PRESENCE OF HISTONES IN *ASPERGILLUS NIDULANS*

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ABSTRACT

Five major histone proteins have been extracted from chromatin isolated from purified nuclei of the fungus, *Aspergillus nidulans*. These proteins had chromatographic properties which were similar to reference calf thymus histones and were purified to electrophoretic homogeneity by gel chromatography on Bio-Gel P10, Bio-Gel P60, and Sephadex G-100. Electrophoresis of these proteins in three different systems (urea-starch, urea-acetic acid polyacrylamide, and discontinuous SDS polyacrylamide) showed that the *A. nidulans* histones H3 and H4 were nearly identical to calf thymus H3 and H4 with respect to net charge and molecular weight criteria, whereas the fungal histones H1, H2a, and H2b were similar but not identical to the corresponding calf thymus histones. Amino acid analysis of *A. nidulans* histones H2a, H2b, and H4 showed them to be closely related to the homologous calf thymus histones. The mobility patterns of *A. nidulans* ribosomal basic proteins in three different electrophoretic systems were distinctly different from those of the fungal histones.

It is a well-established fact that histones are a ubiquitous constituent of chromatin from a wide spectrum of higher plants and animals. The five major histone groups (H1, H2a, H2b, H3, H4) are present in all tissues, and the arginine-rich histones (H3 and H4) especially are remarkably conserved in structure throughout evolution (7, 28, 29, 31, 32).

Histones have also been detected in primitive eukaryotic organisms such as green algae (20), slime molds (6, 17, 27), ciliated protozoans (15, 21), *Volvox* (4), and a dinoflagellate (33). Whether histones are present in the fungi has been unclear. Initial studies of fungi showed the chromatin-associated proteins to be either acidic or neutral, and no proteins were found which were similar to the histones typical of higher species (8, 23, 37). Recent work has shown the presence of several different species of histones in fungi, but in no case have all five histones been identified in a fungus. Hsiang and Cole (19) found two slightly lysine-rich histones in the chromatin of *Neurospora crassa*. In *Achyla* (16), three lysine-rich protein fractions have been identified. Studies on *Saccharomyces cerevisiae* have shown that slightly lysine-rich and arginine-rich classes of basic proteins are associated with the DNA (13, 37, 38); however, no evidence of H1 or H3 has so far been found in this organism.

The filamentous fungus *Aspergillus nidulans* is being used in this laboratory as a model system for genetic and biochemical studies of the molecular events important in mitosis. Since histones may play an important role in determining chromosome structure as well as in the changes in chromosome structure that take place at mitosis (3, 9, 24, 26), we have investigated whether this organism has nuclear basic proteins similar to histones found in higher organisms. We report here the isolation and preliminary characterization

of five major histone proteins present in the nuclei of *A. nidulans*.

MATERIAL AND METHODS

Growth Conditions

Conidial inocula were obtained from diploid cultures of *A. nidulans*, grown on YAG plates (0.5% yeast extract, 1.9% agar, and 2% glucose) at 32°C for 4–5 days. Spores were harvested from the plates by using glass beads and a solution of 0.01% Tween 80 in 0.15 M NaCl. Conidial suspensions at a final concentration of 10^6/ml were inoculated into 1 liter of YG medium (0.5% yeast extract, 2% glucose) in 2-liter Erlenmeyer flasks and rotated at 200 rpm in a gyratory shaker at 32°C for 16 h before harvesting.

Isolation of Nuclei from A. nidulans

Nuclei were prepared according to the method of Gealt et al. (14).

Calf Thymus Histones

Calf thymus histones were obtained from Sigma Chemical Co., St. Louis, Mo.

Preparation of Chromatin and Extraction of Basic Proteins

All steps in the isolation of histones from nuclei were carried out at 0–4°C. The purified nuclei were suspended in 10 vol of 0.01 M Tris buffer, pH 8.0, and homogenized by hand for 5 min with a Teflon-glass homogenizer. The chromatin was sedimented at 12,000 *g* for 10 min and washed by homogenization two additional times in the same buffer in order to remove contaminating ribosomes. Histones were extracted by rehomogenizing the chromatin by hand in 10–20 vol of 0.2 N HCl and agitating for 1 hr, after which the suspension was sedimented at 20,000 *g* for 20 min. The extraction step was repeated once. The extracted histones were pooled, lyophilized, and stored at −80°C, as was the residual protein fraction.

Preparation of Ribosomal Basic Proteins

A. nidulans cytoplasmic ribosomes were prepared from a postnuclear supernate after centrifugation an additional time at 27,000 *g* for 30 min at 4°C by the method of Nomura (10). Overnight dialysis against a solution containing 10 mM Tris, pH 7.4, 30 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol at 4°C was followed by centrifugation through 1.1 M sucrose, 0.5 M NH_4Cl, 20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol for 25 h at 142,000 *g*. The ribosomal pellet was extracted with 0.2 N HCl for 1 h at 0–4°C and centrifuged at 10,000 *g* for 10 min. The supernate was lyophilized and stored at −80°C.

Preparation of Samples for Determination of DNA, RNA, and Protein

Chromatin and ribosomal pellets were dissolved in 0.3 M NaOH. In order to avoid the introduction of systemic errors, RNA, DNA, and protein were not separated before colorimetric analysis by standard methods (5, 25, 34). Synthetic mixtures were used to obtain correction constants for cross-reactivity and, where appropriate, corrections were made.

Column Chromatography

A. nidulans histones were applied to and eluted from a Bio-Gel P10 column (2 × 189 cm) (Bio-Rad Laboratories, Richmond, Va.) equilibrated with 0.01 N HCl (35). Pooled fractions from the Bio-Gel column were additionally purified by gel filtration through Sephadex G-100 (1.5 × 90 cm) (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 0.01 N HCl or Bio-Gel P60, (1.5 × 200 cm) equilibrated with 0.02 N HCl, 0.1 M NaCl (2). Samples from the Bio-Gel P60 column were desalted with a Sephadex G-25 column (1 × 28 cm) with 0.01 N HCl as the eluate.

Electrophoresis of Histones

Urea-starch gels were prepared according to the method of Sung and Smithies (36). Protein samples were dissolved in aluminum lactate buffer (pH 3.1) and subjected to electrophoresis for 15 h at 4°C at 320 V and 26 mA.

Polyacrylamide gel electrophoresis was performed according to the method of Panyim and Chalkley (30), using 15% acrylamide, 2.5 M, or 6.25 M urea gels (0.5 × 8.0 cm) which were pre-electrophoresed overnight (1.0 mA/tube) before sample application and then run at 1.75 mA/tube for up to 5 h before removing and staining the gels with amido-black 10-B.

Histones were also analyzed with the polyacrylamide SDS discontinuous gel system of Laemmli (22). Samples were run at 60 V, 17 mA in the stacking gel, then at 26 mA, 120 V (constant) for an additional 5 h before the gel was removed and stained with Coomassie blue.

Amino Acid Analysis

Samples for amino acid analysis were desalted where appropriate and were handled in base-cleaned glassware. Hydrolysis and amino acid analysis were performed as described by Berg and Prockop (1) with 0.8 M β-mercaptoethanol included in the 6 N HCl (18).

RESULTS

A. nidulans basic nuclear proteins were prepared by acid extraction from washed chromatin obtained from purified nuclei. The chromatin had an RNA/DNA weight ratio of 0.08. This ratio was

similar to the published RNA/DNA ratios for chromatin isolated from other species, including calf thymus (20), rat liver (20) and *N. crassa* (19), and is much lower than the ratio of 3.8 reported by Wintersberger et al. (38) for yeast chromatin. The low RNA/DNA ratio of the *A. nidulans* chromatin indicates that there was no significant contamination of this material by ribosomes. The ratio of acid-extractable protein/DNA for *A. nidulans* chromatin in different experiments ranged between 0.11 and 1.0. Thus, the recovery of acid-extractable protein was variable and usually lower than in other eukaryotes (20). We cannot yet explain the variable recovery; however, the recovery of histone-like proteins from *N. crassa* has also been reported to be unusually low. The residual protein/DNA ratio in *A. nidulans* chromatin was 1.86, higher than that found in rat liver, calf thymus (20), and *N. crassa* (19) chromatins but considerably lower than the ratio of 4.80 reported for *S. cerevisiae* chromatin (38).

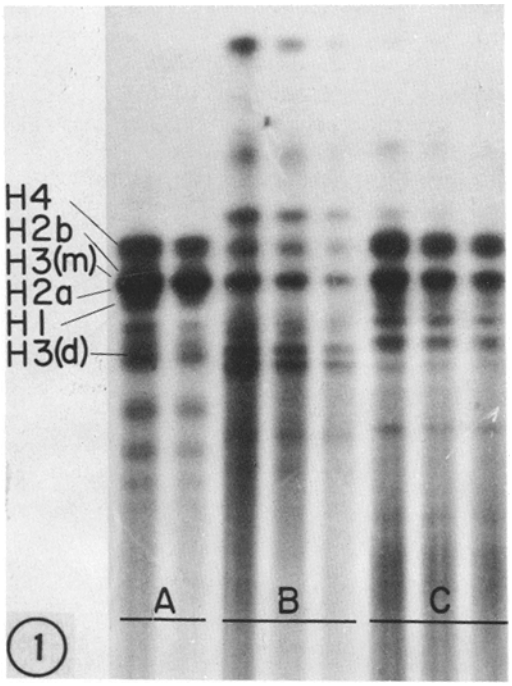

FIGURE 1 Starch gel electropherogram of calf thymus histones, *A. nidulans* ribosomal basic proteins, and *A. nidulans* nuclear basic proteins. (A) calf thymus histones (25 μg, 10 μg); (B) *A. nidulans* ribosomal basic proteins (50 μg, 25 μg, 12.5 μg); (C) *A. nidulans* nuclear basic proteins (50 μg, 25 μg, 12.5 μg). The calf thymus histones are identified to the left of the figure (M. M. Sanders, unpublished observations). The direction of the migration is from bottom to top.

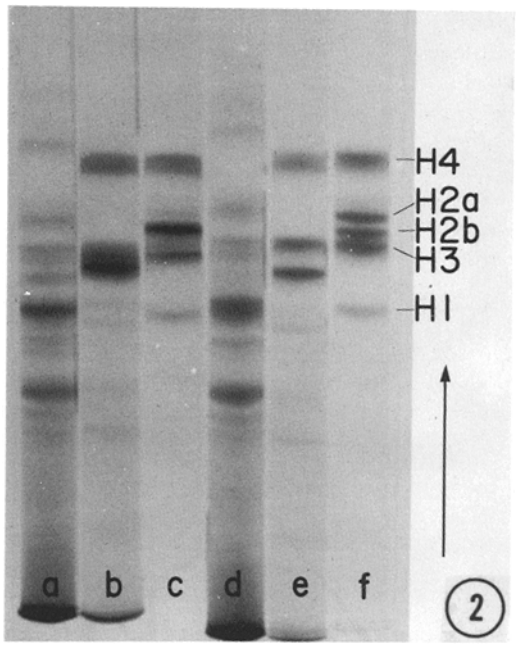

FIGURE 2 Acetic acid-urea polyacrylamide gel electropherograms of *A. nidulans* ribosomal basic proteins, *A. nidulans* nuclear basic proteins, and calf thymus histones. (*a*) *A. nidulans* ribosomal basic proteins (44 μg); (*b*) *A. nidulans* nuclear histones (48 μg); (*c*) calf thymus histones (45 μg), electrophoresed in 6.25 M urea; (*d*) *A. nidulans* ribosomal basic proteins (44 μg); (*e*) *A. nidulans* nuclear histones (48 μg); (*f*) calf thymus histones (45 μg), electrophoresed in 2.5 M urea. The order of migration of calf thymus histones in 2.5 M urea is shown to the right of the figure. The arrow indicates the direction of migration.

The basic proteins extracted from *A. nidulans* chromatin with 0.2 N HCl were analyzed by gel electrophoresis in three different systems, the urea-starch gel system of Sung and Smithies (36), the acetic acid-urea polyacrylamide gel system of Panyim and Chalkley (30), and the discontinuous SDS gel system of Laemmli (22). The *A. nidulans* basic proteins had electrophoretic mobilities similar to those of calf thymus histones in all three systems. The urea-starch gel system separates calf thymus H4 from H1, H2a, H2b and H3, which run close together and are not well-resolved unless previously fractionated (Fig. 1). Polymers of unreduced H3 are also separated from the other histones and are seen as trailing bands in this system. The *A. nidulans* histones give a series of

bands in the general region of the calf thymus histones (Fig. 1). The higher multimers of H3 were conspicuously absent in *A. nidulans*.

The acetic acid 2.5 M urea polyacrylamide gel system of Panyim and Chalkley separates all five calf thymus histones. Calf thymus H4 species migrate most rapidly and give a split band. Calf thymus H2a, H2b, H3, and H1 follow in that order. On 6.25 M urea gels, the order of migration of the calf thymus histones is the same except that H2a and H2b run together as single band (Fig. 2). The *A. nidulans* basic proteins gave four bands on the acid 2.5 M urea gels (Fig. 2). There was a split band which co-migrated with calf thymus H4 species. The next slower band co-migrated with calf thymus H3. A third band which moved behind calf thymus H3 on 2.5 M urea gels broadened on the 6.25 M urea gel and presumably contained H2a and H2b. A weakly staining band migrated more slowly than calf thymus H1 on the 2.5 M urea system.

In the discontinuous SDS acrylamide gels the *A. nidulans* histones were not completely resolved (Fig. 3). Even though this system gives excellent resolution of all five calf thymus histones, it failed to resolve *A. nidulans* H2a, H2b, and H3 from each other although it resolved them well for H1 and H4. *A. nidulans* H1 migrated more rapidly than the calf thymus H1 species in this system. Thus, *A. nidulans* H3 and H4 had electrophoretic mobilities which were identical to those of calf thymus H3 and H4 in all three electrophoretic systems whereas *A. nidulans* H1, H2a, and H2b had mobilities which were similar to but not identical with those of the corresponding calf thymus histones in all of the electrophoretic systems.

Purification of Histones

The putative histones extracted from *A. nidulans* were fractionated by gel filtration on Bio-Gel P10 by the method of Sung and Dixon (35). An elution profile for calf thymus whole histones applied to the same column is shown in Fig. 4, and the species eluting in each peak are indicated. Although the elution behavior of *A. nidulans* nuclear basic proteins was not identical to that of calf thymus histones (Fig. 4), several major features of the profiles were similar. The peak eluting first from the column contained a band which

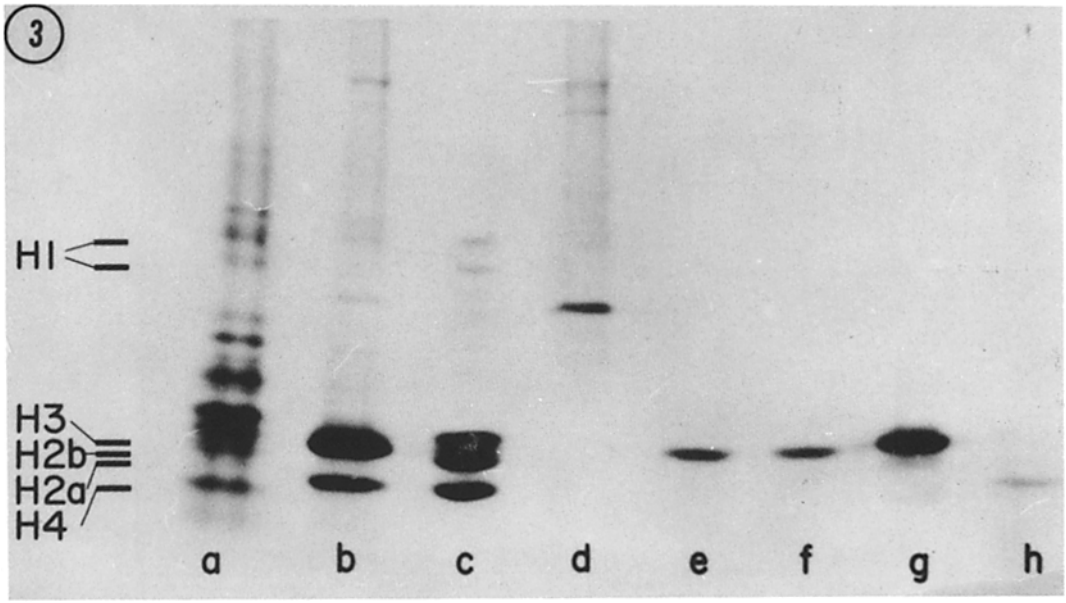

FIGURE 3 Discontinuous SDS polyacrylamide gel electropherogram of *A. nidulans* unfractionated histones, *A. nidulans* purified histones, *A. nidulans* ribosomal basic proteins, and calf thymus histones. (*a*) *A. nidulans* ribosomal basic proteins (19 μg); (*b*) *A. nidulans* unfractionated histones (15 μg); (*c*) calf thymus histones (15 μg); (*d*) *A. nidulans* H1; (*e*) *A. nidulans* H2a; (*f*) *A. nidulans* H2b; (*g*) *A. nidulans* H3; (*h*) *A. nidulans* H4. The order of migration of the calf thymus histones is shown to the left of the figure. Electrophoresis was from top to bottom.

stained and migrated the same as calf thymus H1 (Fig. 4). A major peak apparently corresponding to the calf thymus mixtures of H2a and H2b was eluted next. Electrophoretic analysis of fractions from this peak showed that two species were present which had somewhat slower electrophoretic mobilities on urea-starch gels than calf thymus H2a and H2b in the same system (Fig. 4). The third peak in the *A. nidulans* profile gave two bands in the urea-starch gel system, one of which electrophoresed with a mobility identical to that of calf thymus H3 monomer, and the second of which had a mobility slightly faster than that of calf thymus H3 dimer. The fourth peak from the column gave a split band at the same position as the calf thymus H4. Electrophoretic analysis of the column fractions showed that all five *A. nidulans* histones were resolved in the urea-starch gel system (Fig. 4).

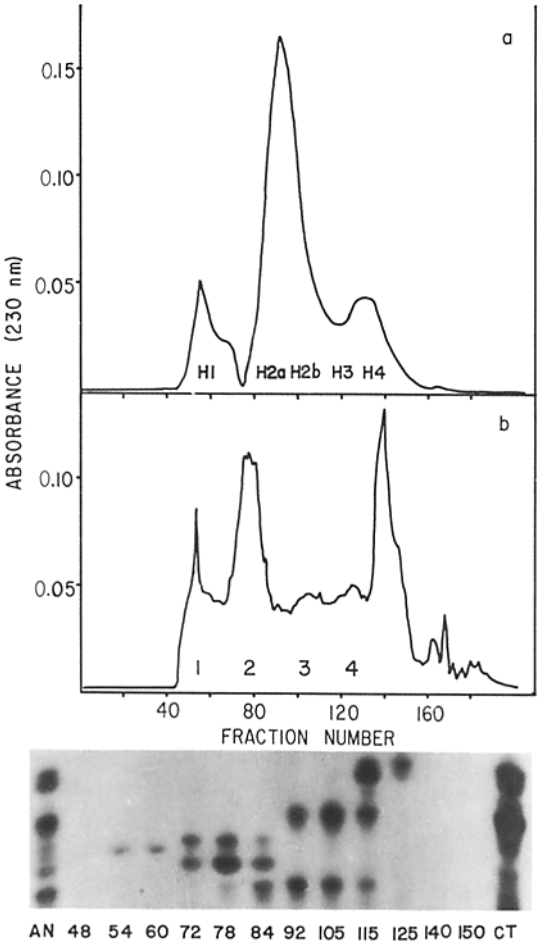

FIGURE 4 Elution profile on Bio-Gel P10 of calf thymus histones (*a*) and *A. nidulans* histones (*b*). The elution positions of the calf thymus and *A. nidulans* histones are indicated. The 2.0 × 189 cm column was equilibrated with 0.01 N HCl, flow rate 43 ml/h. 4.0 ml fractions were collected at room temperature. Samples removed from the indicated fractions of the *A. nidulans* chromatogram were electrophoresed on starch-urea gels and compared with calf thymus histones (*CT*) and the *A. nidulans* (*AN*) histone sample which was applied to the column. Electrophoresis was in the upward direction.

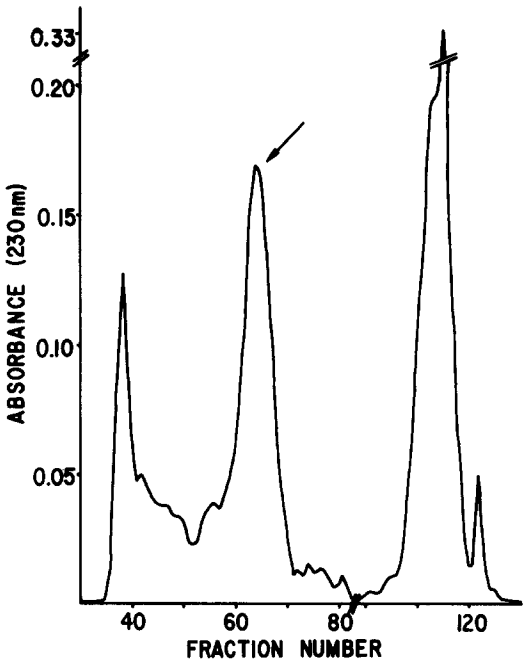

FIGURE 5 Elution profile on Sephadex G-100 of *A. nidulans* peak 4 from the Bio-Gel P10 column shown in Fig. 4 *b*. The 1.5 × 90 cm column was equilibrated with 0.01 N HCl, flow rate 8.4 ml/h. 1-ml fractions were collected at room temperature. The arrow indicates the peak containing *A. nidulans* H4.

Further purification and characterization of the fractions pooled from the Bio-Gel P10 column was carried out on either Bio-Gel P60 or Sephadex G-100 columns. Fig. 5 shows the elution profile for peak 4 from Bio-Gel P10 applied to Sephadex G-100. This peak, which contained *A. nidulans* H4, was fractionated into three peaks on Sephadex G-100. The middle peak had the same chromatographic mobility on this column as calf thymus H4.[1] The material pooled from this peak was electrophoretically pure *A. nidulans* H4 (Fig. 3). Chromatography of peak 3 from the Bio-Gel P10 column on Sephadex G-100 yielded two peaks

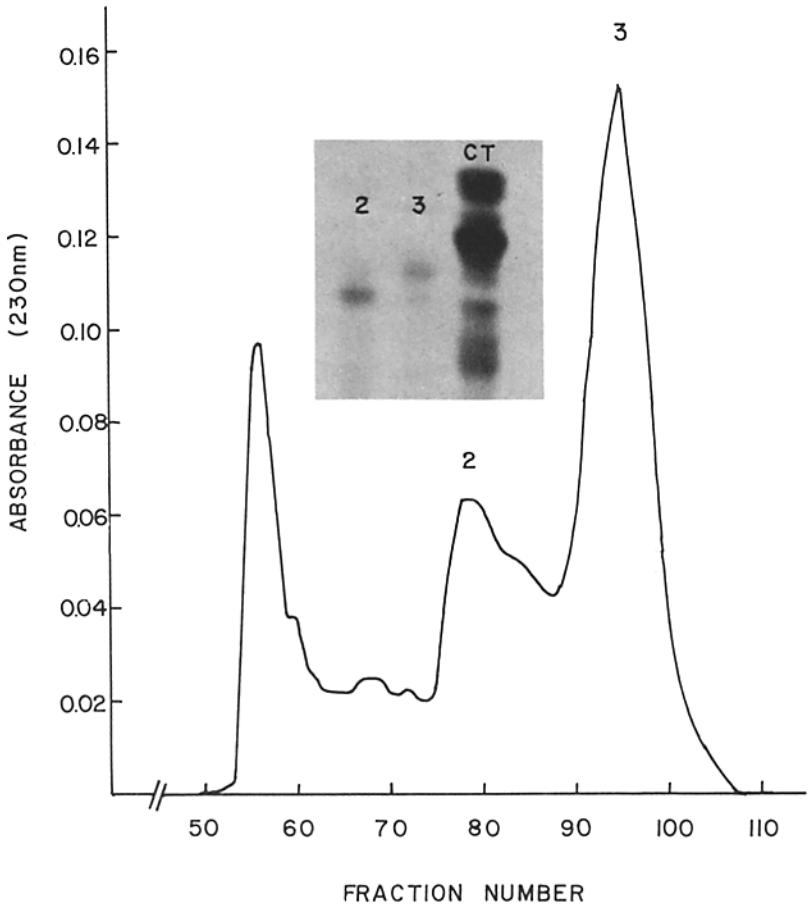

FIGURE 6 Elution profile on Bio-Gel P60 of *A. nidulans* peak 2 from the Bio-Gel P10 column shown in Fig. 4 *b*. The 1.5 × 200-cm column was equilibrated with 0.02 N HCl, flow rate 7.0 ml/h. 1.25-ml fractions were collected at room temperature. The starch gel electropherogram show samples from peaks 2 and 3 compared with calf thymus histones (*CT*).

which eluted in the positions expected for calf thymus H3 in the oxidized dimer and reduced monomer forms.[1] The dimer form moved to the position expected for the monomer after reduction with β-mercaptoethanol in 8 M urea (data not shown). G-100 purified *A. nidulans* H3 migrated as a single band on SDS gel electrophoresis (Fig. 3), indicating that it was free from contamination with H4. Fig. 6 shows the elution profile for peak 2 from the Bio-Gel P10 column rechromatographed on Bio-Gel P60 in the presence of 0.1 M NaCl (2). This chromatographic system which resolved calf thymus histones H2a and H2b resolved two *A. nidulans* proteins (Fig. 6). On the discontinuous SDS gel, the *A. nidulans* proteins had mobilities similar to those of calf thymus H2a and H2b but were not resolved from each other (Fig. 3). On the starch gel the proteins migrated more slowly than calf thymus H2a and H2b and were well-separated (Figs. 4 and 6). The two proteins stained differently with wool fast blue and amido black; the faster moving band on the starch gel stained more intensely with wool fast blue whereas the slower moving band stained more intensely with amido black. Rechromatography on Sephadex G-100 of peak 1 from the Bio-Gel P10 column gave a large peak with a trailing shoulder at the void volume (data not shown). Analysis on SDS acrylamide gels showed that the major *A. nidulans* protein in this shoulder had a molecular weight smaller than

[1] M. M. Sanders. Unpublished observations.

that of calf thymus H1 but had similar staining characteristics.

Amino Acid Analysis of A. nidulans Histones

Sufficient quantities of H2a, H2b, and H4 were recovered to permit preliminary determination of amino acid compositions. The results of two hydrolyses of H2a and H2b showed that these proteins are slightly lysine-rich and are strikingly similar in amino acid composition to the corresponding calf thymus histones (Table I). A single hydrolysis of *A. nidulans* H4 showed that it was rich in arginine with a lysine to arginine ratio almost identical and an amino acid composition similar to that of calf thymus H4.

Comparison of Ribosomal Basic Proteins with Histones

Since ribosomal basic proteins have properties so similar to those of histones that they may be mistaken for histones, we compared *A. nidulans* histones with *A. nidulans* ribosomal basic proteins. Ribosomes were prepared from a postnuclear supernate of *A. nidulans* by the method of Nomura (10). The ribosomes had an RNA/protein weight ratio of 1:1 and a DNA/RNA weight ratio of 0.004, indicating that the ribosomes were free from contamination with chromatin. The ribosomal basic proteins were prepared by acid extraction of the ribosomes. These proteins were then compared with the *A. nidulans* histones in three electrophoretic systems: urea-starch gels (Fig. 1), urea-acrylamide gels (Fig. 2), and discontinuous SDS acrylamide gels (Fig. 3). In all three systems the ribosomal basic proteins exhibited a banding pattern distinctly different from that produced by the *A. nidulans* histones. The differences in electrophoretic banding patterns and the almost complete absence of the most prominent ribosomal bands in the patterns obtained from the *A. nidulans* his-

TABLE I

*Amino Acid Composition**

	A. nidulans (H2a)‡	Calf thymus (H2a)§	*A. nidulans* (H2b)‡	Calf thymus (H2b)§	*A. nidulans* (H4)‖	Calf thymus (H4)§
Aspartic acid	9.0	6.6	5.9	4.8	5.9	4.9
Threonine¶	4.4	3.9	7.6	6.4	8.3	6.9
Serine¶	6.6	3.4	9.8	11.2	4.7	2.0
Glutamic acid	10.6	9.8	9.8	8.0	7.4	5.9
Proline	4.9	4.1	5.2	4.8	0	1.0
Glycine	12.5	10.8	8.3	5.6	18.7	16.7
Alanine	12.9	12.9	14.4	10.4	8.2	6.9
Cysteine	0	0	0	0	0	0
Valine	5.5	6.3	5.8	7.2	7.1	8.8
Methionine	0	0	0	1.6	0.5	1.0
Isoleucine	4.0	3.9	4.9	4.8	5.8	5.9
Leucine	11.2	12.4	6.1	4.8	7.0	7.8
Tyrosine**	0	2.2	0	4.0	0	3.9
Phenylalanine	1.5	0.9	1.6	1.6	2.8	2.0
Lysine	8.6	10.2	14.8	16.0	9.2	10.8
Histidine	2.1	3.1	1.2	2.4	2.3	2.0
Arginine	6.5	9.4	5.0	6.4	12.1	13.7
Basic amino acids						
Acidic amino acids	0.88	1.38	1.34	1.94	1.77	2.45
Lysine/arginine	1.32	1.08	2.96	2.50	0.76	0.79

* Expressed as mole percent.

‡ Average from two 24-h hydrolyses.

§ Data taken from amino acid sequences (9).

‖ Results of a single 24-h hydrolysis.

¶ Not corrected for hydrolytic losses.

** No tyrosine was recovered in any of the hydrolysates, probably because the samples were stored in dilute HCl.

tones indicated that the histones were not significantly contaminated by ribosomal proteins.

DISCUSSION

This report describes the presence of histones in the fungus *A. nidulans*. Because the existence of histones in fungi has been controversial (8, 23, 37), the following stringent criteria were established for the identification of histones in this organism: (*a*) a demonstration that the organism has nuclear basic proteins with properties similar to those of known histones; (*b*) evidence that these proteins are not ribosomal in origin. To meet these criteria, nuclei were isolated from *A. nidulans* and the chromatin prepared from these nuclei was shown to be free from contamination with ribosomal RNA. The acid-soluble proteins extracted from this chromatin resembled calf thymus histones in electrophoretic and chromatographic properties and clearly differed in three gel electrophoretic systems from ribosomal acid-soluble proteins prepared from chromatin-free ribosomes.

Fractionation of *A. nidulans* chromatin basic proteins on Bio-Gel P10, Bio-Gel P60, and Sephadex G-100 resolved five species of histones, two of which were closely related to calf thymus H3 and H4. The *A. nidulans* H3 and H4 had chromatographic properties similar to those of calf thymus H3 and H4 in our column systems and had electrophoretic mobilities identical with those of calf thymus H3 and H4 in all three gel systems. Amino acid analysis of *A. nidulans* H4 indicated that it had a composition similar to that of calf thymus H4 (Table I). Thus, it is likely that the structure of H4, which has been shown to be highly conserved in species as different as peas and cows (11), is also conserved in the fungus *A. nidulans*. Insufficient *A. nidulans* H3 was recovered for amino acid analysis; however, the *A. nidulans* protein was shown to resemble H3 from other sources (12) in its ability to form dimers (Fig. 4) which then could be cleaved to monomers by reduction with β-mercaptoethanol in 8 M urea. The *A. nidulans* H3 dimer had a slightly faster mobility in starch gel electrophoresis than calf thymus H3 dimer. Higher polymers of *A. nidulans* H3 were conspicuously absent. *A. nidulans* H3 is similar in this respect to H3 from pea embryos (9, 12), and thus may have only one sulfhydryl group available for disulfide bond formation. Three additional *A. nidulans* histones, H1, H2a and H2b, were identified and are described in the order in which they eluted from Bio-Gel P10 columns. The first protein off the column resembled calf thymus H1, not only in its elution position from Bio-Gel P10 but also with respect to its electrophoretic mobility on urea-starch gels and its elution position from Sephadex G-100. However, the somewhat faster migration on SDS acrylamide gels suggests the possibility that this protein may be analogous to the lower molecular weight H1 isolated from *Tetrahymena* (15). We were unable to isolate sufficient amounts of this material for amino acid analysis. The second and third histones, H2a and H2b, of *A. nidulans* eluted in a single peak from Bio-Gel P10 and were subsequently resolved by chromatography on Bio-Gel P60. Their elution positions from both columns closely resembled those of calf thymus H2a and H2b. On the basis of these results and electrophoretic mobilities which were similar to those of calf thymus H2a and H2b on discontinuous SDS polyacrylamide gels, we have tentatively identified these species as *A. nidulans* H2a and H2b. Consistent with this identification is the fact that amino acid analysis of these histones showed that they were slightly lysine-rich and that they agreed closely in their composition with the corresponding calf thymus histones (Table I).

Previous reports of fungal histones describe the presence of two slightly lysine-rich histones in *N. crassa* (19) and three histones in *S. cerevisiae* (13, 28). Amino acid analysis of the *N. crassa* histones showed lower ratios of basic to acidic residues compared to the respective histones of calf thymus. Two of the three years histones also had lower basic to acidic amino acid ratios whereas the third histones had a higher ratio than the corresponding calf thymus proteins. Neither H1 nor H3 was detected in yeast. In contrast, *A. nidulans* is similar to the higher eukaryotes in the number and properties of the histones present in its nucleus. Whether these differences in histone number and characteristics between *A. nidulans* and the other fungi are biologically significant or merely the result of technical difficulties involved in isolating and characterizing the fungal histones remains an important question.

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