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Absence of Histones from the Chromosomal Proteins of Fungi

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ABSTRACT **Interphase chromosomes were isolated in good yield from four species of fungi. In no case does the chromatin contain histones such as are characteristic of the chromosomes of other eukaryotic organisms.**

That histones are characteristic chromosomal proteins has been demonstrated for a wide variety of eukaryotic creatures. Thus, the chromosomes of higher plants and animals not only contain histones but, in addition, these proteins are similar in number, chemical properties, and even, in some cases, in primary structure (1, 2). Histones chemically similar to those of higher plants and animals have been found in the green alga *Chlorella* (3) and in the protozoan *Tetrahymena* (4, 5), as well as in a wide variety of invertebrates.

There have been several reports that the nuclei and/or chromatin of fungi lack histones, but contain instead chromosomal proteins of a less basic nature (6–9). We have examined the basic chromosomal proteins of several fungi, using what we believe to be the most rigorous of techniques for both chromatin isolation and histone characterization.

We describe methods for obtaining purified fungal chromatin that result in the recovery of at least 70% of the DNA present in the homogenate. The possibility of proteolytic degradation of histones (if present) is unlikely. We find that histones analogous to those of higher eukaryotes are missing in the fungi we have examined.

MATERIALS AND METHODS

Chromatin isolation

Attempts to isolate chromatin from the fungus *Microsporum gypseum* by the methods suggested for liver and pea (10) were not successful. Fungal nuclei are sheared by these methods, and centrifugal forces sufficient to pellet the chromatin result in gross RNA contamination of the nuclear fraction. A more viscous grinding medium should afford greater protection to the nuclei during the cell breakage step. Stern's glycerol grinding medium admirably satisfied this requirement (11).

Sporulation and spore purification procedures for *Microsporum* have been described previously (12, 13). 2-liter Erlenmeyer flasks, containing 600 ml of glucose (1% w/v) and neopeptone (Difco, 1% w/v) (pH 6.5), were inoculated with 10^6 conidia per ml and shaken at 350 rpm on a New Brunswick controlled environment dry-air shaker (30°C) for 4 days. The mycelia were harvested by suction filtration. The mycelial mat (150 g wet weight) was washed 4 times with 1-liter amounts of ice-cold physiological saline (pH 6.5).

The mycelial mat was resuspended in 350 ml of grinding medium (glycerol 50% w/v, 0.5 M sucrose, 0.001 M $CaCl_2$, and 0.05 M Tris, pH 8). The slurry was poured into an aluminum container and frozen by the addition of liquid nitrogen. Several additions of liquid nitrogen were necessary to completely freeze the material. The frozen slurry was ground to a coarse powder and placed into a Waring blendor. When the temperature of the grinding solution reached −30°C, the blendor was turned to full speed (110 V). Homogenization was continued until the temperature reached 0°C. The homogenate was again poured into an aluminum container and partially frozen (to −20°C) by the addition of liquid nitrogen. The partially frozen slurry subsequently was homogenized at full speed until the temperature reached 0°C. The freezing and homogenization steps were repeated until 70% breakage was observed under a microsope (usually 3–4 cycles). The homogenate was filtered two times through 1 layer of Miracloth and then two times through 3 layers of Miracloth.

The filtrate was centrifuged at 10,000 × *g* for 10 min. The supernatant fraction was then removed with a large-bore pipette and centrifuged at 30,000 × *g* (Sorvall SS-1, 16,000 rpm) for 1 hr. The pellet (90% DNA recovery) was resuspended in 90 ml of grinding solution. The resuspended nuclei were centrifuged again at 10,000 × *g* for 10 min and the supernatant fraction was removed. The 10,000 × *g* supernatant fluid was centrifuged then at 30,000 × *g* for 1 hr. The pellet (90% DNA recovery) was resuspended in 10 ml of 0.01 M Tris, pH 8, and stirred slowly overnight at 0°C. The nuclear lysate was centrifuged at 30,000 rpm (Spinco, 30 rotor) for 20 min. The supernatant fraction was removed and layered onto a discontinuous gradient, consisting of 1 ml of 50% sucrose (in 0.01 M Tris, pH 8) overlaid by 0.5 ml of 20% sucrose (in 0.01 M Tris, pH 8). The chromatin was recovered by centrifugation for 14 hr at 35,000 rpm (Spinco SW-39 rotor). The pellet (80% DNA recovery) was resuspended in 0.01 M Tris, pH 8, and dialyzed (4 hr) against 200 volumes of 0.01 M Tris, pH 8. Aggregated material was removed by centrifugation at full speed in a clinical centrifuge (approx. 3,000 × *g*) for 30 sec. The clear supernatant fluid (70% DNA recovery) was used for all subsequent experiments. This material exhibits the typical ultraviolet absorption spectrum reported for isolated chromatins (10) (Fig. 1). Approximately 200 μg of DNA (as chromatin) was recovered from 150 g (wet weight) of mycelia.

Chromatin was prepared from mycelia of *Neurospora tetrasperma*, just as described above. In the case of *Neurospora crassa*, chromatin was precipitated from French-press homog-

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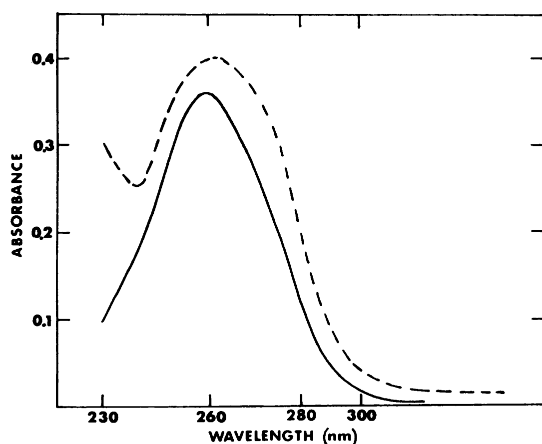

FIG. 1. Ultraviolet absorption spectra of *M. gypseum* chromatin and DNA. - - -, chromatin; ——, DNA. DNA was obtained by pelleting chromatin through 4 M CsCl (35,000 rpm for 24 hr, Spinco 39 rotor) as described by Bonner *et al.* (10).

enates of mycelia by 0.15 M NaCl and the resulting material was purified by centrifugation through 1.8 M sucrose. In the case of *Phycomyces blakesleeanus*, nuclei were readily prepared from squeezed extracts of sporangiophores (stage IV). Chromatin was purified from the lysed nuclei by centrifugation through 1.8 M sucrose.

RESULTS

Table 1 summarizes the chemical composition of *M. gypseum* chromatin and its nuclear fractions. The low ratio of total chromosomal protein to DNA results from a low ratio of basic proteins to DNA in the chromosomal and nuclear fractions. The amounts of nonbasic chromosomal protein and of RNA are similar to those reported for other eukaryotic cells (10). It is our experience that RNA to DNA ratios of greater than 0.05:1 should raise a suspicion of ribosomal contamination. This may explain why we find very low amounts of basic protein in our chromosomal fraction while others have reported larger amounts of "slightly basic" proteins in fungal chromatin (8).

The low ratio of basic protein to DNA suggests either that histone-like proteins are not a major component of chromosomal proteins in this organism or that they are degraded during the isolation procedure. To assess the possibility of degradation, trout testis nucleohistone (gift of K. Marushige) was added to the nuclear fraction and this material was carried through the subsequent isolation steps. The histone to DNA ratio of fish nucleohistone was identical before and after recovery from the *M. gypseum* chromatin fraction. Disc gel electrophoresis profiles of the recovered histones were identical to those reported for this material by Marushige and Dixon (14). Attempts to demonstrate proteolytic activity (as measured by incubation of *M. gypseum* nuclear homogenates with fish histones at 37°C for 1 hr at pH 8 or 5) were negative. It appears unlikely that histones are proteolytically degraded during isolation of chromatin from *M. gypseum*.

TABLE 1. *Chemical composition of M. gypseum chromatin and nuclear fractions**

Source	Total protein	DNA	RNA	Basic protein
Nuclear fraction	15.17 (±0.05)	1	2.00 (±0.02)	0.05 (±0.05)
Chromatin	1.05 (±0.05)	1	0.05 (±0.02)	0.03 (±0.05)

* Protein was estimated by the Folin reaction (16) and DNA by the diphenylamine method (17). RNA was measured by the orcinol reaction (10). Nuclear-fraction values are the average of four experiments. Chromatin values are the average of six experiments. Basic protein was extracted with 0.4 N H_2SO_4, 0.2 N HCl, or with 1 M $CaCl_2$ (10, 17). All of these procedures gave equivalent values.

Figs. 2 and 3 show disc electropherograms of the basic and acidic protein fractions of *M. gypseum* chromatin. Gel electrophoresis of the small amount of extractable basic protein (either from chromatin or nuclei) did not demonstrate any bands that migrated in the histone area. Sodium dodecyl sulfate–gel separation of the nonhistone chromosomal proteins indicated the presence of 6 major bands and 5 minor bands (apparent as small peaks or shoulders on the scan). This profile is reproducible and suggests the limited heterogeneity of fungal acidic chromosomal proteins (as detectable by our separation methods), as is the case with the chromatin of other eukaryotes (15).

Fig. 4 shows the template activity for RNA synthesis of *M. gypseum* chromatin in the presence of added *Escherichia coli* RNA polymerase. *M. gypseum* chromatin possesses 40–50% of the template activity of deproteinized DNA. The chromatin used caused no detectable hydrolysis of tritiated mouse RNA (a gift of Dr. J. Hudson) and did not, therefore, contain any considerable amount of RNase that might have decreased or prevented template activity. The high template activity of fungal chromatin is perhaps to be expected, since

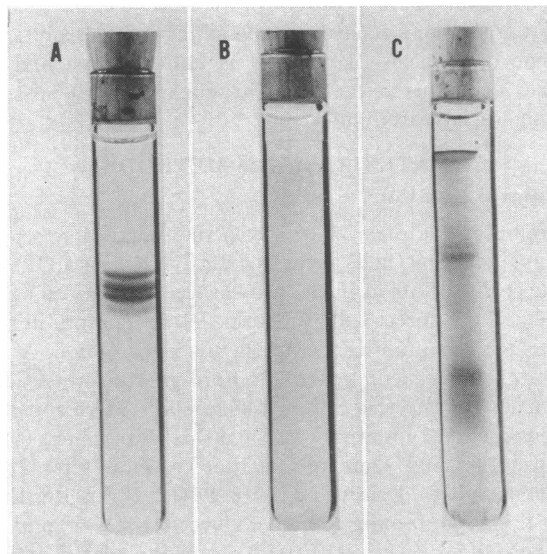

FIG. 2. Gel electrophoretic separation of *M. gypseum* chromosomal proteins. The origin is at the top in all photographs. (*A*) Fish histone (20 μg separated on polyacrylamide–urea gels according to Bonner *et al.* (10). (*B*) *M. gypseum* acid-soluble proteins (10 μg) separated on polyacrylamide–urea gels according to Bonner *et al.* (10). (*C*) *M. gypseum* nonhistone chromosomal proteins separated on sodium dodecyl sulfate–polyacrylamide–urea gels (18, 19). The method is a modification of that of Elgin and Bonner (15).

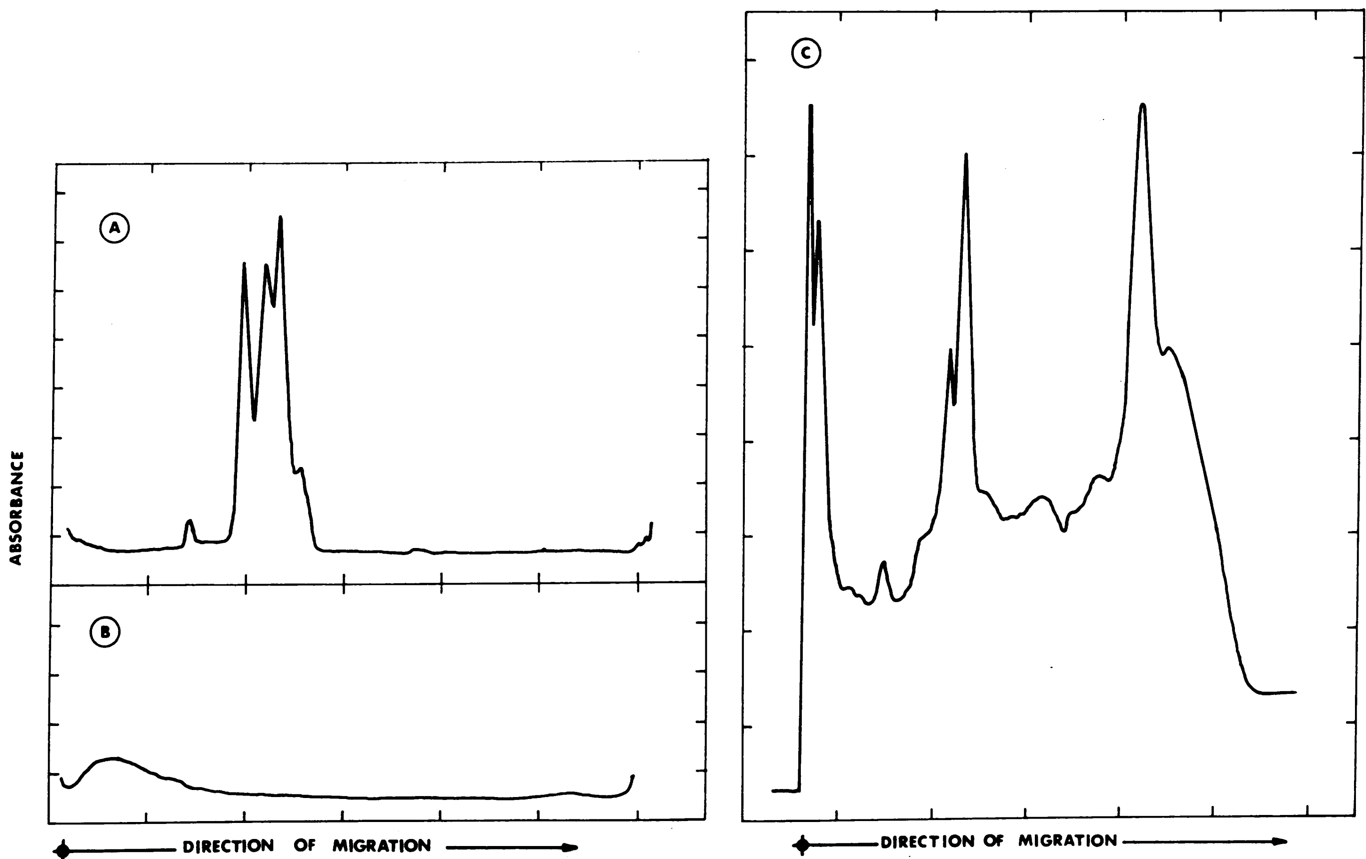

FIG. 3. Densitometric tracings of *M. gypseum* chromosomal proteins (see Fig. 2). The origin is on the left in all figures. Gels were scanned with a Gilford linear transporter (Model 2410) fitted to a Model 2400 recording spectrophotometer. 6-cm gels were scanned at 2 cm per minute (560 nm). A 0.1 × 2.36-cm aperture plate was employed to maintain band resolution.

histones are apparently absent from it. Van der Vliet *et al.* (6) have reported that yeast chromatin also exhibits high template activity for RNA synthesis. These results suggest

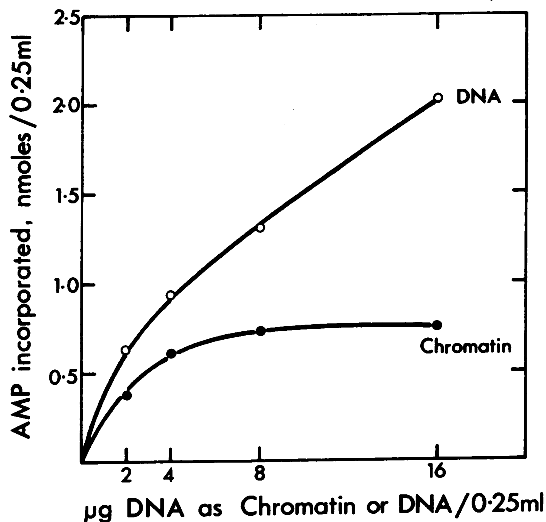

FIG. 4. RNA synthesis directed by *M. gypseum* chromatin and DNA, as a function of template concentration. Template activity was determined as described by Marushige and Dixon (14). DNA was prepared as in Fig. 1 (10). 114 μg of *E. coli* RNA polymerase was present per 0.25-ml reaction mixture. Incorporation by the enzyme alone has been subtracted.

that a larger portion of the fungal genome is available for transcription than is the case in higher eukaryotic cells.

The chromatin of *Neurospora tetrasperma* was studied by methods identical to those outlined above. This chromatin, as well as that prepared from lysed *Phycomyces* nuclei, was purified by sucrose density gradient centrifugation and extracted with acid as outlined above. Such extracts also failed to yield detectable histone as judged by disc-electrophoretic mobility.

The chromatin of *Neurospora crassa*, prepared as described above, was extracted with 0.4 N H_2SO_4, and the resulting (very sparse in amount) acid-soluble proteins were prepared for disc electrophoresis as described by Bonner *et al.* (10). Such electrophoresis revealed no proteins with mobilities characteristic of histones.

DISCUSSION

We have found histones to be absent from the chromatins of *Microsporum gypseum* (fungi imperfecti), *Neurospora tetrasperma* and *N. crassa* (ascomycetes), and *Phycomyces blakesleeanus* (phycomycete). Dwivedi *et al.* (8) have found histones to be absent from the chromatin of *Neurospora crassa;* we confirm their result. Histones appear to be absent from the chromatins of ascomycetes and lower fungi; we have not yet investigated any basidiomycete.

Histones of similar amino acid composition, end groups, electrophoretic mobilities, and primary structure are found in higher plants and animals, as well as in invertebrates, the green alga *Chlorella*, and the protozoan *Tetrahymena*. The

fungi that we have investigated seem to differ from other eukaryotes in that they lack these characteristic chromosomal proteins, which suggests that the fungi may have branched at an early level from the main stream of eukaryote evolution.

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1. Fambrough, D., and J. Bonner, *Biochemistry*, **5**, 2563 (1966).
2. DeLange, R., D. Fambrough, E. Smith, and J. Bonner, *J. Biol. Chem.*, **244**, 5669 (1969).
3. Iwai, K., in *The Nucleohistones*, ed. J. Bonner and P. O. P. Ts'o (Holden-Day, San Francisco, 1964), p. 59.
4. Iwai, K., H. Shiomi, T. Ando, and T. Mita, *J. Biochem.*, **58**, 312 (1965).
5. Griffith, J., Ph.D. thesis, California Institute of Technology (1969).
6. Van der Vliet, P., G. Tonino, and T. Rozijn, *Biochim. Biophys. Acta*, **195**, 473 (1969).
7. Tonino, G., and T. Rozijn, *Biochim. Biophys. Acta*, **124**, 427 (1966).
8. Dwivedi, R., S. Dutta, and D. Bloch, *J. Cell Biol.*, **43**, 51 (1969).
9. Stumm, C., and J. Van Went, *Experientia* (*Basel*), **24**, 1112 (1968).
10. Bonner, J., G. Chalkley, M. Dahmus, D. Fambrough, F. Fujimura, R. C. Huang, J. Huberman, K. Marushige, H. Ohlenbusch, B. Olivera, and J. Widholm, *Methods Enzymol.*, **12**, 3 (1968).
11. Stern, H., *Methods Enzymol.*, **12**, 100 (1968).
12. Leighton, T., and J. Stock, *Appl. Microbiol.*, **17**, 473 (1969).
13. Leighton, T., and J. Stock, *J. Bacteriol.*, **101**, 931 (1970).
14. Marushige, K., and G. Dixon, *Develop. Biol.*, **19**, 397 (1969).
15. Elgin, S. C., and J. Bonner, *Biochemistry*, **9**, 4440 (1970).
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
17. Mohberg, J., and H. Busch, *Arch. Biochem. Biophys.*, **134**, 577 (1969).
18. Shapiro, A., E. Viñuela, and J. Maizel, *Biochem. Biophys. Res. Commun.*, **28**, 815 (1967).
19. Maizel, J. V., *Science*, **151**, 988 (1966).