ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 256, No. 1, July, pp. 167-178, 1987

Histone Variants and Acetylated Species from the Alfalfa Plant *Medicago Sativa*

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Received November 21, 1987, and in revised form January 22, 1987

The histones from the alfalfa plant *Medicago sativa* have been characterized in terms of type variants and levels of acetylation. Histones were isolated directly from total plant tissue (callus), eliminating the need to develop methods for nuclear isolation. An acid-urea-polyacrylamide gel with a transverse Triton X-100 gradient resolved and identified in a single gel at least one type of histone H4, two variant forms of histone H2B, two variant forms of histone H3, and four variant forms of histone H2A from a crude histone preparation. Histone H4 was present 25% in an unmodified state and 75% as monomodified, presumably as monoacetylated histone. Both histone H3 variants displayed five bands, consistent with up to four internal sites of acetylation. The two H3 variants differed in their steady-state level of acetylation, suggesting that they may reside in different chromatin environments. Several histone H1 species were identified by solubility and cross-reactivity with antiserum raised against the globular part of bovine $H1^0$, indicating conservation of epitopes between histone H1 of mammals and higher plants. © 1987 Academic Press, Inc.

Histones of higher plants have been studied in a limited number of plant species including tobacco, wheat, rye, barley, and corn, sometimes only by SDS[2] gel electrophoresis (1-4). This gel system is not able to provide information on the occurrence of histone variants and on the extent of histone acetylation, a modification thought to function in chromatin by allowing chromatin unfolding for gene transcription, chromatin assembly, and during spermatogenesis (5). Information available on plant chromatin and histones is limited (6). It is unknown whether one can extrapolate processes observed in mammalian cells and lower eukaryotes into higher plant systems, e.g., mitotic chromatin condensation by phosphorylation of histone H1, dynamic core histone acetylation in transcriptionally active chromatin, or the occurrence of specific histone variant species in specific chromatin domains (5, 7).

We have developed a simple method to prepare plant histones from total plant tissue which has allowed us to obtain preliminary information on histone complexity and extent of histone acetylation. It obviates the need to develop methods for the isolation of nuclei and of chromatin, methods often tedious and frequently unreliable (8, 9). In this work, we utilize this method to characterize the histones of *Medicago sativa* grown in callus culture. The crude histone preparation is analyzed electrophoretically in a complex acid-urea gel in which a gradient of Triton X-100 is able to differentiate between core histones and other protein species. We identified four core histone classes, the occurrence of variant histone forms, and the presence of histone acetylation. Several alfalfa protein

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[2] Abbreviations used: SDS, sodium dodecyl sulfate; PCA, perchloric acid; AUT, acid-urea-Triton; HMG, high mobility group; CTAB, cetyltrimethylammonium bromide; AUC, acid-urea-CTAB; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

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species were detected that are thought to be histone H1 on the basis of their solubility in 5% perchloric acid and antigenic reactivity of a wide spectrum anti-H1^0 antiserum.

EXPERIMENTAL PROCEDURES

Alfalfa culture. Alfalfa tetraploid cultivar strains of *M. sativa* were grown as callus culture for 28 days from inoculation on nutrient agar (10). Callus size ranged from 1 to 2 cm. Callus cultures were collected, frozen at −70°C, and used within a few days for the preparation of histones.

Histone preparation. GuCl buffer is a solution of guanidine hydrochloride in 0.1 M potassium phosphate buffer, adjusted to pH 6.8 by KOH. Two milliliters of 40% GuCl buffer per gram of fresh weight of frozen callus was added. Callus was allowed to thaw on ice and was sonicated on ice for 2 to 5 min at medium intensity until essentially all tissue clumps had disappeared. The sonicate was cleared by centrifugation for 5 min at 1250*g* at 4°C and the large pellet was resonicated with up to 1 ml of 40% GuCl buffer per gram of callus and was recentrifuged. The combined supernatants were further clarified by centrifugation for 10 min at 30,000*g* at 4°C. The clear supernatant was acidified with concentrated hydrochloric acid to 0.25 N, and DNA and acidic proteins were precipitated for 15 min on ice. The cloudy solution was clarified by centrifugation for 30 min at 40,000*g* at 4°C, diluted with 0.1 M potassium phosphate buffer, pH 6.8, to a refractive index equal to that of 5% GuCl buffer, and adjusted to pH 6.8 by concentrated KOH.

Bio-Rex-70 resin, 100–200 mesh (Bio-Rad), was prepared in advance by extensive washing in 5% GuCl buffer until the refractive index and pH of the effluent were identical to that of 5% GuCl buffer. When small amounts of histones were prepared, a minimum column volume of 0.5 ml was maintained by the addition of Sephadex G-25 Fine, swollen and equilibrated in 5% GuCl buffer. The amount of Bio-Rex resin used was optimized experimentally for complete binding of all histone species without binding major amounts of nonhistone proteins which were not present under conditions of limiting amounts of Bio-Rex resin. The optimum amount of Bio-Rex-70 was identical to that observed during histone preparation from isolated nuclei of *Physarum polycephalum* (11), i.e., 1 ml of settled resin per 1 mg of total histone. This translated into the use of 1 ml of resin per 30 g of callus fresh weight.

The callus extract was incubated overnight at room temperature under constant mixing with the appropriate amount of Bio-Rex resin. The resin was washed at least three times with large volumes of 5% GuCl buffer, the resin was allowed to settle, and the supernatant was discarded. The resin was packed in a small polypropylene column (Bio-Rad 731-1550), washed with at least 8 vol of 5% GuCl buffer, and eluted with 10 vol of 40% GuCl buffer at elution rates not exceeding 1 column volume per 5 minutes. For column volumes exceeding 5 ml, slow elution with absorbance monitoring of the effluent at 230 nm allowed collection of the eluted histones in a smaller volume (11). The eluate was dialyzed against 100 vol of 5% acetic acid twice for 1 h and once overnight and was frozen and lyophilized.

Lyophilized histones were extracted into 5% perchloric acid (PCA) from a histone solution of 1 mg/ml in water by the addition of 1 vol of 10% PCA, precipitation on ice for 30 min, and centrifugation for 15 min at 10,000*g* at 4°C. The PCA supernatant extract and the PCA-insoluble pellet, solubilized in 5% acetic acid, were extensively dialyzed against 5% acetic acid and lyophilized.

Histone gel electrophoresis. SDS-polyacrylamide gels (18% acrylamide–0.09% bisacrylamide) were used as described by Thomas and Kornberg (12). Discontinuous acid–urea–Triton (AUT) gels at 15% acrylamide, 0.1% bisacrylamide, 8 M urea, and 8 mM Triton X-100 were run according to Mende *et al.* and Bonner *et al.* (11, 13). All gels were stained overnight with 0.1% Coomassie brilliant blue R-250 in 7% acetic acid, 20% methanol and were destained in 7% acetic acid, 20% methanol.

Coomassie-stained gel bands were quantitated in freshly destained wet gels using a Varex universal interface 12-bit analog-digital converter for data collection from an EC-910 densitometer and analyzed on an Apple IIe computer using software developed specifically for this analysis ((14); J. H. Waterborg and R. E. Harrington, unpublished). Programs in BASIC are available on request from J.H.W.

The molecular weights of the alfalfa histones were calculated from their mobility in SDS gels with calf thymus histones used as molecular markers exactly as described (11).

The standard AUT gel was modified to allow detection of core histone species among nonhistone proteins. The standard gel solution contained 15% acrylamide, 0.1% bisacrylamide, 1 M acetic acid, 50 mM ammonium hydroxide, 8 M urea, 0.5% TEMED, and 0.0003% riboflavin 5′-phosphate. A gel 240 mm wide by 160 mm high was prepared in the dark between two 300 × 175-mm glass plates. These were spaced 1 mm apart by one long and two short Teflon spacers and the assembly was positioned sideways. The gel was prepared by first pouring 25 mm gel solution containing 20% glycerol and 10 mM Triton X-100 followed by a linear gradient from 20% glycerol and 10 mM Triton X-100 to 0% glycerol and 0 mM Triton X-100 to a height of 110 mm and finally 25 mm gel solution without glycerol or Triton X-100. The gel was polymerized using a fluorescent light box and was then reoriented to an upright position by the removal of a short spacer strip and the addition of a long one. A

standard 4% acrylamide stacking gel with a 110-mm-long preparative sample slot was added and polymerized. An alfalfa histone preparation containing 1 mg histones was loaded in 0.9 ml sample buffer. Electrophoresis was for 16 h at 300 V under constant voltage conditions with gel staining and destaining as described above.

Histone Bio-Gel chromatography. A 400-ml column (2.1 × 116 cm) of Bio-Gel P-60 (Bio-Rad) in 20 mM HCl, 0.02% sodium azide, 50 mM NaCl was prepared (11, 15) and aged for several months. An alfalfa histone preparation containing 2.5 mg of histones was loaded in 0.5 ml of 8 M urea, 20 mM HCl, 1% 2-mercaptoethanol and eluted at a constant flow rate of 11 ml/h. Fractions of 3.5 ml were collected, monitored for absorbance at 210 nm, pooled, dialyzed against 5% acetic acid, and lyophilized. Concentrates from 1- and 2-ml fraction volumes were analyzed by electrophoresis on short (110 × 150 × 1 mm) SDS and AUT gels, respectively, and concentrates from 5-ml column eluates (except for 2.1 ml of Fraction 13) were analyzed on long (300 × 150 × 1 mm) AUT gels. Identification of protein bands A–E in SDS gels (Fig. 4C) with protein bands in AUT gels (Fig. 4D) was possible for bands A, B, and C based on their relative abundance and distribution over the fractions. It is likely that the set of bands labeled E (Fig. 4D) is identical to the smear below band C in SDS gels. Band D is not clearly identified in AUT gels. It may comigrate among the bands marked E, or below them.

Western blotting. SDS gels with alfalfa histones were electroblotted to nitrocellulose (0.2-μm pore size) as prescribed for the Bio-Rad transblot cell in 25 mM Tris, 190 mM glycine, pH 8.3, 10% methanol, and 0.1% SDS. AUT gels were diffusion-blotted onto nitrocellulose (0.2-μm pore size) in 1% acetic acid after two 5-min rinses of the gels in 1% acetic acid. Paper towels were used as wicks for a total buffer flow of 4 ml of transfer buffer per squared centimeter of gel. The blots were quenched with 5% Carnation dry milk in 10 mM Tris, pH 7.4, 0.15 M NaCl, 0.5% Triton X-100, 0.1% SDS. They were incubated in the same buffer with 1:100 diluted rabbit serum raised against the globular part of beef liver $H1^0$ (16) and the immunocomplex was detected with ^{125}I-protein A after an exposure of several days at −70°C with sensitized XAR-2 Kodak film (17). The serum detects calf H1 variants at a sensitivity of 0.25 relative to $H1^0$ and detects H1 and $H1^0$ of *P. polycephalum* with approximately equal sensitivity.

RESULTS

Histone Preparation

The methods described here allowed the preparation of histones from total plant callus tissue in order to assess histone composition and complexity in a plant without the need for nuclear and chromatin preparative methodology. Using these methods, we can cope with large amounts of cellular carbohydrates and obtain a protein preparation essentially free of carbohydrates. The technique derives from a method developed originally for the preparation of histones from isolated nuclei of the acellular slime mold *P. polycephalum*, free of the nuclear slime of this organism (11). Plant tissue is homogenized in a strongly dissociating and chaotropic solution of guanidine hydrochloride which maximally solubilizes cellular components, inhibits proteolytic activity, and dissociates chromatin constituents. After acid precipitation of DNA, the strongly basic histones are allowed to adsorb to the weak cation exchanger Bio-Rex-70. Unadsorbed proteins and soluble carbohydrate compounds are removed from the resin by washing, and the adsorbed proteins are batch-eluted by a high concentration of guanidine hydrochloride.

Using tetraploid callus cultures of alfalfa, *M. sativa*, SDS gel analysis of the proteins bound to and eluted from the Bio-Rex resin showed that a limited number of proteins were present (Fig. 1, lane 1). Some of the major protein bands comigrated with known core histone species such as histones H4 and H3 of calf thymus (Fig. 1, lane 2). The remainder of the major bands showed somewhat higher apparent molecular weights, similar to those observed for H2-type core histones in tobacco, wheat, rye, barley, and corn (1–4, 18). A major part of the remaining protein bands comigrated approximately with the calf thymus H1 histone species (Fig. 1, lanes 1 and 2). The use of higher-than-standard amounts of resin (see Experimental Procedures) to bind the solubilized nonacidic proteins resulted in the detection of many more protein species in the final preparation (Fig. 1, lane 3), while reduction in the resin-to-chromatin ratio appeared to lead to lower histone recoveries without a major increase in relative histone content of the preparation (results not shown).

Quantitation of the amounts of histones in our preparation was accomplished by

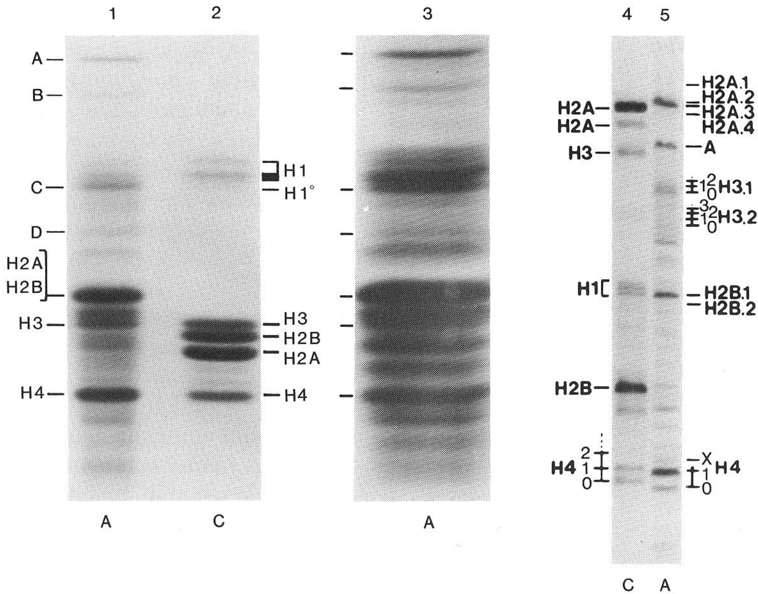


FIG. 1. SDS gel electrophoresis of alfalfa histone preparations. Alfalfa histones were prepared with 1 ml Bio-Rex-70 resin per guanidine hydrochloride extract from 30 g (lane 1) and 20 g (lane 3) of fresh callus, respectively. The histone preparation shown in lane 1 was also analyzed on a 30-cm-long AUT gel (lane 5). Calf thymus histones (Worthington) were used as markers in lanes 2 and 4. Alfalfa core histone species are labeled according to the identification and nomenclature determined as shown in Figs. 2–5. Protein species, soluble in 5% PCA (Fig. 7), are labeled A–D.

scanning and peak quantitation of the Coomassie brilliant blue stain present in known amounts of calf thymus histone H4 bands compared to the band in the alfalfa histone preparation that comigrated with H4. Histone H4 was selected on the basis of the extreme sequence conservation of this histone among all known species (5). The amounts of histone H4 obtained per gram of fresh callus wet weight ranged in different preparations from 10 to 14 μg, suggesting that total histone recovery per gram of callus was approximately 50 to 70 μg. Approximately the same amount of DNA can be prepared per gram of callus with standard phenol–chloroform extraction procedures.

Identification of Alfalfa Core Histones

In the absence of prior isolation of alfalfa nuclei and chromatin, the histone preparation obtained is not pure and histone species cannot be easily identified. This is especially true if one wants to identify the histones among the multiple bands seen on a long acid–urea–Triton X-100 gel (Fig. 1, lane 5) with separation between histone species that differ slightly from each other, as, for example, those variants that differ by single amino acid substitutions. Such variants and histones that differ by a single charge, e.g., by differential acetylation of internal lysine residues, are separated in this gel system. Complicated multiband patterns arise even for purified histone preparations, as that of calf thymus (Fig. 1, lane 4). Tentative identification can be made on the basis of coelectrophoresis between alfalfa and calf thymus histones only for the most conserved histone, histone H4, and even this comigration is not precise. The well-known separation between H4 species that differ in levels of modification by single acetyl or phosphoryl moieties is seen for the H4 histones of calf and alfalfa.

We have used the observation that the electrophoretic mobility of core histones in acid–urea gels can be affected in a species-specific manner by the presence of Triton

X-100 (13, 19, 20). This effect is absent for other proteins such as histone H1 species and for nonhistone proteins including the high mobility group (HMG) proteins (20). We used a gradient acid–urea gel similar to one described before (18) which incorporates, from side to side, a linear 0 to 10 mM Triton gradient. At one side, the gel is a regular discontinuous acid–urea gel with 8 M urea while at the other side it is an AUT gel (13). The result of such a gel is shown in Fig. 2. It is clear that the electrophoretic mobility of ionic species in the gel varies across the Triton and glycerol gradient. Measurements of the relative mobility of all protein bands showed that, with the exception of a limited number of bands that were identified by this characteristic as core histones (18), all protein species showed relative mobilities that were constant from 0 to 10 mM Triton.

Figure 3E shows the derivative of the relative mobility of selected reference protein bands (marked in Fig. 2 as bands a–g) in relation to the Triton concentration. These proteins do not adsorb Triton and therefore display a constant gel mobility. Four protein bands start to bind Triton at 2.5 mM and appear saturated at approximately 6 mM Triton (Fig. 3A). Two multiple protein species start to adsorb Triton at 3 mM and are nearly saturated at 10 mM (Fig. 3B). Two protein species bind Triton and reach half-saturation at 10 mM (Fig. 3C)

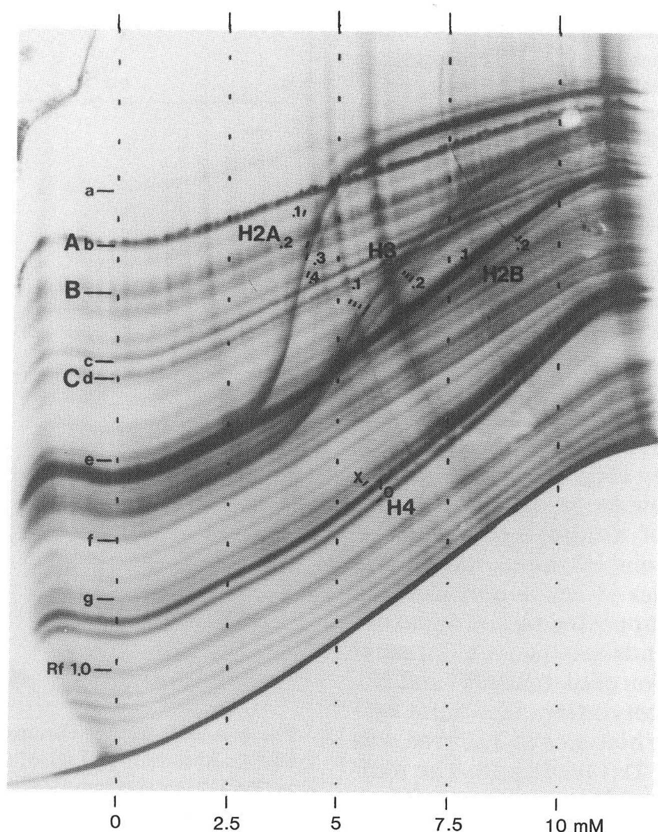


FIG. 2. Electrophoresis of alfalfa histones on an acid–urea gel with Triton gradient. Alfalfa histones were separated by electrophoresis on a 30-cm-long discontinuous acetic acid, 8 M urea gel with a linear Triton X-100 gradient from 0 to 10 mM from left to right. All core histone species identified by this gel are named. Protein bands A, B, and C were soluble in 5% PCA (Fig. 7) and bands A and B stained reddish with Coomassie blue. R_f 1.0 indicates the protein band used to determine the relative mobility (R_f) of all bands. Bands a–g were used to plot the derivative of the relative mobility of nonhistone proteins (Fig. 3E).

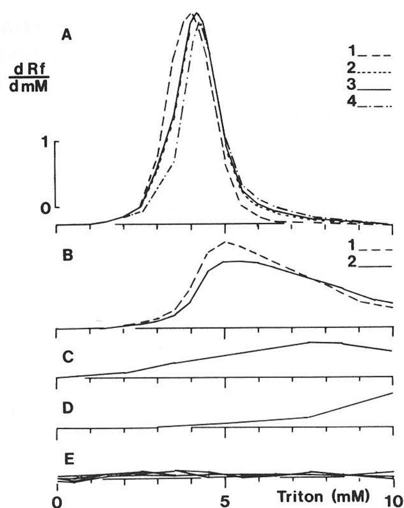


FIG. 3. Relative mobility of protein bands in the acid–urea–Triton gradient gel of Fig. 2. The derivative of the relative band mobility (R_f) with concentration gradient of Triton (mM) is plotted in arbitrary units. R_f was standardized on the protein band indicated by R_f 1.0 in Fig. 2. (A) H2A histones, 1–4 mark the four variant forms H2A.1, H2A.2, H2A.3, and H2A.4, respectively. (B) H3 histones, 1 and 2 mark the two variant forms H3.1 and H3.2, respectively. (C) H2B histones (the data for the two variants coincide). (D) H4 histones with identical differential mobility for the non- and monoacetylated forms. (E) The derivative of the relative mobility of protein bands a–g (Fig. 2).

and a triplet of two major bands and one minor band bind Triton with the lowest measurable affinity (Fig. 3D). These specific affinities (18) allow us to identify the core histone species of alfalfa into groups of histone H2A, histone H3, histone H2B, and histone H4 species (Figs. 1 and 2). This identification is supported by the apparent multiplicity of bands arising from internal histone modification of histones H4 and H3, presumably by acetylation. Optimum resolution of all core histones in 8 M urea was obtained at 8 mM Triton (Fig. 2). The multiple species of the histones are numbered according to their relative mobilities under these "standard" AUT gel conditions (Fig. 1, lane 5; see also Figs. 4D and 5).

Bio-Gel Chromatographic Separation of Histones

Fractionation of the crude histone preparation by Bio-Gel chromatography was used for a preliminary assessment of the relative amounts of the individual histone variants and for quantitation of the extent of modification of the core histones. The order of alfalfa histone elution was histone H1, histones H2A and H2B, histone H3, and histone H4, as shown by SDS (Fig. 4C) and AUT (Fig. 4D) gel electrophoresis. This elution sequence is seen for histones from other species with similar molecular weights of the histones (3, 11, 15, 18). Slight differences in column retention were noted among variants (Fig. 4B). All core histone bands were identified in both gel systems. The major protein bands A–C (Figs. 4C and 4D) at and near the Bio-Gel void volume that could possibly be H1 histones (see Fig. 7A) were identified in both types of gels.

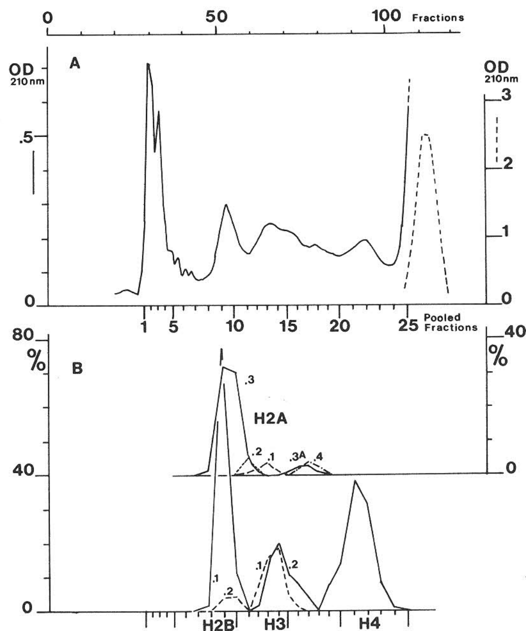
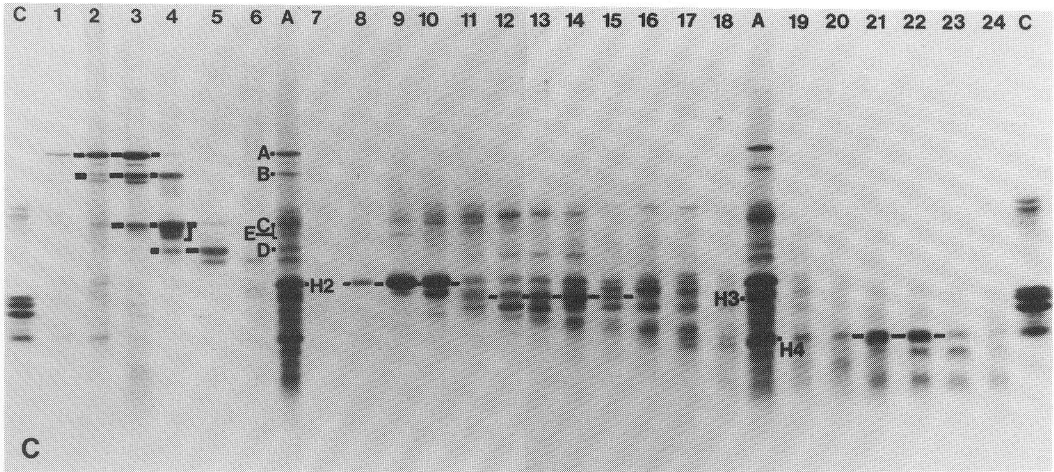


FIG. 4. Alfalfa histone chromatography on Bio-Gel P-60. (A) Absorbance of fractions. The peak around Fraction 112 (total column volume) contained 2-mercaptoethanol and urea but no protein. Fractions were combined as indicated and analyzed on SDS (Panel C) and AUT gels (Panel D) with calf thymus (C) and alfalfa histones (A) as markers. (B) The percentage elution of each histone species, differentiated by variant form as quantified by scanning the gels in Figs. 4 and 5. The H2A elution peaks are offset relative to the other peaks for clarity. (C and D) The gel lanes are marked by the numbers of the pooled Bio-Gel fractions.



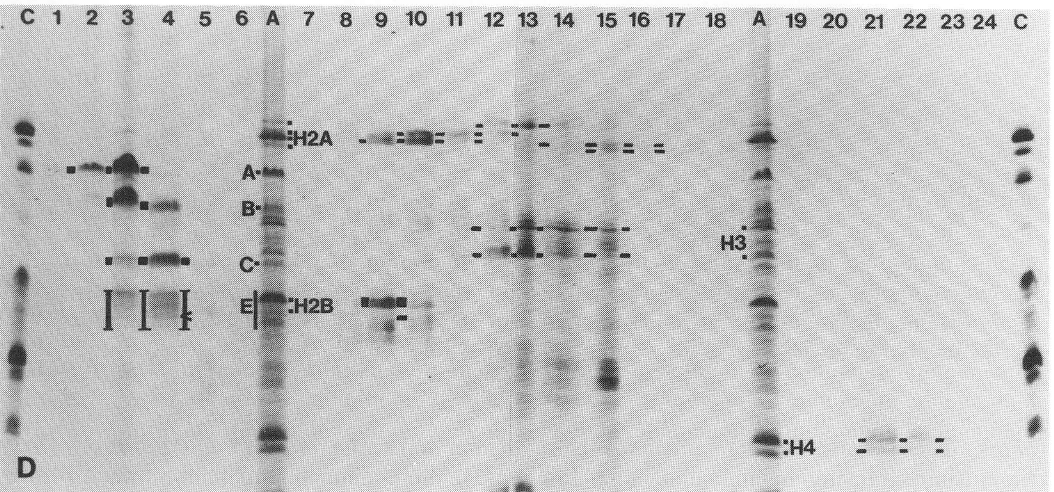


FIG. 4—*Continued.*

The heterogeneous bands in both gels that were labeled E are probably the same, while the migration of band D in AUT gels is uncertain. In the SDS gels, the apparent molecular weights of histones H3 and H4 were essentially identical to those of their calf thymus counterparts. All H2-type histone species comigrated at an apparent molecular weight of 16,900 ± 100 (n = 6). They also comigrate in acid–urea gels as shown in Fig. 2 at 0 mM Triton.

The elution of H4 could be analyzed on the short SDS and AUT gels, but elution behavior and fine band structure for the H2A, H2B, and H3 species required the longer high-resolution AUT gel shown in Fig. 5. The H2B variants H2B.1 (91%) and H2B.2 (9%) and the major H2A form, H2A.3, eluted almost simultaneously from the Bio-Gel column. The minor H2A forms, recognized in Fig. 2, eluted later, and an additional variant form, H2A.3A (Fig. 5), comigrated with H2A.3 in AUT gels. The band indicated by the arrows could possibly be a fifth H2A species, or it may be identical to the reference band with the lowest mobility in Fig. 2, lane a, which clearly is not a core histone. The relative amounts of H2A variants were 7, 6, 70, 8, and 9% for H2A forms 1, 2, 3, 3A, and 4, respectively. Small but detectable amounts of minor bands just above the major H2A and H2B

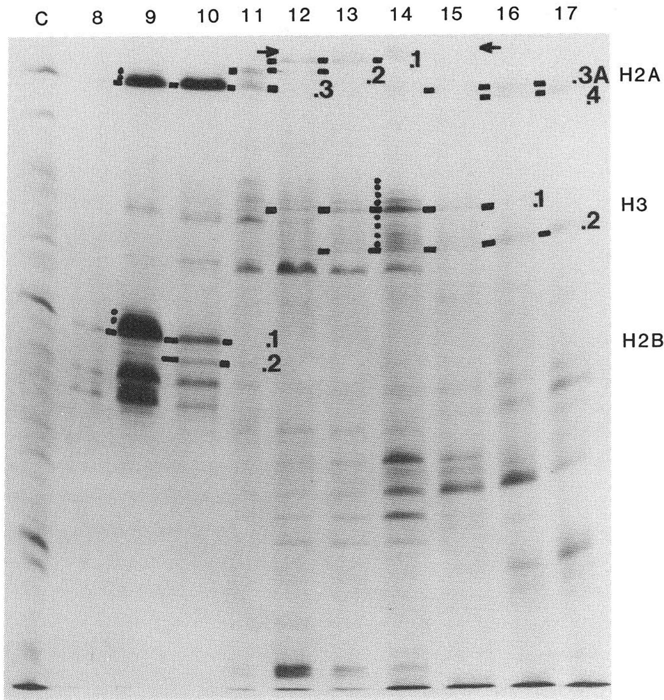


FIG. 5. High-resolution AUT gel electrophoresis of selected Bio-Gel fractions. Electrophoresis of Bio-Gel Fractions 8–17 on a 30-cm-long AUT gel (calf thymus histone markers, C). All identified core histone species are labeled. The band above H2A.1, marked by arrows, could possibly be an additional H2A variant. The position of the nonacetylated forms of the histone species is connected by the lines between the lanes. In selected lanes, the acetylated forms of histones H2A, H2B, and H3 are marked by solid circles.

forms, H2A.3 and H2B.1, indicate that these histones may be modified to a low extent, probably by acetylation (Fig. 5).

The two H3 variants eluted very closely together. The more extensively modified species eluted slightly earlier than the less modified forms. This phenomenon has been observed also during fractionation of the histones of *P. polycephalum* on Bio-Gel columns (J. H. Waterborg and H. R. Matthews, unpublished) and supports the idea that the detected modification is acetylation rather than phosphorylation. Histone H3.1 with 48% of the mass of H3 and an average content of 0.8 acetate groups per molecule was clearly less modified than histone H3.2 with, on average, 1.6 acetate groups (Fig. 6).

Histone H4 eluted from the Bio-Gel column free of any other histone contamination. In AUT gels, 75% appeared in a form with one positive charge less than the form with the highest gel mobility (Fig. 4D). We presume that these bands represent mono- and nonacetylated forms. No trace of H4 species differing by two or more positive charges from the fastest component could be detected. The minor band marked with x, observed in Fig. 2 just above monomodified H4 at a position expected to be midway between mono- and diacetylated H4, was not detected in this gel. This band may represent a minor H4 variant on the basis of its affinity for Triton X-100, or it could be a form of the single H4 histone modified to give a mobility shift of less than one full charge difference as caused by methylation (21).

Identification of Alfalfa Histone H1 Species

Noncore histones such as H1 cannot be identified as histones by their binding of

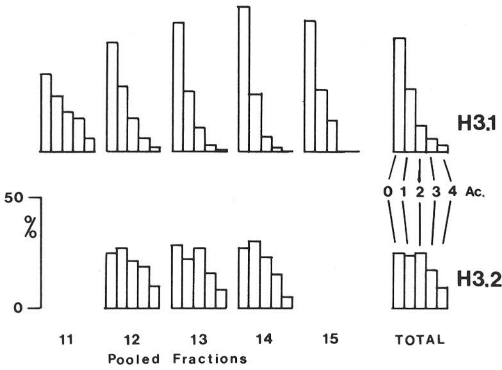


FIG. 6. Extent of histone acetylation in histone species H3.1 and H3.2. The extent of acetylation modification of H3 histones was determined by scanning of the gel shown in Fig. 5 followed by quantitation of the individual bands. H3.1 and H3.2 are drawn on the same scale.

Triton. Therefore, histone H1 species were identified by exploiting two H1 characteristics: (i) histone H1 is soluble in 5% PCA and (ii) histone H1 shows a remarkable sequence conservation, although not as extensive as the core histones, and alfalfa H1 might therefore be detectable by heterologous antisera. In addition, it is known for calf thymus and *P. polycephalum* histones that staining SDS and AUT gels with fresh solutions of Coomassie brilliant blue R-250 results in a red to purple tinge to bands of histone H1. SDS-gel electrophoresis detected four discrete protein bands that were solubilized from a total alfalfa histone preparation in 5% PCA (Fig. 7, lane 2, bands A–D) and some lesser discrete protein bands (F). Bands A–D are also the four major protein species in the Bio-Gel void volume (Fig. 4C, lanes 1–6). Bands A and B are present in larger amounts than band C (Fig. 1, lane 5; Fig. 2). Bands A and B stain purple with Coomassie in SDS and AUT gels, while the third component of E (see arrow in lane 4 of Fig. 4D) stains purple in AUT gels. These initial results suggest that bands A–D, and possibly also heterogeneous bands E and F, could be histone H1 species or proteolytic fragments of these histones.

An antiserum raised against the globular part of bovine liver $H1^0$ was used subsequently to identify possible alfalfa H1 species. This serum had been shown to have reactivity for bovine H1 variants and for both H1 and $H1^0$ species of the lower eukaryote *P. polycephalum* (16). In blots of SDS gels of alfalfa histones, the serum detected primarily band D. Longer exposures (Fig. 7, lane 5) detected reaction with band A and a low-molecular-weight band F, in addition to a band between C and D, not seen by Coomassie staining. This latter band comigrates with the protein bands labeled E in Fig. 4C. In blots of AUT gels, which contained lanes equivalent to lanes 2–4 of Fig. 4C, reactivity with the antiserum was seen with bands A, B, and C (Fig. 7, lanes 6–8). Band D was not detected.

Thus the four major proteins A–D, initially recognized as possible H1 species on the basis of solubility in 5% PCA and/or purple staining with Coomassie, were all detected by the antiserum. The cross-reactivity clearly differed with the state of the protein: D reacted most strongly after denaturation with SDS, while the others reacted stronger when blotted from native (AUT) gels. The signal on a nitrocellulose blot from AUT gels is weak due to the interference of Triton with histone transfer and absorption (22). The identity of the minor cross-reacting bands E and F as H1 or proteolytic H1 fragments will require further study.

The apparent molecular weights of the four major protein species that are likely H1 variants were determined in SDS gels to be approximately 28,600, 26,200, 20,500, and 18,100 (± 200, $n = 6$) for bands A, B, C, and D, respectively, as measured relative to the known calf thymus histones. The larger molecular weights of the major H1 species A and B are consistent with the larger molecular weights for histone H1 often seen in plants and lower eukaryotes relative to those of mammalian cells (2, 4–6, 23). Only band D is smaller than calf thymus H1 species. We cannot exclude that this minor species is a proteolytic part of a histone H1 which may explain the higher cross-reactivity seen for this band in SDS gel blots (Fig. 7, lane 5).

DISCUSSION

Identification of histone species, especially if they do not comigrate with

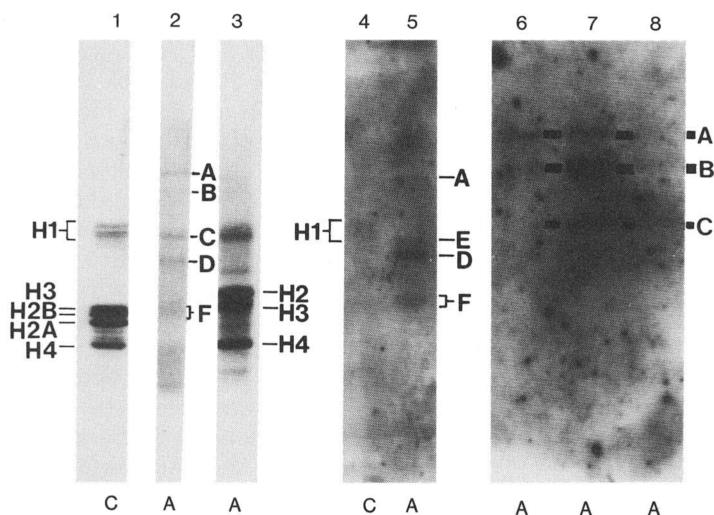


FIG. 7. Survey of possible histone H1 species. Alfalfa histone analysis by electrophoresis on SDS gels after separation into a fraction soluble (lane 2) and insoluble in 5% PCA (lane 3). Protein band F was less discrete than soluble bands A–D. Lane 5 is a Western blot of an SDS gel lane similar to lane 2, and lane 4 is a similar gel lane with calf thymus histones. Lanes 6–8 are a Western blot of an AUT gel with the samples also run in lanes 2–4 of Fig. 4D. C, calf thymus histones. The blots were incubated with antiserum raised against bovine globular $H1^0$, and immunocomplexes were detected with ^{125}I-protein A.

known histones from other organisms, is generally possible only after extensive purification and amino acid analysis (3, 8, 11). Based on their extreme sequence conservation, histone H3 and H4 bands in SDS gels can often be identified by electrophoretic comigration with calf histone species. Other protein bands prepared from nuclei or chromatin can often be only tentatively identified and are sometimes collectively called H2s (1, 4).

In this work, we have shown that we can easily identify core histones by their specific abilities to bind Triton X-100 with affinities that are clearly different among the various histone species (Fig. 3). This method of preliminary identification has been used previously for purified barley and chicken histones and confirmed by purification to homogeneity and amino acid analysis (18). The specific binding characteristics of each of the core histones for Triton has been observed in all species so far examined (11, 13, 19, 20). We show this identification to be possible even in relatively crude histone preparations (Fig. 2). An AUT gel with a transverse gradient of urea at a constant concentration of Triton X-100 has been described to distinguish histone variants of the sea urchin *Parechinus angulosus* (24). This gel system, while similar in concept to ours, lacks the power of histone identification on the basis of affinity to Triton X-100. It is not possible to identify histone H1 species in our gel system because these histones do not display the characteristic of binding Triton X-100.

Histone variant species are generally identified only after separation in a two-dimensional gel system: a first-dimension AUT gel followed by acid-urea-CTAB or SDS electrophoresis (13, 25). The latter option, in particular, requires a protein preparation free of nonhistone proteins to allow variant identification. The Triton gradient gel used in this work incorporates into a one-dimensional gel all the features of the two-dimensional AUT/AUC system and thus allows histone identification. It is superior to existing methods for variant detection since it combines the ease of a one-dimensional gel with the avoidance of oxidative artifacts and the acceptability of non-histone-contaminating protein species. An additional advantage of this ap-

proach is the easy way in which the optimum amount of Triton X-100 required for histone variant resolution in standard AUT gels is determined. Disadvantages are the requirement for a larger sample size (1 mg of histones as compared to 0.1 mg in other methods) and, in the event that major amounts of nonhistone proteins are observed, the difficulty in variant quantitation by densitometric scanning of the gel. However, such quantitation is also difficult in two-dimensional gel systems.

One of the functions of the urea in AUT gels is its dissociative power between histones and Triton. If a gel similar to that shown in Fig. 2 is run at a lower concentration of urea, then binding of Triton, reduction of electrophoretic mobility, and histone saturation by Triton will occur at lower Triton concentrations (18). Thus the histone identification gel of Fig. 2 can also be obtained if the concentrations of urea and of the Triton gradient are both reduced.

The method for histone preparation from crude cell lysates by selective adsorption to Bio-Rex-70 resin, used in this case with alfalfa callus cultures, has been used previously with total plasmodia and even with enucleated cytoplasm of *P. polycephalum* (21). The easy transfer of this technology among organisms suggests that it can also be used with other plant or animal tissues for preparation of histones and analysis of histone variants.

We have used this and associated methods to describe and identify multiple histone variants and levels of modification, presumably acetylation, from a higher plant. The multiple alfalfa histone species detected in Fig. 2 have been called variants, but definitive proof for this assignment will require purification to homogeneity, amino acid analysis, and sequence determination (8). This is the only way to exclude the possibility that some of the histone bands that have been called variants are not in fact modified forms of the same primary histone species.

One such possible modification is the carboxy-terminal addition of ubiquitin to histones H2A and H2B, observed at levels of 10 and 1.5%, respectively, in L cells (26, 27) and at 7 and 6% in *P. polycephalum* (28). Based upon electrophoretic mobility, only histone H2A.1 could be such a modified form of the major H2A histone, H2A.3. All other minor species detected have mobilities below or too close to the major form to be compatible with such a modification (29). At the moment we cannot exclude the possibility that some of the lower mobility forms among the H2A variants in the AUT gel might have arisen by phosphorylation. The absence of proteolytic degradation of the histones during preparation is suggested by the results in Fig. 2. We cannot detect bands that bind Triton like the various core histone species but that have clearly lower molecular weights. Therefore, the discrete minor histone species detected are probably not derived from the major forms by proteolytic fragmentation. The absence of oxidized dimeric H3 forms in Fig. 2 suggests that the minor variant forms observed are not oxidized derivatives of the major histone species, as may be found after two-dimensional gel analysis without proper precautions (13).

The differences in relative amounts between variants of a histone species could arise from differential rates of gene expression or from differences in gene copy level within the histone gene family. Such differences may be important to supply the different histone forms required for different chromatin environments, as was noted for the more hydrophobic H2A variant in mice which was preferentially present in heterochromatin (7). In these terms, it was particularly interesting to observe that H3 variant H3.2 showed a much higher level of steady-state acetylation than H3.1 (Fig. 6). H3.2 could be preferentially localized in chromatin domains that actively transcribe genes or that have the potential to do so. The use of nondifferentiated clonal callus cultures makes the alternative explanation of different H3 variant specificity for different tissues unlikely.

The presence of acetylated lysine residues in histone species with lower mobilities in AUT gels has not been proven (Fig. 5). The assignment of this modification was based on identical patterns and distributions observed in histones of higher and lower eukaryotes which have been shown to contain acetylation of lysine residues in

the amino-terminal domain of the histones (5). To our knowledge, this is the first time that histone H3 acetylation up to the level of tetraacetylation has been described in plants. This is equivalent to the maximum levels of acetylation generally observed in mammalian cells after hyperacetylation by butyrate (5, 9). The acetylation of alfalfa histones H3 and H4, and of pea and tobacco H4, supports the expectation that this modification is a general feature in chromatin, possibly necessary for transcriptional activity (5, 30).

The finding of multiple H1 variants in alfalfa is consistent with data from other plants and lower eukaryotes, showing some increases in molecular size as compared to mammalian cells (6, 11). At the same time, the observed cross-reactivity of alfalfa H1 histone bands with antiserum raised against the globular portion of bovine $H1^0$ indicates conservation of epitopes in histone H1 from animals to higher plants.

ACKNOWLEDGMENTS

We thank Thomas J. McCoy of USDA/ARS, College of Agriculture, University of Nevada Reno (present address: Department of Plant Sciences, University of Arizona, Tucson) for the source of alfalfa callus. We are also grateful to L. Mende and E. M. Bradbury, Department of Biological Chemistry, University of California, Davis, for the opportunity to probe alfalfa histones with their anti-globular-$H1^0$ antiserum. This work was supported in part by U.S. Public Health Service Grant GM 33435 and by Hatch Projects 131 (REH) and 136 (IW) through the College of Agriculture, University of Nevada Reno.

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