Enhanced Stability of Histone Octamers from Plant Nucleosomes: Role of H2A and H2B Histones†

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Received March 26, 1992; Revised Manuscript Received June 30, 1992

ABSTRACT: Gel filtration and sedimentation studies have previously established that the vertebrate animal core histone octamer is in equilibrium with an $(H3–H4)_2$ tetramer and an H2A–H2B dimer [Eickbush, T. H., & Moudrianakis, E. N. (1978) *Biochemistry 17*, 4955–4964; Godfrey, J. E., Eickbush, T. H., & Moudrianakis, E. N. (1980) *Biochemistry 19*, 1339–1346]. We have investigated the core histone octamer of wheat (*Triticum aestivum* L.) and have found it to be much more stable than its vertebrate animal counterpart. When vertebrate animal histone octamers are subjected to gel filtration in 2 M NaCl, a trailing peak of H2A–H2B dimer can be clearly resolved from the main octamer peak. When the plant octamer is subjected to the identical procedure, there is no trailing peak of H2A–H2B dimer, but rather a single peak containing the octamer. A sampling across the octamer peak from leading to trailing edge shows no change in the ratio of H2A–H2B to $(H3–H4)_2$. Surprisingly, the plant octamer shows the same stability at 0.6 M NaCl, a salt concentration in which the vertebrate animal octamer dissociates into dimers and tetramers. Equilibrium sedimentation data indicate that the assembly potential of the wheat histones in 2 M NaCl is very high at all protein concentrations above 0.1 mg mL^{-1}. In order to disrupt the forces stabilizing the plant histone octamer at high histone concentrations, the concentration of NaCl must be lowered to approximately 0.3 M. Because histones H3 and H4 are essentially identical proteins in plants and animals, it is logical to assume that the H2 histones (H2A and H2B) are responsible for the enhanced stability of plant histone octamers. Reconstitution of octamers from acid-extracted histones provides evidence that this is true. Wheat H2A and H2B histones form an octamer of enhanced stability with either wheat or chicken H3 and H4 histones.

The histone core complex is an octameric assembly of two each of the four core histones (H2A, H2B, H3, and H4) around which nuclear DNA wraps to form nucleosomes, the elementary unit of chromatin structure (van Holde, 1989). A variety of physical chemical experiments support the concept of the core histone octamer having a tripartite organization in solution, with two H2A–H2B dimers flanking a central $(H3–H4)_2$ tetramer complex (Eickbush & Moudrianakis, 1978; Godfrey et al., 1980; Butler & Olins, 1982; Benedict et al., 1984). The H2A–H2B dimer and $(H3–H4)_2$ tetramer subunits are each stabilized by strong, hydrophobic interactions which can be disrupted only under extreme conditions (Eickbush & Moudrianakis, 1978). Dimer–tetramer interactions are relatively weak in comparison; the limited number of hydrogen bonds between these subunits can be disrupted by changes in temperature or pH or by the addition of urea (Eickbush & Moudrianakis, 1978). The large exothermic heats associated with histone assembly support hydrogen bonding as the primary source responsible for stabilizing dimer–tetramer interactions (Benedict et al., 1984).

These studies of the isolated histone octamers have used mammalian or avian chromatin as the source of the experimental material. We have long been interested in the role of chromatin structure in the regulation of gene expression in plants and in the question of whether the differences in plant and animal H2 histones result in biochemical and physiological differences in plant and animal chromatin. Histones H3 and H4 presumably cannot contribute to any structural differences in plant and animal chromatin as these are the archetypal examples of evolutionarily conserved proteins. The plant and animal histones are of the same size and have sequences with identical amino acids at 98% (H4) and 97% (H3) of the positions (Wu et al., 1986). Although the H2A and H2B histones are also considered to be conserved, they exhibit considerable differences between plants and animals. Plant H2A and H2B are larger than their animal counterparts (Spiker, 1988). Additionally, plants possess numerous H2A and H2B variants that differ from one another in molecular weight (Spiker, 1982; Langenbuch et al., 1983; Moehs et al., 1988). Most histone variants of vertebrate animals, in contrast, have the same molecular weight and differ from one another only by amino acid point substitutions (Wu et al., 1986).

Relatively little sequence information is available for plant H2 histones. Two wheat H2A variants and one wheat H2B variant, however, have been sequenced by protein sequencing methods (Rodrigues et al., 1985, 1988; Brandt et al., 1988). Deduced sequences for H2 histones have recently appeared based on cDNA sequences from parsley, tomato, and pea H2A (Spiker et al., 1990; Koning et al., 1991) and wheat H2B (Yang et al., 1991).

Compared to the most abundant calf thymus H2A histone variant (129 amino acid residues), one of the wheat H2A variants has an extension of 19 amino acids at its C-terminus. A second wheat H2A variant has an N-terminal extension of 8 amino acids and a C-terminal extension of 14 amino acids. The parsley, tomato, and pea H2A histones have N- and C-terminal extensions in the same range as the second wheat

† This work was supported in part by grants from the National Science Foundation (S.S.) and the National Institutes of Health (E.N.M.).

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0006-2960/92/0431-10844$03.00/0

H2A. In a central block of 99 amino acid residues, in which the major calf and chicken H2A variants have identical sequences, the wheat H2A variants have amino acids identical to calf and chick at 79% of the positions.

The sequenced wheat H2B variant has an N-terminal extension of 24 amino acids compared to calf thymus H2B (Brandt et al., 1988). The 90 C-terminal residues of calf and wheat H2B are identical at 79% of the positions.

These differences between plant and animal H2A and H2B histones have no detectable effect on pairwise histone–histone interactions as studied by fluorescence polarization, light scattering, and circular dichroism (Spiker & Isenberg, 1977, 1978). Neither do they appear to affect the ability of the histone core to form nucleosomes in vitro (Liberati-Langenbuch et al., 1980). They apparently do, however, have an effect on the conformation of the nucleosome core (the complex of 146 base pairs of DNA and the histone octamer). On native polyacrylamide gels, plant nucleosome cores have a much lower electrophoretic mobility than their vertebrate animal counterparts (Arwood & Spiker, 1990). This lower electrophoretic mobility cannot be accounted for simply by differences in charge or by the differences in sizes of the H2 histones. We thus have assumed it is due to a conformational change. Recently, the extended C-terminal tail of wheat H2A has been shown to interact with linker DNA between nucleosome cores (Lindsey et al., 1991).

In view of these data comparing plant and animal histones and complexes involving histones, we have asked whether the differences between plant and animal H2A and H2B have an effect on the associative behavior of the H2A–H2B dimer and $(H3–H4)_2$ tetramer in forming the core histone octamer. Wheat histone complexes were analyzed by both gel filtration and equilibrium sedimentation in order to compare their behavior to that of calf thymus and chicken erythrocyte histones (Eickbush & Moudrianakis, 1978; Godfrey et al., 1980). These data suggest that the isolated wheat histone octamer is a considerably more stable assembly over a greater range of ionic strengths than is its animal counterpart. For example, at 0.6 M NaCl, the wheat histones remain associated in a stable octamer, but the chicken octamers are dissociated into H2A–H2B dimers and $(H3–H4)_2$ tetramers.

Reconstitution of histone octamers with mixtures of acid-extracted histones has provided direct evidence for the role of H2 histones in the enhanced stability of the plant octamers. Exclusion chromatography shows that wheat histone octamers reconstituted from acid-extracted histones have a stability indistinguishable from that of salt-extracted octamers; e.g., they are stable in 0.6 M NaCl. In contrast, octamers reconstituted with wheat H3+H4 and chicken H2A+H2B dissociate into dimers and tetramers at a concentration of 0.6 M NaCl in a manner identical to that of octamers reconstituted solely with chicken histones. Finally, octamers reconstituted with chicken H3+H4 and wheat H2A+H2B remain intact in 0.6 M NaCl. Thus, the enhanced stability of wheat histone octamers is due to properties of the H2A and H2B histones.

EXPERIMENTAL PROCEDURES

Isolation of Chromatin and Histone Octamers. Wheat embryo chromatin was isolated as previously described (Spiker, 1984). Wheat germ was obtained from General Mills, Inc., Minneapolis, MN. All solutions contained 15 mM β-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF),[1] and 12 mM sodium bisulfite; all operations were carried out at 4 °C. Purified chromatin was extracted with 0.35 M NaCl to remove non-histone chromosomal proteins. The chromatin was then washed twice in deionized water, and the resulting gel was sheared. Histone octamers were extracted from the sheared chromatin by adding an equal volume of 4 M NaCl, 20 mM Tris-HCl, and 0.2 mM PMSF. The DNA was pelleted by centrifugation at 44 000 rpm for 12 h in a Beckman Ti50 rotor. Histone octamers were subsequently recovered from the supernatant.

Chromatin and histone octamers were also isolated from chicken erythrocytes. Whole chicken blood was collected from mature white Leghorn chickens at a local processing plant. Erythrocytes were isolated by the method of Rill et al. (1978), and nuclei were prepared according to Sandeen et al. (1980). All solutions contained 15 mM β-mercaptoethanol and 0.1 mM PMSF. Non-histone chromosomal proteins were removed from the nuclei with 0.35 M NaCl, and histone complexes were extracted from the DNA as described above. Salt-extracted wheat and chicken octamers could be stored at 4 °C for several weeks with no signs of degradation.

Gel Filtration. Histone complexes were fractionated on several Sephadex G-100 (Pharmacia LKB) columns (Eickbush & Moudrianakis, 1978). Two larger columns (2.5 × 150 cm) were equilibrated with 2 or 0.3 M NaCl. A smaller column (2.5 × 100 cm) was equilibrated with 0.6 M NaCl. All buffers contained 10 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF. Histone complexes were concentrated to 7 mg mL^{-1} using either an Amicon ultrafiltration device with a YM-10 membrane or an Amicon Centricon-10 device. The histone complexes were applied to the columns and eluted at a flow rate of 10 mL h^{-1}. Sephadex columns were run at 4 °C, and 5.3-mL fractions were collected.

Histone fractions were also separated by denaturing exclusion chromatography on a Bio-Gel P-60 column (2.5 × 170 cm; Bio-Rad) equilibrated with 0.01 N HCl and run at room temperature as previously described (Spiker, 1982).

Histone Stoichiometry. The stoichiometry of the individual histones within the wheat octamer was determined by subjecting the purified octamers to denaturing chromatography on Bio-Gel P-60. The concentration of protein in the well-resolved H2, H3, and H4 peaks was determined spectrophotometrically (Spiker & Isenberg, 1977). The concentration of protein in the H2 peak was corrected for differences in absorbance by using refractive indices. Molecular weights of 15 324 for H3 and 11 282 for H4 were used to convert mass ratios to molar ratios (DeLange et al., 1969, 1973). Since H2A and H2B cannot be resolved by Bio-Gel P-60 chromatography, we directly determined only (H2A–H2B)/H3 and (H2A–H2B)/H4 ratios. In order to assign individual molar ratios for H2A and H2B, we assumed that these proteins were present in equimolar ratios (Spiker, 1982). An average molecular weight of 16 111 was determined for all of the wheat H2 histones based on combining the data from the known sequences of two of the wheat H2A variants (Rodrigues et al., 1985, 1988) and one of the wheat H2B variants (Brandt et al., 1988) and the mobilities of the variants on $NaDodSO_4$–polyacrylamide gels (Spiker, 1982).

Histone stoichiometry was also estimated from Coomassie Blue-stained proteins separated by $NaDodSO_4$–polyacrylamide gel electrophoresis. The wheat H2A and H2B variants, which differ in molecular weight, migrate to different positions in the gel and thus appear as multple bands rather than the single band for H2A and the single band for H2B that occur with vertebrate animal histones. Because any individual band of wheat H2A or H2B does not stain as intensely as the H3

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

and H4 bands, visual inspection gives the impression that H2A and H2B are underrepresented (e.g., see Figure 1A, lane b). However, scans of stained gels indicate that the total area under the H2A–H2B peak is approximately equal to the H3–H4 areas. In experiments in which we wished to determine if there were changes in ratios of histones across a peak eluted from a Sephadex column, we scanned Coomassie Blue-stained gels. Because the H2 histones are not base-line-resolved from histone H3, we calculated ratios of the combined H2–H3 peak area relative to H4. These values did not give us absolute ratios of the histones, but allowed us to determine if their ratios were different in the leading edge, center, and trailing edge of the octamer peak.

Analytical Ultracentrifugation. Meniscus depletion sedimentation equilibrium runs were performed in a Beckman Model E analytical ultracentrifuge equipped with Rayleigh interference optics following procedures outlined in Godfrey et al. (1980). Briefly, all octamer solutions were dialyzed overnight into either 2.0 M or 0.6 M NaCl, each containing 10 mM HEPES, pH 7.5, and 1 mM EDTA, to assure dialysis equilibrium. Double-sector cells with 12-mm aluminum-filled epon centerpieces and quartz windows were filled to a column height of 2.7 mm (100 μL). Sedimentation was performed at 20 000 rpm for 24 h using a Beckman An-F (four-hole) rotor at 18 °C. Plates were read on a Nikon microcomparator, and apparent number-, weight-, and z-average molecular weights were calculated by the method of Roark and Yphantis (1969) on a Hewlett-Packard minicomputer.

Acid Extraction and Fractionation of Histones for Reconstitution Studies. Chromatin isolated from wheat germ and chicken erythrocytes as described above was washed with 0.35 M NaCl to remove non-histone chromosomal proteins and subsequently swelled in distilled water. The chromatin was sheared (Brinkmann Polytron), and the histones were extracted by making the chromatin 0.4 N in sulfuric acid. The DNA was pelleted by centrifugation, and the supernatant was dialyzed against 95% ethanol to precipitate the histones. After the histones were washed twice in a mixture of 6 parts acetone and 1 part 0.1 N HCl followed by two acetone washes, they were dried and stored at −20 °C.

Fifty milligrams of chicken or wheat histones was separated on a Bio-Gel P-60 column as described above. Fractions obtained were used in reconstitution experiments.

Reconstitution of Histone Octamers. Octamers were reconstituted as described in Greyling et al. (1983). Fractions from the Bio-Gel P-60 column containing H2A, H2B, H3, and H4 were combined and concentrated to 3 mg mL^{-1} with an Amicon ultrafiltration device and a YM-10 membrane. Solid urea was added to a concentraton of 8 M, and the histone mixtures were dialyzed into 2 M NaCl, 10 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF. Following dialysis, the histone complexes were concentrated to 12 mg mL^{-1} with an Amicon Centricon-10 device and subjected to exclusion chromatography at 4 °C on a Sephadex G-100 column (2.5 × 150 cm) equilibrated in 2 M NaCl, 10 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF. In the case of octamers reconstituted from mixtures of wheat and chicken histones, the H3 and H4 histone fractions of one species were mixed with the H2A and H2B histones of the other species, and the reconstitution was carried out the same as the homologous octamer reconstitution. After elution of the octamer reconstitution mixture from the Sephadex G-100 column equilibrated in 2 M NaCl, 10 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF, the fractions containing octamers were combined, dialyzed into a buffer containing 0.6 M NaCl, 10 mM Tris-HCl, and 0.1 mM PMSF, and fractionated at 4 °C on a Sephadex G-100 column equilibrated in the 0.6 M NaCl-containing buffer.

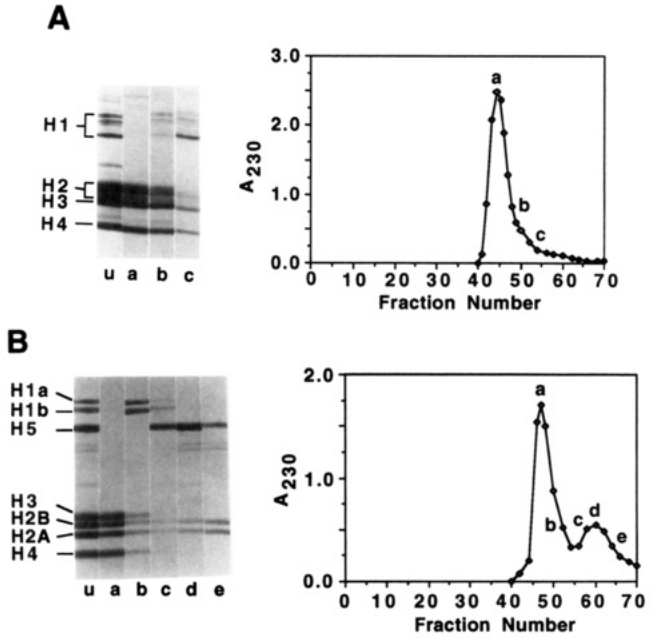

FIGURE 1: High-salt Sephadex G-100 chromatography and NaDodSO$_4$ gels of (A) wheat histone complexes and (B) chicken histone complexes. Wheat and chicken chromosomal proteins extracted from purified chromatin with 2 M NaCl were loaded onto a column equilibrated with 2 M NaCl and 10 mM Tris-HCl, pH 7.5, and eluted with the same buffer. The fractions denoted on the elution profile from the column (points closest to identifying letter) correspond to the lanes in the NaDodSO$_4$ gels. Lane u (unfractionated) denotes the extract before being loaded onto the column.

Gel Electrophoresis. Histones were analyzed on 0.5-mm microslab gels as described by Matsudaira and Burgess (1978). NaDodSO$_4$–polyacrylamide gels (18%) (Thomas & Kornberg, 1975) were used to examine wheat histones and histone complexes containing wheat H2A and H2B histones, while 15% NaDodSO$_4$–polyacrylamide gels (Laemmli, 1970) were used to examine chicken erythrocyte histones and histone complexes containing chicken H2A and H2B histones. The different systems are used to give optimal resolution of all histones.

RESULTS AND DISCUSSION

Histone complexes extracted from wheat embryo chromatin by 2 M NaCl and 10 mM Tris-HCl, pH 7.5, were fractionated on a Sephadex G-100 column equilibrated in the same buffer (Figure 1A). At the left in Figure 1A is an 18% polyacrylamide gel of selected fractions from the column. In order to be able to directly compare our data with previously published work, we extracted both wheat embryo chromatin and chicken erythrocyte chromatin following the procedure of Eickbush and Moudrianakis (1978). The elution profile and 15% polyacrylamide gel of chicken histone complexes passed over the same G-100 column are shown in Figure 1B. Our results with the chicken histone complex are essentially identical to the previously published work of Eickbush and Moudrianakis (1978).

In contrast to the chicken histone complex shown in Figure 1B, which elutes in two peaks, the wheat histone complex elutes as a single peak. The leading edge of the peak contains the histone octamer (Figure 1A, lane a), while the trailing edge of the peak contains mostly H1 (Figure 1A, lane c). Including H1 in the protein mixture that was applied to the column allows us to confirm two points based on previous work with animal systems. First, because H1 does not elute

with the main peak, H1 is not bound to the octameric complex. Second, because H1 is larger than any of the core histones, yet elutes later than the core histones, the wheat core histones must be involved in a stable complex of molecular weight greater than H1. The assumption that the wheat core histone complex is indeed an octamer is bolstered by the observation that the apex of the peak comes consistently at fraction 44. The apex of the chicken octamer peak is found consistently at fraction 46 using the same column (Figure 1B). This is in line with the expectation that the wheat octamer should be slightly larger than the chicken octamer due to the increased sizes of the H2 histones.

There is no evidence for any trailing H2A–H2B dimer peak for the wheat histones (Figure 1A) as there is for the chicken histones (Figure 1B). It would appear from these data that the equilibrium between the H2A–H2B dimer, the $(H3–H4)_2$ tetramer, and the octamer complex is pushed toward formation of a stable octamer in wheat. It might be argued that a substantial fraction of the wheat histones do exist as free H2A–H2B dimers but that they cannot be detected because the larger plant dimers are not resolved from the $(H3–H4)_2$ tetramer. To exclude this possibility, we have carefully assessed the H2A–H2B–H3/H4 ratios across the peak and have found the ratios to remain constant (data not shown). Additionally, at lower ionic strength (0.3 M NaCl), free H2A–H2B dimers can indeed be resolved from the $(H3–H4)_2$ tetramer (Figure 4C).

As mentioned above, the H2 histones in plants are larger than their animal counterparts and exist as several molecular weight variants (Spiker, 1982). They are resolved into several bands which migrate just above H3. The $NaDodSO_4$ gel of the wheat chromatin extract and the wheat octamer (Figure 1A, lanes u and a) and also acetic acid–urea–Triton X-100 gels (Zweidler, 1978) and two-dimensional gels (Moehs et al., 1988) reveal that all of the wheat H2A and H2B variants are present in the wheat chromatin extract and that all are incorporated into the wheat histone octamer (data not shown). The ratios of the variants do not change across the octamer peak in 2 M NaCl.

The fact that the wheat chromatin extract elutes as a single peak at 2 M NaCl, in contrast to the chicken chromatin extract which elutes in two peaks, suggests that the wheat octamer is more stable than the chicken octamer. This increased stability is not due to excess H3 and H4 in the wheat extract. We established the stoichiometry of the histones in the leading and trailing edges of the complex eluted in 2 M NaCl by scanning $NaDodSO_4$ gels of these fractions (Figure 1A, lanes a and b; scans not shown). Because some of the H2 variants comigrate with H3, H3 appears to be present in greater than stoichiometric amounts on this Coomassie Blue-stained gel. Thus, we combined the areas of the H3 and H2 regions of the gel and found that together they comprise approximately 3 times the area of the H4 band. This ratio is the same in Figure 1A, lanes a and b. These data, along with previously published data (Spiker et al., 1987), indicate that the four core histones are present in equimolar ratios.

An additional method for determining the stoichiometry of the wheat histone octamer is presented in Figure 2. Fractions 42–45 from the Sephadex column of Figure 1A were combined and dialyzed into 0.01 N HCl. The histones were then concentrated and loaded onto a Bio-Gel P-60 column under denaturing conditions. The elution profile (Figure 2) shows the fractionation of the histones into three peaks containing H2, H3, and H4. Areas under these peaks were used to determine the relative mass concentrations of the histones in the core complex. The areas under the peaks were normalized

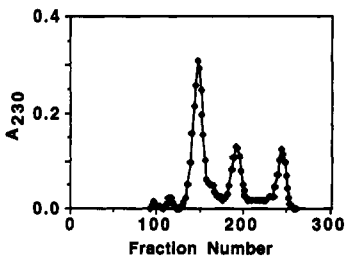

FIGURE 2: Wheat histone octamer dissociated into histone fractions by dialysis into 0.01 N HCl and elution from a Bio-Gel P-60 column equilibrated in 0.01 N HCl. Wheat octamer was obtained by eluting the 2 M NaCl extract of wheat chromatin from a Sephadex G-100 column (as shown in Figure 1) and combining the fractions from the leading edge of the peak. After dialysis into 0.01 N HCl, the proteins were applied to a Bio-Gel P-60 column equilibrated with 0.01 N HCl and eluted with 0.01 N HCl. Areas under peaks were normalized for the differences in absorbances of the core histones at 230 nm to determine histone stoichiometry. H2 (H2A and H2B) elutes around fraction 150, H3 elutes around fraction 190, and H4 elutes around fraction 240.

to account for the differences in the absorbances of H2, H3, and H4 at 230 nm, and molar ratios were calculated as described under Experimental Procedures. We calculate molar ratios of H2/H3/H4 = 2.00/1.28/1.08 in the core complex. Although this analysis suggests that there is a slight enrichment in H3 and H4 in the wheat octamer, it cannot account for the increased stability of the wheat octamer as compared to the chicken octamer. Similar measurements on the chicken octamer resulted in ratios of H2A/H2B/H3/H4 = 1.00/1.04/1.34/1.38 (Godfrey et al., 1980).

Wheat histones were subjected to high-speed (meniscus depletion) equilibrium sedimentation in order to better describe the assembly potential of wheat dimers and tetramers into an octameric complex. In 2 M NaCl, the distribution of mass for the wheat histones remained fairly constant at ~100 kDa, with protein concentration having little effect on the molecular weight moments (Figure 3A). These values are significantly higher than those obtained using calf histones under the same experimental conditions; for calf, the weight-average molecular weights range from 65K at low protein concentratioin to 90K at high protein concentration (Figure 3A). Since the molecular weight moments for the wheat histones do not change sufficiently over the concentration range observed, reliable curve fitting cannot be performed. It is obvious from the molecular weight moments alone that the assembly potential of the wheat histones in 2 M NaCl is very high at all protein concentrations greater than 0.1 mg mL^{-1}; the formation of the wheat octamer is greatly favored in the equilibrium, much more so than for the calf octamer under identical conditions.

Eickbush and Moudrianakis (1978) found that for both calf and chicken histone octamers, the equilibrium between the histone subunits allowed significant amounts of H2A–H2B dimers and $(H3–H4)_2$ tetramers to be detected at 0.6 M NaCl. Since the wheat octamer is so much more stable in 2 M NaCl than the chicken octamer, we tested its stability in both 0.6 and 0.3 M NaCl. Purified octamers from the leading edge of the Sephadex column eluted with 2 M NaCl (Figure 1A) were dialyzed against 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF, concentrated with an Amicon Centricon-10 device, and loaded onto a Sephadex G-100 column equilibrated into the same buffer. Surprisingly, the wheat octamer appears to remain intact at 0.6 M NaCl; indicated fractions (Figure 4A, lanes a–c) contain the full complement of wheat histones. On the basis of this profile, there appears to be no dissociation of the wheat complex into tetramers and dimers. The contrast between this profile and the one obtained by applying chicken octamers to the same

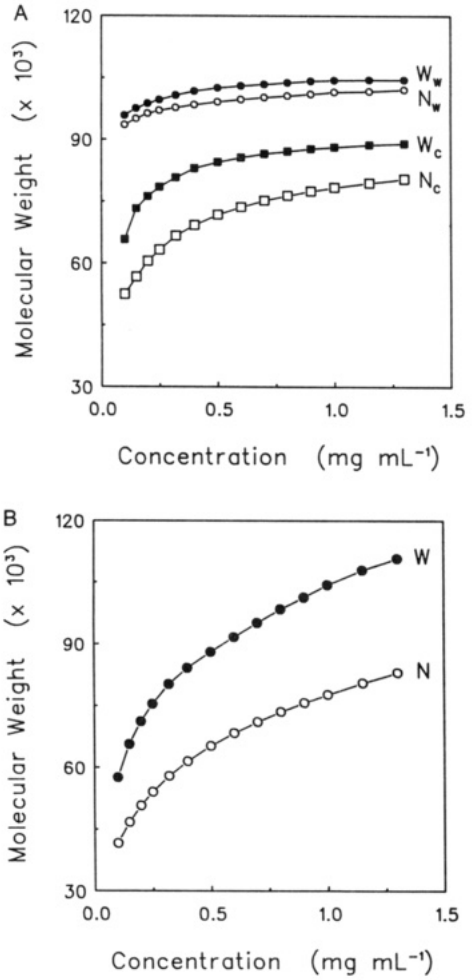

FIGURE 3: Meniscus depletion equilibrium sedimentation of wheat and calf thymus core histones. Run conditions: 0.35 mg mL^{-1} initial loading concentration, 20 000 rpm, 24 h, 18 °C. (A) Apparent number- and weight-average molecular weight moments for wheat (N_W, W_W) and calf thymus (N_C, W_C) histones in 2 M NaCl, 10 mM HEPES, pH 7.5, and 1 mM EDTA. The calf thymus data are fitted to a tripartite model for the octamer which assumes an H2A–H2B–$(H3–H4)_2$ hexamer as an intermediate (Godfrey et al., 1980). The molecular weight moments for the wheat histones do not change sufficiently over the concentration range to allow for reliable curve fitting. (B) Apparent number- (N) and weight-average (W) molecular weight moments for wheat histones in 0.6 M NaCl, 10 mM HEPES, pH 7.5, and 1 mM EDTA.

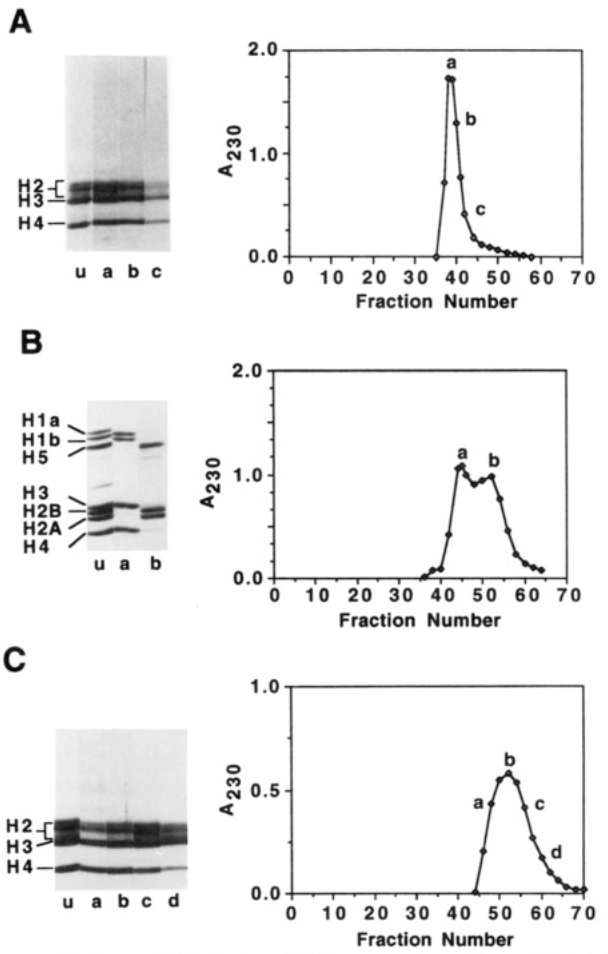

FIGURE 4: Low-salt Sephadex G-100 chromatography and $NaDodSO_4$ gels of wheat histone complexes (A and C) and chicken histone complexes (B). Chicken histone complexes were isolated as in Figure 1. Histone H1 was removed from wheat histone complexes by chromatography in 2 M NaCl as in Figure 1. Both complexes were dialyzed against column buffer before being loaded onto columns. The column in (A) and (B) was equilibrated with 0.6 M NaCl and 10 mM Tris-HCl, pH 7.5. The column in (C) was equilibrated with 0.3 M NaCl and 10 mM Tris-HCl, pH 7.5. The fractions denoted on the elution profiles from the columns (points closest to identifying letter) correspond to the lanes in the $NaDodSO_4$ gels. Lane u (unfractionated) denotes the extract before being loaded onto the column.

column is striking (Figure 4B). Histone H1 was not removed from the chicken octamers shown in this figure. Chromatography of chicken octamers from which H1 had been removed gave the same results (data not shown). The protein composition of the two eluted peaks (Figure 4B, lanes a and b) shows that the chicken octamer is dissociated into tetramers and dimers at this salt concentration. The peak of the chicken tetramer is at fraction 44 on this column, which is indicative of the smaller size of the histone complexes at 0.6 M NaCl as compared to 2.0 M NaCl. The peak of the wheat octamer is at fraction 38, and by fraction 44, nearly all of the wheat complexes have been eluted (Figure 4A).

Wheat histones were also analyzed by equilibrium sedimentation in 0.6 M NaCl at neutrality (Figure 3B). The number- and weight-average molecular weight moments indicate that a mixed population of dimers, tetramers, and octamers exists in the equilibrium at this ionic strength, with the averages increasing greatly with increasing protein concentration. This is consistent with the gel filtration studies described above (Figure 4A), which were performed at a protein concentration of 7 mg mL^{-1}. Large values for the z-average molecular weights even at low protein concentration indicate that some aggregation is occurring within the system at this ionic strength; therefore, curve fitting to a hexameric intermediate model was not attempted.

At 0.3 M NaCl, the wheat octamer appears to be destabilized (Figure 4C). Wheat Hl was removed from the octamer as described above, and the octamer was subsequently dialyzed against the 0.3 M NaCl column buffer, concentrated, and loaded onto the column. While there is not complete resolution of histone tetramers and dimers, $NaDodSO_4$ electrophoresis of selected fractions (Figure 4C, lanes a–d) clearly reveals different ratios of the core histones eluting at different positions across the peak. H3 and H4 are prominent in the leading edge of the peak (lanes a and b). H2 histones dominate in the trailing edge (lane d). Furthermore, the gels indicate that the larger H2A and H2B histones primarily elute in the front half of the peak while the smaller H2A and H2B variants primarily elute in the back half of the peak (e.g., the higher molecular weight H2 histones are visible in lane a where the lower molecular weight H2 histones do not appear, and the lower molecular weight H2 histones are enriched in lane d). Acetic acid–urea–Triton X-100 gels confirm these results (data not shown). The size of the complex eluted in 0.3 M NaCl also shows that little or no octamer is present. Histones applied

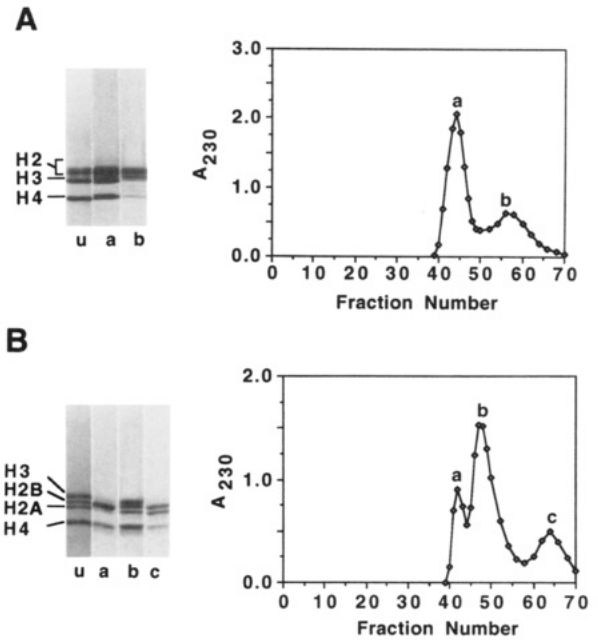

FIGURE 5: Wheat and chicken octamers reconstituted from acid-extracted histones. Reconstitution mixtures of (A) wheat and (B) chicken histones were applied to a Sephadex G-100 column equilibrated in 2 M NaCl and 10 mM Tris-HCl, pH 7.5, and eluted with the same buffer. $NaDodSO_4$ gels of the reconstitution products: Lane u (unfractionated) represents the mixture before it was loaded on the column. The other lanes on the gels represent fractions from the elution profile (points closest to identifying letter).

to identical columns in 0.3 or 2.0 M NaCl both elute as a single peak; however, the protein elutes from the high-salt column earlier than it does from the low-salt column (compare Figure 1A with Figure 4C).

The studies outlined above indicate that the plant histone octamer is a much more stable structure than the vertebrate animal histone octamer. This enhanced stability would not have been anticipated from earlier studies on pairwise interactions of histones. Following the work of D'Anna and Isenberg (1974), who examined the interactions of calf thymus histones using circular dichroism, light scattering, and fluorescence anisotropy, the pairwise interactions of pea histones were examined using the same techniques (Spiker & Isenberg, 1977). The association constants of the pea H2A–H2B histone pairs were indistinguishable from those of their calf thymus counterparts. Also, interkingdom hybrid histone complexes could be formed between these pea and calf histones, and the association constants were indistinguishable from those of the calf complexes or the pea complexes (Spiker & Isenberg, 1978). Similar results were obtained with yeast histones (Mardian & Isenberg, 1978) and Tetrahymena histones (Glover & Gorovsky, 1978).

Thus, it appears as though the interactions that stabilize the $(H3–H4)_2$ tetramer and the H2A–H2B dimer have been evolutionarily conserved. However, because the interactions responsible for formation of the octameric complex have resulted in a plant octamer with greater stability, it appears that there may have been evolutionary changes in these interactions. The changes logically are a result of the differences in sizes and sequences of the H2 histones. In order to provide direct evidence for this possibility, we have reconstituted octamers from acid-extracted histones. Acid-extracted histones were separated on a Bio-Gel P-60 column (Spiker, 1982), and the fractions obtained were variously recombined to reconstitute wheat octamers, chicken octamers, and octamers with mixtures of wheat and chicken histones.

Figure 5A shows the elution of the wheat histone reconstitution mixture from the same Sephadex G-100 column used to characterize the salt-extracted octamers. Histone fractions H2, H3, and H4 were combined and concentrated. Solid urea was added to 8 M, and the histones were then dialyzed into 2 M NaCl and 10 mM Tris-HCl, pH 7.5. The reconstitution mixture was eluted from the column equilibrated in the same buffer. Figure 5B shows the results of the same procedure carried out with the acid-extracted chicken histones. In both cases, complete reconstitution of octamers was not achieved. Nevertheless, the octamer peak was the major peak in each case (Figure 5A, lane a; Figure 5B, lane b). The peak fraction of both the wheat and the chicken octamers was the same for the salt-extracted and the reconstituted octamers. Characteristically, the peak of the wheat octamers was found at fraction 44, while the peak of the chicken octamers was found at fraction 47. This difference in elution volume is presumably due to the larger size of the wheat H2 variants. It may also partly be the result of differences in the conformation of wheat and chicken octamers (Arwood & Spiker, 1990).

In Figure 5A, a trailing peak of wheat histones H2A and H2B elutes after the octamer peak. We presume the existence of this peak is due to a slight excess of H2 histones in the reconstitution mix and to incomplete formation of octamers. The existence of this peak does not appear to signify a difference in stability of octamers reconstituted from salt- and acid-extracted histones, because when the octamer peak is collected and resubjected to chromatography on the same column, no trailing peak appears (data not shown).

During the reconstitution of octamers from wheat histones, no appreciable aggregation occurs (Figure 5A). In contrast, when octamers are reconstituted from chicken histones, an aggregate that contains mainly H2B and H4 is formed and elutes ahead of the octamers (Figure 5B, lane a). Other authors have observed the formation of an aggregate when they have used this procedure to reconstitute chicken octamers (Lindsey et al., 1983). The aggregate they observed, however, consisted primarily of H3 and H4. We have no explanation for the difference between their observations and ours.

As we have shown above, native, salt-extracted wheat histone octamers are stable in 0.6 M NaCl while vertebrate animal octamers are essentially completely dissociated into H2A–H2B dimers and $(H3–H4)_2$ tetramers. In order to assess the relative stability of reconstituted wheat and chicken octamers, we determined if they were dissociated into tetramers and dimers at 0.6 M NaCl. Fractions 42–48 of the reconstituted wheat octamers (Figure 5A) were combined, dialyzed into 0.6 M NaCl and 10 mM Tris-HCl, pH 7.5, concentrated, and eluted from a Sephadex column equilibrated in the same buffer. Fractions 46–54 of the reconstituted chicken octamers (Figure 5B) were treated in the same manner. The results are presented in Figure 6A,B. The reconstituted wheat octamers exhibit the same stability as the salt-extracted wheat octamers (compare to Figure 4A). The acid-extracted reconstituted octamer peak exhibits slightly more trailing than the salt-extracted native octamer, but there is no apparent change in histone ratios across the peak (Figure 6A, lanes a and b). In contrast, the reconstituted chicken octamers, like the salt-extracted chicken octamers, are completely destabilized in 0.6 M NaCl. They elute in two peaks representing tetramer and dimer (Figure 6B, lanes a and b).

We presume that the difference in stability of the plant and vertebrate octamers is a function of the properties of the H2 histones. In order to provide experimental evidence for that assumption, we reconstituted histone octamers with mixtures of wheat and chicken histones. The results are shown in Figure 7A,B. When octamers are reconstituted with wheat H3+H4

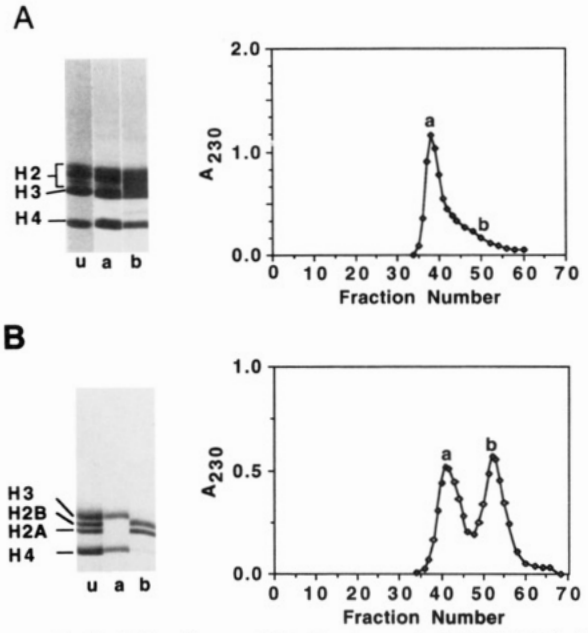

FIGURE 6: Stability of reconstituted octamers in 0.6 M NaCl. (A) Wheat octamers and (B) chicken octamers reconstituted from acid-extracted histones were dialyzed into a buffer containing 0.6 M NaCl and 10 mM Tris-HCl, pH 7.5, and eluted from a Sephadex G-100 column equilibrated in the same buffer. $NaDodSO_4$ gels of the fractions: Lane u (unfractionated) shows the proteins before they were loaded onto the column. Lanes a and b show the proteins from two different regions of the elution profile.

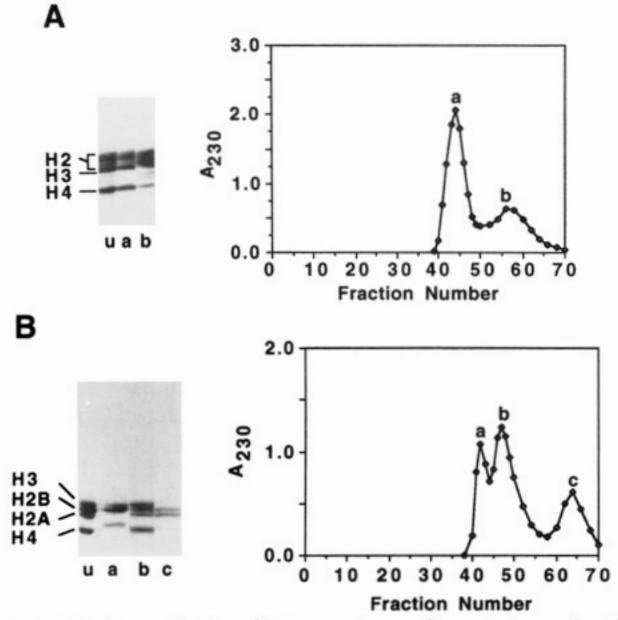

FIGURE 7: Reconstitution of histone octamers from mixtures of acid-extracted wheat and chicken histones. (A) Chicken H3+H4 and wheat H2A+H2B. (B) Wheat H3+H4 and chicken H2A+H2B. The reconstitution mixtures were eluted from a Sephadex G-100 column equilibrated in 2 M NaCl and 10 mM Tris-HCl, pH 7.5. The elution profiles and $NaDodSO_4$ gels of the eluted peaks are shown.

and chicken H2A+H2B (Figure 7B), the results were quite similar to the reconstitution of octamers with chicken histones alone (Figure 5B). As in the reconstitution of octamers with chicken histones, an aggregate consisting of H2B and H4 is formed. Substantial amounts of H3 also appear in this aggregate (Figure 7B, lane a). In addition, a trailing peak consisting of chicken H2A and H2B is observed. In contrast to the aggregative properties of the heterologous octamers reconstituted with chicken H2A+H2B, the heterologous octamers reconstituted with wheat H2A+H2B do not contain aggregates and exhibit an elution profile similar to the elution

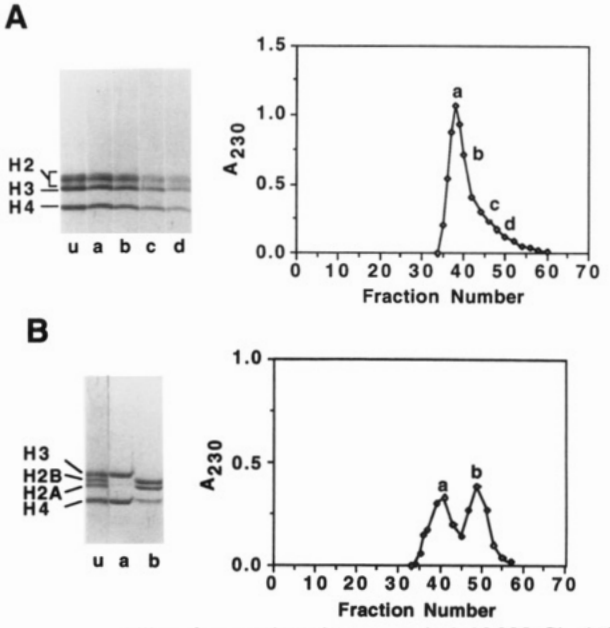

FIGURE 8: Stability of reconstituted octamers in 0.6 M NaCl. (A) Histone octamers reconstituted from chicken H3+H4 and wheat H2A+H2B (obtained from peak a, Figure 7A) were dialyzed into a buffer containing 0.6 M NaCl and 10 mM Tris-HCl, pH 7.5, and eluted from a Sephadex G-100 column equilibrated in the same buffer. (B) Histone octamers reconstituted from wheat H3+H4 and chicken H2A+H2B (obtained from peak b, Figure 7B) were treated identically. $NaDodSO_4$ gels of the octamers before they were loaded onto the column (u) and fractions across the elution profiles (points closest to identifying letter) are shown.

profile of octamers reconstituted entirely with wheat histones (compare Figure 7A and Figure 5A). As noted earlier, two minor H2A variants are enriched in the trailing peak as compared to the octamer peak (Figure 7A, lane b).

The stabilities of the reconstituted heterologous octamers in 0.6 M NaCl were also determined. Octamers from each of these two reconstitution mixtures (Figure 7A, lane a; Figure 7B, lane b) were dialyzed into 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF and eluted from a Sephadex column equilibrated in this buffer. Figure 8A,B demonstrates that octamers reconstituted with wheat H3+H4 and chicken H2A+H2B dissociate into tetramer and dimer in 0.6 M NaCl (Figure 8B) while the octamers reconstituted with chicken H3+H4 and wheat H2A+H2B remain stable in 0.6 M NaCl (Figure 8A).

These results provide experimental evidence that the wheat H2A and H2B histones are responsible for the increased stability of the wheat histone octamer. What features of these plant proteins might account for the increased stability of the octamer? The central core regions of the wheat H2A and H2B histones have sequences that have the same amino acid residue as their vertebrate animal counterparts in approximately 80% of the positions. In these core regions, the sequences of vertebrate animal histones are identical. Thus, even thouigh 80% sequence identity would be considered highly conserved, amino acid replacements could be responsible for the differences in octamer stability. A more likely possibility is the N- and C-terminal regions of the H2A and H2B histones. In these regions not only do the sequences diverge drastically but also the plant histones exhibit extensions of several amino acids (Rodrigues et al., 1985, 1988; Brandt et al., 1988; Spiker et al., 1990; Koning et al., 1991; Yang et al., 1991). Exactly what sequences in these regions should be considered candidates for the determinants of increased octamer stability are at present unknown. However, one intriguing sequence does exist. All plant H2A histones for which sequence

information exists have C-terminal regions containing repeats of the motif "SPKK". This motif (SPbasic,basic) is found in the termini of H1 histones and in the N-terminus of sea urchin sperm H2B and has been suggested to function in binding to the minor groove in A/T-rich DNA (Churchill & Suzuki, 1989). Despite this suggestion, it is possible that this sequence in the C-terminal region of plant H2As has a role in increased octamer stability. The experiments of Hatch et al. (1983) implicate the C-terminal region of H2A as playing a role in the H2A–H2B dimer, $(H3–H4)_2$ tetramer interface. They treated the isolated calf thymus histone octamers with trypsin under conditions of 2 M NaCl and neutral pH. In this case, when the histones are strongly interacting and the octamer is the predominant structure, the C-terminal region of H2A is protected from attack by trypsin. However, when the salt concentration is reduced and the octamer destabilized, the C-terminus of H2A becomes susceptible to attack by trypsin. These results suggest that sequences in the C-terminus of H2A play a role in the interaction of H2A–H2B dimers with $(H3–H4)_2$ tetramers. Thus, the extended C-termini of plant H2A histones may play a role in the increased stability of the plant octamer.

It is unknown at the present time what effect the increased stability of the plant histone octamer may have on the physiology of plant chromatin. The high percentage of repeated and nontranscribed DNA in wheat suggests that a high proportion of the DNA must be packaged into transcriptionally inert chromatin. A highly stable histone octamer may participate in such packaging. The previously-reported low electrophoretic mobility of the isolated wheat nucleosome may also reflect properties that lead to packaging of transcriptionally inert chromatin (Arwood & Spiker, 1990). The uniform packaging of wheat chromatin as evidenced by extended nucleosome ladders (Spiker, 1985) may also be due in part to the properties of the histone octamer.

Experiments on the properties of octamers from invertebrate animals, fungi, and protists have not been reported, but the question now arises whether the stability of the plant octamer or the vertebrate animal octamer will be more typical of eukaryotes. Of particular interest would be the stability of the octamer in an organism such as *Saccharomyces cerevisiae*, whose genome is essentially entirely in a transcriptionally poised state (Lohr & Hereford, 1979), and in sea urchin sperm chromatin, whose H2B has been shown to contain the "SPbasic,basic" motif (Strickland et al., 1978).

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