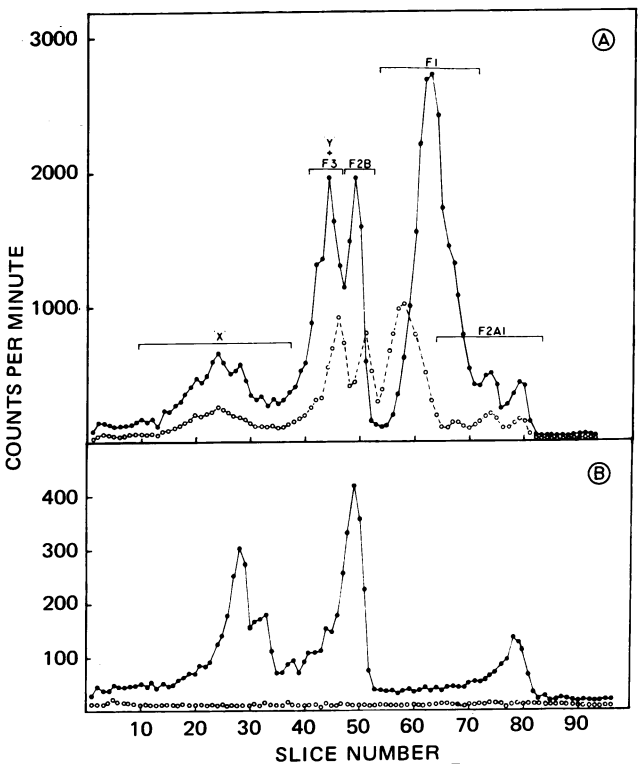

FIG. 1. Electrophoretic comparison of macro- and micronuclear histones. (A) [^{3}H]Lysine-labeled macronuclear histones (●) from strain WH-6 and [^{14}C]lysine-labeled histones (○) from strain GL. (B) [^{3}H]Lysine-labeled histones (●) from micronuclei of strain WH-6 and [^{14}C]lysine-labeled histones from strain GL (○). The identification of the electrophoretic fractions (except for X and Y, which remain uncharacterized) of histones extracted from macronuclei of strain WH-6 has been described (10, 35). The differences in the electrophoretic profiles of histones isolated from macronuclei of strains GL and WH-6 will be discussed elsewhere (C. A. Johmann and M. A. Gorovsky, in preparation). Other differences between the histone complements of macro- and micronuclei, such as the absence of fractions F3 and Y and of acetylated subspecies, are the subject of other communications (ref. 25; M. A. Gorovsky and J. B. Keevert, in preparation).

Comparison of Micronuclear Chromatin with F1-Deficient Macronuclear Chromatin. The thermal denaturation and buoyant density of DNA in chromatin are greatly influenced by the amount of protein with which the DNA is associated (32–35). Therefore, we compared the thermal denaturation properties and the buoyant density of micronuclear chromatin with macronuclear chromatin and with macronuclear chromatin from which histone F1 was removed. Table 1 and Fig. 3 demonstrate that the half melting temperature (T_m) of micronuclear chromatin is essentially the same as that of macronuclear chromatin, and is distinctly different from that of macronuclear chromatin from which histone F1 has been removed by 0.5 M NaCl. Washing micronuclei with 0.5 M NaCl prior to isolating chromatin results in only a small change in its thermal denaturation properties (Fig. 3).

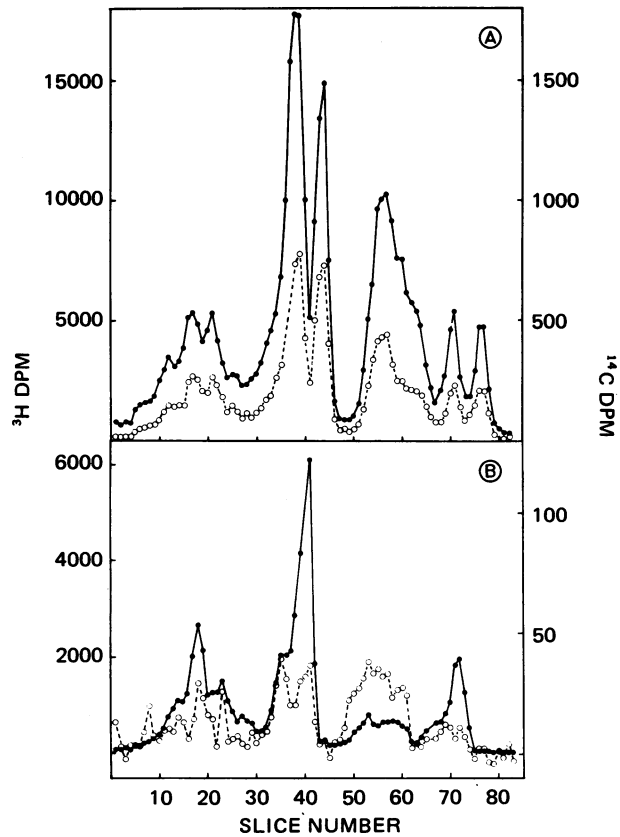

FIG. 2. Electrophoretic comparison of macro- and micronuclear histones. (A) [^{3}H]Lysine-labeled macronuclear histones (●) from strain WH-6 and [^{14}C]lysine-labeled histones (○) from added macronuclei of strain WH-6. (B) [^{3}H]Lysine-labeled histones (●) from micronuclei of strain WH-6 and [^{14}C]lysine-labeled histones (○) from added macronuclei of strain WH-6. All samples in panel B were counted for 10 min. The small deviations below zero in this figure result from the fact that all vials containing NCS and fluor were first counted before the gel slices were added and digested. Background counts were then subtracted for each vial.

Fig. 4 demonstrates that the buoyant density of formaldehyde-fixed micronuclear chromatin in CsCl is different from that of macronuclear chromatin from which histone F1 has been removed. The difference in buoyant densities of macro- and micronuclear chromatin seen in Fig. 4 may be due to different amounts of non-histone protein associated with the two types of chromatin (see below). Washing micronuclei with 0.5 M NaCl results in little change in the buoyant density of micronuclear chromatin.

Histone:DNA Ratios of Macro- and Micronuclear Chromatins. Since the thermal denaturation properties and the buoyant density of micronuclear chromatin were unlike those of macronuclear chromatin devoid of histone F1, it seemed likely that increases in the amounts of other micronuclear histones served to compensate for the absence of histone F1. To see if this was the case, we have compared the amounts of acid-soluble material extracted from macro- and micronuclear chromatin which migrates in histone regions of polyacrylamide gels with the amounts of DNA of those chromatins. In Exps. 1 and 2 (Table 2) histones were measured by quantitative microdensitometry of gels stained with fast-green (31). Because macro- and micronuclei contain different histone fractions (Fig. 1; ref. 27) and the color yields of the individual histone–fast-green complexes are unknown, we also compared the histone:DNA ratio of the two

Table 1. Summary of thermal denaturation properties of macro- and micronuclear chromatin

Chromatin	No. of exps.[a]	T_m	Hyperchromicity[b]
Macronucleus	4	69.4	24.7
Micronucleus	4	69.2	26.7
Macronucleus salt-washed	3	65.1	29.9
Micronucleus salt-washed	3	67.8	21.6
DNA[c]	11	34.0	40.3

[a] Values are for independent isolations of chromatin. Values for DNA represent 11 experiments on the same sample.
[b] Calculated as % increase in absorbance at 260 nm at 86° over that at room temperature. Values are uncorrected for thermal expansion of water.
[c] DNA was from isolated macronuclei. The thermal denaturation properties of macro- and micronuclear DNAs are indistinguishable.

chromatins by isotopic techniques (Exp. 3, Table 2); it seemed unlikely that the incorporation of a mixture of radioactive amino acids would lead to the same systematic errors as the staining technique. The data of Table 2 show that the histone:DNA ratios of macro- and micronuclear chromatins are essentially the same.

DISCUSSION

In some experiments we noted that histones extracted from isolated micronuclear preparations contained less F1 than might be expected from the amounts of macronuclear contamination. Similarly, when histones were extracted from small quantities of macronuclei, less F1 (relative to the other histone fractions) was often recovered than if histones were extracted from a larger number of nuclei. The reason for this failure to recover small amounts of F1 is unknown. Histone F1 is also highly susceptible to proteolytic degradation (36) and is the easiest histone to dissociate from the chromatin with salts (37) and with acids (38). Therefore, it was necessary to demonstrate that the absence of F1 in histones extracted from micronuclei was not due to artifactual loss during isolation of nuclei or extraction of histones. In this regard, it should be noted that *(i)* comparisons have only been made between macro- and micronuclei isolated from the same cells, *(ii)* the methods used to separate macro- and micronuclei during isolation are physical techniques (centrifugation, filtration) which would not be expected to dissociate any histones from chromatin, and *(iii)* the isolation procedures are known to inhibit proteolysis of histone F1 in macronuclei (10).

We have also performed a mixing experiment in which ^{14}C-labeled macronuclei were added to a micronuclear

Table 2. Histone/DNA in macro- and micronuclear chromatin

Exp. no.	Macronucleus[a,b]	Micronucleus[a]
1[c]	1.00	0.98
2[d]	1.00	0.86
3[e]	1.00	1.25

[a] Values in arbitrary units.
[b] Value set = 1.0.
[c] DNA by diphenylamine; histone by fast green.
[d] DNA by A_{260}; histone by fast green.
[e] DNA by [^{14}C]thymidine; histone by [^{3}H]aminoacid mixture.

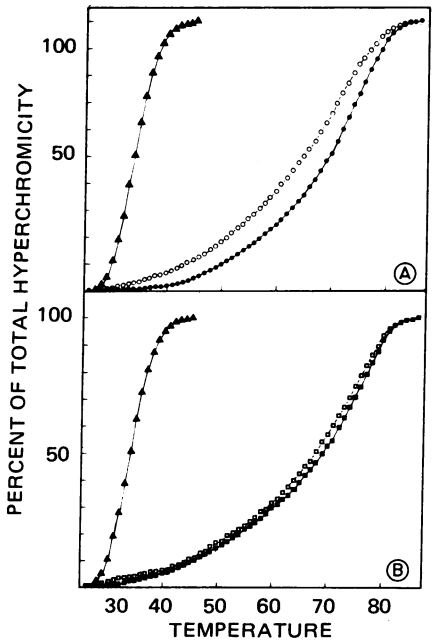

FIG. 3. Thermal denaturation profile of macro- and micronuclear chromatins. (A) Denaturation of macronuclear chromatin (●) has been compared with that of macronuclear chromatin isolated from nuclei that had been washed with 0.5 M NaCl to remove histone F1 (○). (B) Thermal denaturation profiles of micronuclear chromatin (■) and micronuclear chromatin from salt-washed nuclei (□) are similarly compared. The thermal denaturation profile of purified macronuclear DNA (▲) is included for comparison with the various chromatins. The thermal denaturation profile of purified micronuclear DNA is indistinguishable from that of macronuclear DNA (M. -C. Yao and M. A. Gorovsky, unpublished observations). All data points are average values from at least three experiments (see text and Table 1 for additional details).

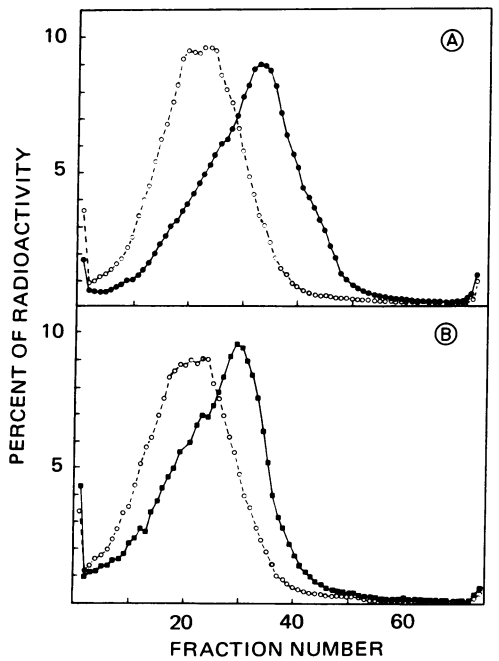

FIG. 4. Buoyant density of macro- and micronuclear chromatin in CsCl. (A) [^{3}H]Thymidine-labeled macronuclear chromatin (●) and [^{14}C]thymidine-labeled chromatin from salt-washed macronuclei (○). (B) [^{3}H]Thymidine-labeled micronuclear chromatin (■) and [^{14}C]thymidine-labeled chromatin from salt-washed macronuclei (○). The gradient contained 38.5% CsCl in 0.2 mM EDTA. Centrifugation was in a Spinco SW-50 rotor at 32,000 rpm for 63 hr at 18°. Approximately 37,000 ^{3}H counts and 19,000 ^{14}C counts were in gradient A, while approximately 23,000 ^{3}H counts and 18,000 ^{14}C counts were in gradient B.

preparation that had been labeled with a tritiated amino acid. In this experiment, care was also taken to add large amounts of carrier (unlabeled) macronuclei to insure good recovery of F1. The ^{3}H profile of gels containing histones from such a mixture of nuclei showed a typical micronuclear pattern, while the ^{14}C profile (and the absorbance) showed a typical macronuclear pattern, with normal amounts of histone F1 (Fig. 2). We conclude from this mixing experiment that the absence of F1 from micronuclei reflects an intrinsic difference between these two nuclei. In addition, the thermal denaturation properties and the buoyant density of micronuclear chromatin are different from those of macronuclear chromatin from which histone F1 has been removed by salt, and the histone:DNA ratios of macro- and micronuclear chromatins are similar. We have also examined the (unlikely) possibility that micronuclear chromatin contains histone F1, but in a form that is not easily extracted with acids. Accordingly, we have extracted the acid-insoluble residues of macro- and micronuclear chromatins with 0.1% sodium dodecyl sulfate. No additional histone-like material was found when these extracts were examined on polyacrylamide gels containing dodecyl sulfate.

Taken together, these results argue strongly against the possibility that the absence of histone F1 from micronuclear chromatin is a preparative artifact. Thus, while studies on isolated cellular components cannot *unequivocally* rule out the possibility that, *in vivo*, micronuclear chromatin also contains histone F1 and has a higher histone:DNA ratio than macronuclear chromatin, there is no evidence that micronuclear chromatin is in any way deficient in its histone content when compared to macronuclear chromatin. It is more likely that histone F1 has been replaced in micronuclear chromatin by increased amounts of the other micronuclear histones (X, F2B, and unacetylated F2A1; the properties of histone F3 and Y that also seem to be absent in micronuclei will be discussed in a later communication).

The observation that chromatin isolated from micronuclei does not contain any detectable histone fraction F1 reinforces our previous suggestion (10) that histone F1 and its phosphorylation do not function uniquely in the process of mitotic chromosome condensation (16, 21–23). Thus, histone fraction F1 is present and is phosphorylated in macronuclei, which divide amitotically, with little or no change in chromatin organization; F1 is absent in micronuclei, which divide mitotically and show distinct changes in chromatin condensation during the cell cycle (2–7). Similarly, the absence of histone F1 from micronuclear chromatin makes it unlikely that this histone fraction (or its phosphorylation) plays an indispensable role in the process of chromosome replication (18), or in the sorting out of sister chromatids (20). Recent reports (39, 40) that F1 is not found in the histone complement of some lower eukaryotes also support these conclusions. Of course, it is possible that, if present, F1 must be phosphorylated for any or all of these processes to occur.

Our findings of extensive phosphorylation of F1 in macronuclei coupled with the absence of F1 in micronuclei would seem to argue against the recent suggestion that phosphorylation of F1 is an important step in the initiation of mitosis (21, 41). However, since macronuclei control the vegetative functions of *Tetrahymena*, it is still possible that phosphorylation of F1 plays some role in the control of cell divi-

sion. Such a role, however, would have to be independent of whether the nuclear division occurs by a mitotic or an amitotic mechanism.

Finally, because macro- and micronuclei show extreme differences in their genetic activity, we are tempted to suggest that histone F1 functions in the control of genetic activity in eukaryotic nuclei. Phosphorylation of histone F1 is clearly correlated with the rate of cell division in genetically active nuclei of a variety of cell types, including *Tetrahymena* (10–16). This process could be associated with an event, occurring once per cell cycle, during which the specific DNA sequences that code for RNA within a genome might be altered. Such a function for F1-phosphorylation would account for its correlation with cell division and for the fact that it seems to occur at different times in the cell cycle in different cell types. It should be noted that the phosphorylation of F1 itself is probably not gene specific (20). It could, nonetheless, be a necessary step in changing the state of activity of specific genes. In this respect, it is interesting to note that cells frequently must undergo a normal division cycle before differentiating (42).

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