IMMUNOFLUORESCENCE EVIDENCE FOR THE ABSENCE OF HISTONE H1 IN A MITOTICALLY DIVIDING, GENETICALLY INACTIVE NUCLEUS

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ABSTRACT

Antibodies directed against whole histone and purified lysine-rich histone H1 extracted from isolated macronuclei of the ciliate *Tetrahymena* were obtained and conjugated to fluorescein isothiocyanate. The fluorescein-antibody conjugates were used to directly label *Tetrahymena* cells. Both macro- and micronuclei were visibly fluorescent in cells stained with anti-whole histone conjugate. However, the anti-H1 conjugate only labeled macronuclei. This *in situ* demonstration of the lack of positive immunofluorescent staining of micronuclei with anti-H1 conjugate provides further evidence for the absence of H1 in the genetically inactive, mitotically dividing *Tetrahymena* micronucleus.

The lysine-rich histone, H1, has been implicated in a variety of nuclear processes. Recent evidence links H1 and its phosphorylation in a number of cell types with the alteration of the structure of interphase chromatin throughout the cell cycle (16, 18), including the conversion of nonproliferating cells to proliferating ones (17), rates of cell replication (1, 2, 28), chromosome condensation during mitosis (4, 5, 25), premitotic separation of sister chromatids (26), and the control of genetic activity (19, 24, 27).

Studies done on the histones of the ciliate *Tetrahymena* have indicated that H1 is present, in multiple molecular species each capable of being phosphorylated, in the amitotically dividing, genetically active macronucleus of this organism (14).[1] However, the mitotically dividing, genetically inactive micronucleus apparently lacks histone H1 (12). These findings impose certain limits on the speculations concerning the biological role(s) of H1 in chromatin structure and function (12, 13).

Since histone H1 is highly susceptible to proteolytic degradation (3, 30) and is easily dissociated from chromatin (10), the absence of H1 in isolated *Tetrahymena* micronuclei conceivably could be the result of a preparative artifact. Data obtained by Gorovsky and Keevert (12) argue strongly against such artifactual loss of H1 from micronuclei. However, studies done on isolated nuclei and chromatin cannot unequivocally dismiss the possibility that in vivo micronuclei contain H1. Thus, an immunofluorescence analysis was undertaken to determine the presence or absence of histone H1 in the macro- and micronuclei of *Tetrahymena* strain BVII. Antibodies directed against BVII whole histone and purified H1 were isolated, conjugated to fluorescein isothiocyanate, and used to stain BVII cells. The results confirm our previous conclusion that micronuclei lack histone H1 (12).

[1] Johmann, C. A., and M. A. Gorovsky. Manuscript in preparation.

MATERIALS AND METHODS

Preparation of Histone

Whole histone was extracted from macronuclei isolated from the highly inbred strain, B-1868-VII, of *Tetrahymena pyriformis* during deceleratory growth phase, as previously described (15). The lysine-rich histone, H1, was extracted from whole histone by solubilization in 0.5 M perchloric acid (PCA) (23). The purity of the histone samples was determined by quantitative urea-acrylamide gel electrophoresis (22, 29).

Immunological Procedures

Rabbits were immunized against either whole histone or fraction H1. Equal amounts of histone and yeast RNA (Sigma Chemical Co., St. Louis, Mo.) were complexed in 0.14 M NaCl, pH 6.8, then emulsified with 1.5 vol of complete Freund's adjuvant (Calbiochem, San Diego, Calif.) by vigorous blending on a VirTis homogenizer (VirTis Co. Inc., Gardiner, N. Y.) (31). Each rabbit was injected intramuscularly with 1.25 ml containing 0.5 mg of H1 or 1.0 mg of whole histone. Booster injections, identical with the immunizing injection, were given 7, 14, and 30 days later. Serum was collected by cardiac puncture 10 days after the final booster.

The specificity of the antisera was determined by the precipitin ring test (9). Normal serum (serum obtained before immunization) and both antisera (termed anti-whole histone and anti-H1) were tested against various compounds (Table I).

TABLE I

*Specificity of Antisera by the Precipitin Ring Test**

Test compound	Anti-whole histone antiserum	Anti-H1 antiserum	Normal serum
BVII whole histone	++	++	−
BIV whole histone‡	++	NT§	NT§
Calf thymus whole histone	+	±	−
BVII H1‖	++	++	−
BVII PCA-insoluble histone	++	−	−
Yeast RNA	−	−	−
Buffer	−	−	−

* Ring test performed by overlaying the undiluted antiserum with the test compound dissolved in borate-buffered saline, pH 7.8.

‡ The histones of strains BVII and BIV are electrophoretically identical (23).

§ NT = not tested.

‖ The test compound, BVII H1, was a PCA-soluble sample further purified by chromatography on Bio-Rex 70,[1] and contained no detectable non-histone protein contaminants on either urea-acrylamide or SDS acrylamide gels.

Preparation of Fluorescein Conjugates

γ-Globulin fractions were isolated from the sera by $(NH_4)_2SO_4$ precipitation (9). Conjugation of the three types of γ-globulin (normal, anti-whole histone, and anti-H1) with fluorescein isothiocyanate (FITC: Nutritional Biochemical Corp., Cleveland, Ohio) was achieved by usng the procedure given by Goldman (11). Unreacted dye was removed from the fluorescein-γ-globulin conjugates by chromatography on Sephadex G-50 (coarse, 100–300 mesh, Sigma Chemical Co.), with phosphate-buffered saline, pH 7.5 (PBS), as eluent. To obtain conjugates of each type with optimal ratios of bound fluorescein to protein concentration (F/P ratio), suitable fractions from the Sephadex column were chromatographed on DEAE-cellulose (DE52, Whatman Biochemicals Ltd., Maidstone, Kent, England) according to Goldman (11). F/P ratios were calculated with equations given by Goldman (11) and Wood et al. (32).

Immunofluorescence Microscopy

SLIDE PREPARATION: BVII cultures were suspended in sterile 10 mM Tris, pH 7.4, for at least ½ h. The cells were collected and resuspended in 30 mM glycerol-1 mM $CaCl_2$-0.8 mM $MgCl_2$, pH 6.2, for 20 min at 4°C. This treatment resulted in the loss of cilia, partial breakdown of the plasma membrane and "rounding up" of cells. The cells were then suspended in distilled H_2O for 2–5 min at 4°C. After this treatment, the cells were intact, though round and rather clumped, the nuclei distinct and the cytoplasm vacuolated. Drops of cells were placed on slides, dried at 37°C, and fixed in 100% methanol for 5 min at −20°C.

STAINING OF SLIDES: Cells without significant levels of autofluorescence were stained directly by flooding the slides with specific conjugated antibodies and incubating for 5 h in a humidity chamber at 22°C. Slides were extensively rinsed in PBS, mounted with 10% glycerol in PBS, and viewed with a Leitz microscope with an Osram HBO 200 mercury arc lamp and a dark-field condenser (E. Leitz, Inc., Rockleigh, N. J.). The exciting light was passed through a heat-absorbing filter (BG38) and a blue excitation filter (BG12). Two barrier filters (OG1, Wratten no. 12) were placed between the stage and the oculars.

ACRIDINE ORANGE STAINING: Fluorescein-labeled slides, which had been properly viewed and photographed, were then stained with acridine orange (0.125 mg/ml in PBS; Fisher Scientific Co., Pittsburgh, Pa.) for 5 min at 22°C, extensively rinsed in PBS and H_2O, dried, mounted, and viewed as above.

PHOTOGRAPHY: Pictures were taken with a Leitz 35 mm camera and Kodak Tri-X-Pan film (ASA 400, Eastman Kodak Co., Rochester, N. Y.).

RESULTS

Whole histone and fraction H1 were contaminated by less than 5% nonhistone protein (Fig. 1). In

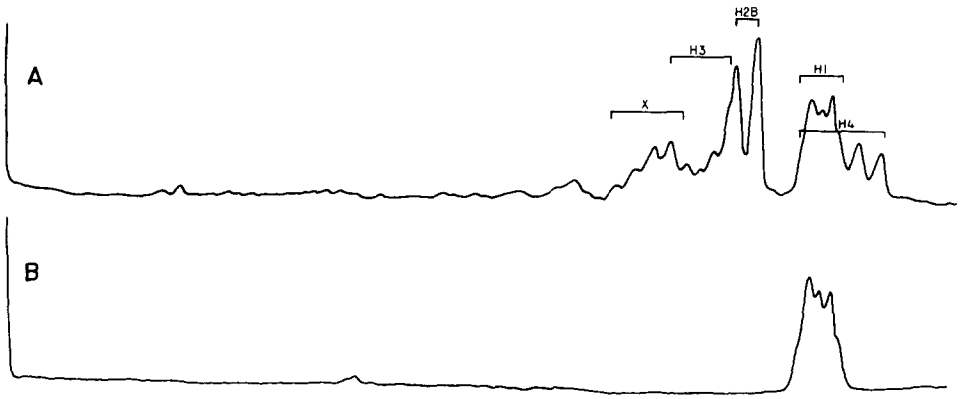

FIGURE 1 Densitometer tracings of high resolution, low pH urea-acrylamide gels containing (A) BVII macronuclear whole histone and (B) BVII macronuclear PCA-soluble histone (H1). The origin of the gels is on the left. The identities of the electrophoretic groups are given in reference 22.

addition, the H1 sample was not contaminated by any other histone.

Antisera directed against whole histone and purified H1, along with normal serum from a nonimmunized rabbit, were analyzed for specificity by the precipitin ring test. The results, given in Table I, indicate that anti-whole histone serum reacted strongly with whole histone from strains BVII and BIV, and with both PCA-soluble and PCA-insoluble BVII histone. Anti-H1 serum reacted with BVII whole histone and BVII H1, but not with PCA-insoluble histone. Neither antiserum reacted with RNA or buffer. No positive reactions were observed with normal serum.

γ-Globulins from all three sera were isolated and conjugated to FITC conjugates. The coupled γ-globulins were then chromatographed on DEAE-cellulose (the elution profiles and calculated F/P ratios are given in reference 21). Since overcoupled antibody (i.e. high F/P ratio) has a tendency to bind nonspecifically to cells, only samples with low F/P ratios ($\leq$2.0) and a protein concentration of at least 0.2 mg/ml were used to stain cells.

The autofluorescence of BVII cells prepared and fixed in methanol was extremely pale, even after 5 h incubation under PBS. Slides stained with two different preparations of conjugated normal γ-globulin (F/P = 0.8 or 10.0) displayed general, nonspecific fluorescence over the entire cell, only slightly greater than the background level of autofluorescence.

Cells treated with conjugated anti-whole histone or conjugated anti-H1 γ-globulins are shown in Figs. 2 and 3, respectively. The anti-whole histone conjugate positively stained both the macro- and micronuclei of the cells; the micronucleus was frequently visible even in cells where it was embedded in the macronucleus. However, in cells which were stained with anti-H1 conjugate alone (Fig. 3*a*), no fluorescence was observed over any structure which resembled a micronucleus, although the macronuclei were visibly stained.

Acridine orange, a dye which stains DNA yellow-green and RNA orange-red, was used as an independent method of localizing the nuclei in cells which have previously been labeled with one of the fluorescein conjugates. Cells stained with anti-whole histone or anti-H1 conjugates and restained with acridine orange (data not shown) exhibited brightly fluoresced macro- and micronuclei. In Fig. 3, the same cells are shown in which treatment with FITC-conjugated anti-H1 (Fig. 3*a*) resulted in labeled macronuclei only, while restaining with acridine orange (Fig. 3*b*) resulted in fluoresced micronuclei, as well as stained macronuclei.

Numerical analyses of the staining properties of cells treated with anti-H1 conjugate, anti-whole histone conjugate, or acridine orange are given in Table II. Only those cells containing clearly stained macronuclei were scored. Micronuclei were seen in only 54–60% of the cells stained with acridine orange, probably because the micronucleus is not always separate, and thus distinguishable from the macronucleus. A similar percentage (55%) of cells had fluorescent micronuclei after staining with anti-whole histone conjugate. Thus, the failure to visualize micronuclei in all of the

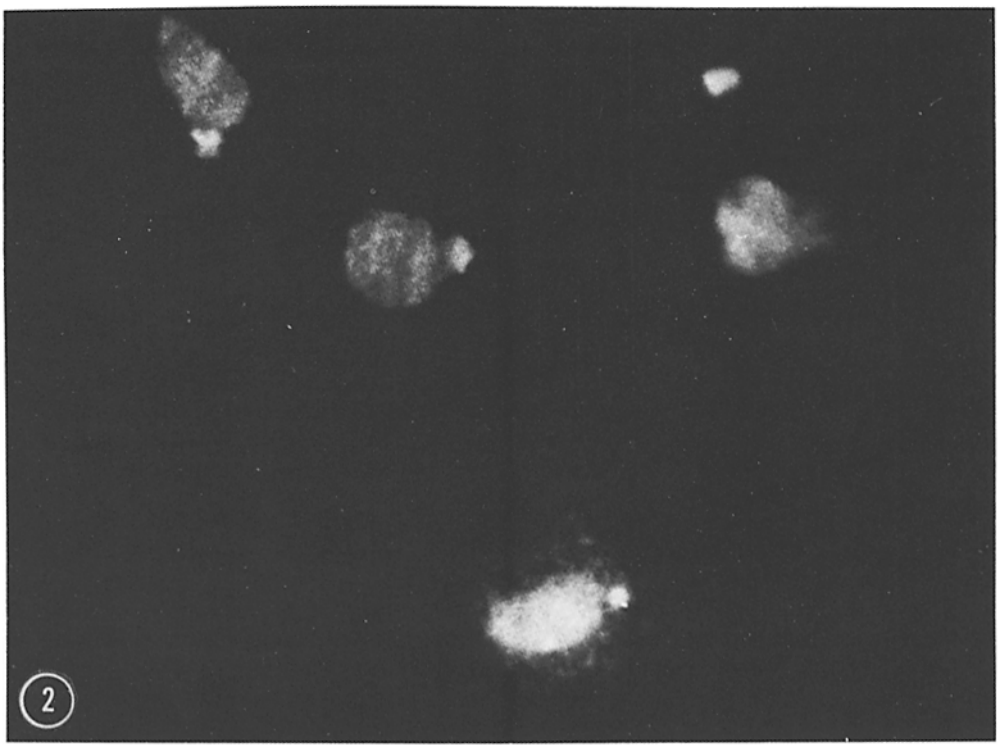

FIGURE 2 Fluorescein-conjugated anti-whole histone antibody staining of BVII cells fixed in methanol. Film exposure, 1 min. × 1,200.

cells after treatment with anti-whole histone conjugate probably is due to the quality of the cytological preparations, rather than to the actual absence of histone in some micronuclei or to any artifact of the antibody staining procedure. No fluorescent micronuclei were ever detected in cells treated solely with anti-H1 conjugate. This quantitative observation verifies the conclusion drawn from Fig. 3 that micronuclei were not stained by FITC-labeled anti-H1 γ-globulin.

As additional controls on the specificity of the anti-H1 preparation, cells were treated either with 0.5 M PCA to extract H1 or with unconjugated anti-H1 γ-globulin, before incubation with anti-H1 conjugate. In both cases, no fluorescence above background autofluorescence was observed. When PCA-extracted cells were subsequently treated with anti-whole histone conjugate, both the macro- and micronuclei were clearly stained (data not shown, see reference 21).

DISCUSSION

The demonstration that, in the same cell, the micronucleus is unlabeled by anti-H1 conjugate but can be visibly stained by acridine orange (Fig. 3) strongly supports the conclusion (12) that micronuclei of *Tetrahymena* strain BVII lack detectable levels of H1. Since the necessary controls were carried out, with the expected results, and since the specificity of the anti-H1 γ-globulin preparation was established, there appears to be no trivial reason for the lack of positive staining of micronuclei with anti-H1 conjugate. The one possible preparative problem, i.e. the loss of H1 from micronuclei during the treatment of the cells, is unlikely since the macronuclear H1, under the same conditions, was not lost. Therefore, unless the H1 of micronuclei is so different from macronuclear H1 that it has very different solubility properties, the lack of positive immunofluorescent staining for H1 in micronuclei cannot be a preparative artifact.

It must be noted, however, that there are two important immunological considerations concerning the absence of H1 staining in micronuclei. First, the antigenicity of any H1 in micronuclei may be different from that of macronuclear H1 used for the immunization. It is probable that

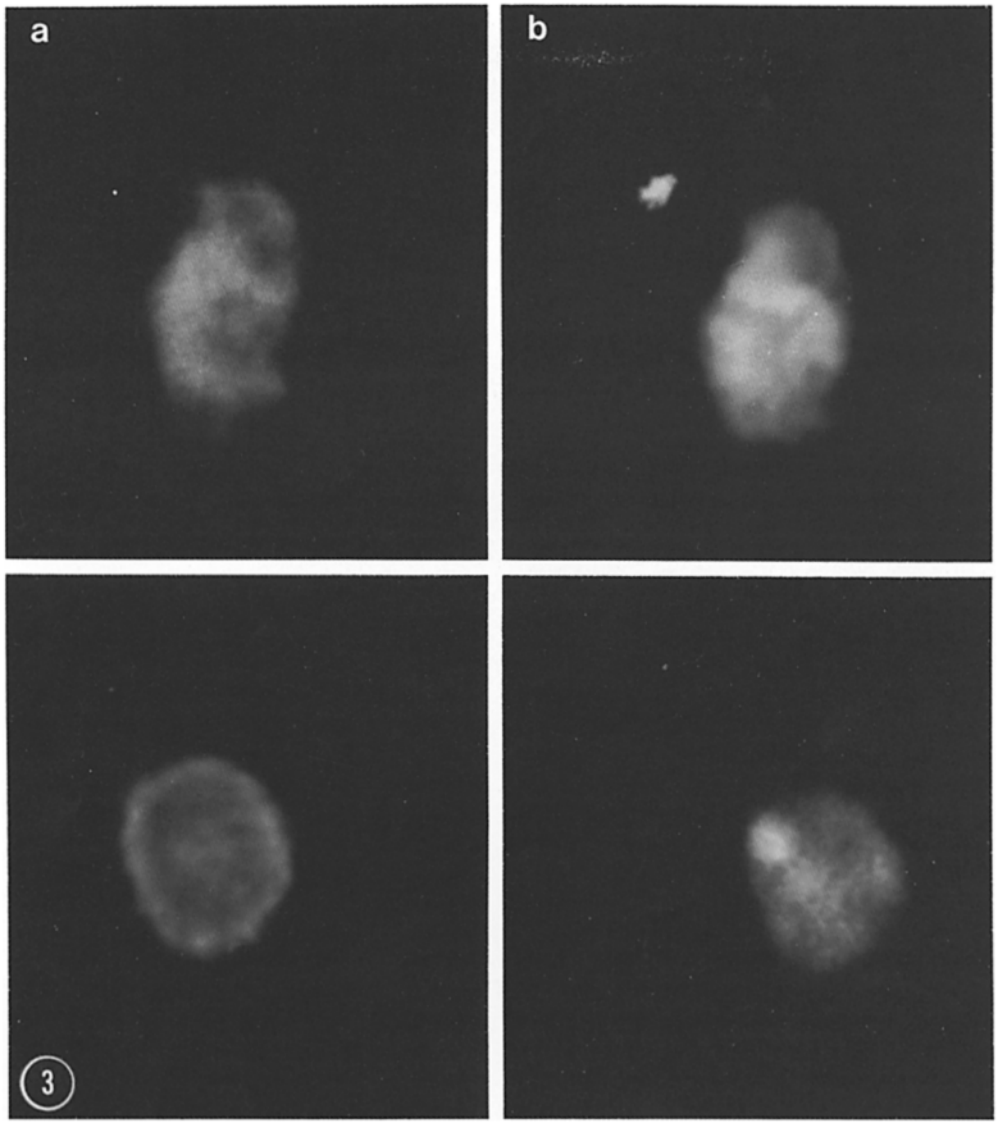

FIGURE 3 BVII cells stained with (*a*) fluorescein-conjugated anti-H1 antibody alone and (*b*) anti-H1 conjugate plus acridine orange. Film exposure, 5 min (*a*) and 25 s (*b*). × 2,400.

primary and higher orders of the structure of histones are involved in their antigenicity. All levels of structural properties could be changed in relatively minor ways, resulting in related but immunologically distinct H1 molecules. In fact, it has been demonstrated that the different H1 components of a single organism are immunologically distinguishable (6, 7) and that H1 components contain both common and specific antigenic determinant sites (8, 20). The second immunological problem deals with the possibility that, if H1 exists in micronuclei, it may have a different conformation in micronuclear chromatin, so that its antigenic determinant sites are not exposed *in situ*. In regard to this problem, Zick et al. (33) have examined the exposure of each H1 component in rat thymus chromatin using antisera directed against each H1 molecule. Their results suggest that in rat chromatin the five H1 components are arranged in a similar manner and, moreover, that the H1 antigenic determinants which are exposed in chromatin are the determinants

TABLE II

Numerical Analysis of Nuclear Fluorescent Staining

Treatment*	Cells observed‡	Cells with fluorescent micronuclei	Cells with fluorescent micronuclei
			%
Acridine orange	53	30	57
Anti-whole histone conjugate	189	105	55
+ Acridine orange	71	38	54
Anti-H1 conjugate	63	0	0
+ Acridine orange	49	29	60

* BVII cells suspended in Tris for <2 h (dividers still present) were incubated for 5 h with antibody conjugates and/or stained with acridine orange as described in Materials and Methods.

‡ All cells, in randomly chosen fields, with positively stained macronuclei were counted.

shared by all five H1s. Thus, it seems unlikely, even though the present work is concerned with two different nuclei, that micronuclei could contain an H1 without a single exposed antigenic determinant in common with macronuclear H1.

Aside from these considerations, the data given by Gorovsky and Keevert (12) and the immunofluorescence evidence presented here overwhelmingly support the proposal that micronuclei lack detectable levels of H1 during most of the cell cycle. The possibility that a transient form of H1 appears in the micronucleus at some stage in the life cycle of the cell has not been rigorously disproven, although no micronuclei were observed to be stained by anti-H1 conjugate even when cells were used which were still undergoing division (Table II). It should be noted, however, that unequivocal identification of dividing cells was not possible after glycerol treatment and methanol fixation. Thus, it is not known whether any of the cells examined were dividers. In conclusion, unless a transient form of H1 does exist, it seems as though histone H1 and its phosphorylation cannot be required for the processes of chromosome replication, condensation, or separation at mitosis (Discussion, reference 12).

Tetrahymena strain BVII is capable of conjugation, during which the macronuclei degenerate while the micronuclei undergo meiosis and fertilization to form a synkaryon (zygotic nucleus). The synkaron divides twice more, resulting in two anterior and two posterior nuclei in each conjugant. The anterior nuclei eventually differentiate into macronuclei (macronuclear anlagen) and the posterior nuclei ultimately give rise to vegetative micronuclei. The technique of immunofluorescent staining could be used to determine when H1 first appears in macronuclear anlagen during conjugation. Even though vegetative micronuclei may lack H1, at some point during the development of macronuclei the accumulation of H1 must occur and should be detectable by the immunofluorescence method. Thus, it may be possible to correlate the appearance of H1 with some particular structural or functional change as micronuclei differentiate into macronuclei.

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