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Isolation and Characterization of the Histone Variants in Chicken Erythrocytes†

Michael K. Urban,* Samuel G. Franklin, and Alfred Zweidler

ABSTRACT: Chicken erythrocyte histones 2A, 2B, and 3 can be resolved into nonallelic primary structure variants by polyacrylamide gel electrophoresis in the presence of Triton X-100. These variants were isolated and characterized by analysis of their tryptic and thermolytic peptides. The major variants of chicken H2A and H2B differ from the analogous component of calf thymus by a small number of conservative amino acid substitutions in the basic terminal regions, which interact with DNA. This moderate rate of allelic evolution of the slightly lysine-rich histones contrasts with the complete conservatism found in the arginine-rich histones. Chicken H4 and both chicken H3 variants are identical with their corresponding components in mammals. The amino acid substitutions distinguishing histone variants are located within the highly conserved hydrophobic regions, which are involved in histone–histone interactions.

Eucaryotic chromatin is arranged into similar repeating units, nucleosomes, each consisting of ca. 200 base pairs of DNA associated with two molecules each of histones 2A, 2B, 3, and 4 and variable amounts of H1 and/or H5 (Kornberg, 1974; Olins & Olins, 1974; Oudet et al., 1975; Van Holde et al., 1974). While the nucleosomal "spacer region", which is associated with H1–H5, is variable between cells of the same species and different species, the nucleosomal "core region", which contains the histone octamer (H2A, H2B, H3, H4)$_2$ and 140 base pairs of DNA, is extremely uniform (Felsenfeld, 1978). It is therefore not surprising that H1-like histones have been found to be quite variable, while the nucleosomal core histones have been highly conserved in evolution (De Lange, 1978; De Lange & Smith, 1975; Elgin & Weintraub, 1975). This conservatism is particularly stringent within the hydrophobic regions of the histones, which may be involved in important intermolecular interactions (Kootstra & Bailey, 1978).

We have previously shown that mammalian histones 2A, 2B, and 3 can be resolved into nonallelic primary structure variants by polyacrylamide gel electrophoresis in the presence of the nonionic detergent Triton X-100 (Franklin & Zweidler, 1977; Zweidler, 1976, 1978; A. Zweidler and S. Franklin, unpublished experiments). Since the H2A and H3 variants appear to have been preserved unchanged throughout the evolution of mammals, it was important to determine if the same variants already existed in nonmammalian species. We report here on the isolation and characterization of two variants each of chicken erythrocyte histones 2A, 2B, and 3.

Materials and Methods

Preparation of Cells and Isolation of Nuclei. All procedures were conducted at ~2 °C unless specified. Blood from a White Leghorn rooster was collected from a severed jugular

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vein into one-sixth volume of 1% (w/v) sodium heparin in Seligmann's balanced salt solution (SBSS). Red blood cells were separated from serum by centrifugation at 1600*g* for 10 min, the buffy coat was removed by aspiration, and blood cells were resuspended and centrifuged twice more in SBSS.

Preliminary experiments involved isolation of nuclei from cells lysed by the following different methods: 0.05% saponin in buffer A (10 mM potassium–Tris–maleate, pH 7.4, 5 mM $MgCl_2$, and 50 mM glycine); 1% Triton X-100 in buffer A; 10 mM potassium–Tris–maleate, pH 7.4, 25 mM glycine, and 1 mM $CaCl_2$ (buffer B); the method of A. Zweidler, M. Urban, and P. Goldman (unpublished experiments). All buffers also contained 1 mM TLCK[1] and 1 mM TPCK (proteolytic inhibitors) and 1% (v/v) TDG and 1 mM DTE (antioxidants). The last method, which was used for all subsequent nuclear isolations, includes the following: direct homogenization of −70 °C frozen cells, without thawing, in 30 mL of buffer A at 3300 rpm for 1 min in a Waring blender fitted with a foam adaptor. In all cases, the lysate was centrifuged at 300*g* for 5 min, the supernatant was aspirated, and the crude nuclear pellet was rehomogenized in buffer A by blending at 7000 rpm for 30 s and centrifuged as above through a 5-mL underlayer of 5% (w/v) sucrose in buffer A. This pellet was homogenized at 4000 rpm for 30 s in 1% (w/v) Triton X-100 in buffer A and centrifuged as above. The pellet was then suspended in buffer B.

Histone Extraction. After washing in buffer B, pelleted nuclei were extracted with 0.27 M NaCl–10 mM trisodium ethylenediaminetetraacetate (EDTA), pH 7.2, and centrifuged at 9000*g* for 10 min. The pellet was suspended in 1 M NaCl, centrifuged as above, made 0.25 N in HCl, and recentrifuged to remove acid-insoluble material. The histone extract was judged to be free of cytoplasmic and nuclear membrane protein by polyacrylamide gel electrophoresis (Jackson, 1976a,b). Alternatively, nuclei in buffer B were digested for 15 min at ~2 °C with 30 units/mL micrococcal nuclease (Sigma), frozen at −70 °C, quick-thawed in 2 mM EGTA, and then extracted with 1 M NaCl, 0.25 M HCl, 1% TDG, and 100 μg/mL protamine sulfate (Sigma) in order to ensure quantitative recovery of all histone components.

The acid-soluble proteins were precipitated by the addition of one-fifth volume of 120% (w/v) trichloroacetic acid (Cl_3AcOH). Pellets were washed with 20% Cl_3AcOH and 0.4% (v/v) HCl in acetone and with acetone alone (with 1% TDG) by resuspension and centrifugation and dried in a vacuum desiccator.

Isolation of Histone Variants. After removal of H1 and H5 from the histone extract with 5% (v/v) perchloric acid (Johns & Diggle, 1969), the remaining proteins were dissolved in 8 M urea in 0.1 M sodium acetate buffer, pH 4.85, and fractionated at room temperature by ion-exchange chromatography on a 1.5 × 25 cm carboxymethylcellulose column (Whatman, CM-52) equilibrated with the above buffer containing 4 M urea. Elution was carried out with the following concentrations of guanidinium chloride (Research plus) in the equilibrium buffer: 1.8% (200 mL), a linear gradient of 1.8–2.5% (200 mL), and finally a 5% wash (100 mL). Individual histone components were isolated from the above fractions enriched in individual histone classes by preparative polyacrylamide gel electrophoresis in the presence of Triton X-100 (A. Zweidler and S. Franklin, unpublished experiments).

[1] Abbreviations used: TPCK, L-1-tosyl-2-phenylalanyl chloromethyl ketone (Sigma); TLCK, L-1-tosyllysyl chloromethyl ketone (Sigma); DTE, dithioerythritol (Pierce); TDG, thiodiglycol (Pierce).

Gel Electrophoresis. Protein fractions were analyzed on 12% polyacrylamide gels containing 6 mM Triton X-100 and 3–7.5 M urea (Zweidler, 1978) and on 15% polyacrylamide gels with 2.5 or 6 M urea (Panyim & Chalkley, 1969). Electrophoresis was also performed in 18% polyacrylamide–0.1% $NaDodSO_4$ gels according to Laemmli (1970).

Cyanogen Bromide Cleavage. Typically, an excess of cyanogen bromide in 100 μL of 70% (v/v) formic acid, flushed with nitrogen, was used to cleave 50 μg of protein at room temperature for 12 h. The reaction mixture was lyophilized, dissolved in 100 μL of doubly distilled water, and relyophilized.

Amino Acid Analysis. Ampules were cleaned in chromic–sulfuric acid and rinsed with 1% (v/v) TDG and 5% (v/v) mercaptoacetic acid before use. Samples were hydrolyzed under nitrogen in constant-boiling 6 N HCl, that was distilled from ninhydrin, for 24 h at 110 °C. For performic acid oxidation of cysteine, a mixture of 10 volumes of 30% (v/v) hydrogen peroxide and 88% (v/v) formic acid was permitted to stand at room temperature for 1 h. This mixture was then reacted with the protein sample for 4 h at 0 °C, lyophilized, and hydrolyzed as above.

Hydrolysates were lyophilized, dissolved in 0.1 M sodium citrate buffer, pH 2.2 (Pierce), and analyzed in a Durrum D-500 amino acid analyzer. Analysis was performed in triplicate and normalized to cohydrolyzed histone standards as a correction for hydrolytic losses.

Peptide Mapping [According to Franklin & Zweidler (1977) and A. Zweidler and S. Franklin (Unpublished Experiments)]. Histones were digested with trypsin in 0.1 M ammonium bicarbonate, pH 8.1 (ratio of 1:50 enzyme to substrate, added twice), for 4 h at 37 °C. The samples were lyophilized, suspended in electrophoresis buffer (see below), and centrifuged to recover the tryptic core. Thermolytic digestion of the core (ratio of 1:500 enzyme to substrate) was in 0.1 M ammonium acetate buffer, pH 7.0, for 2.5 h at 37 °C.

Electrophoresis for both tryptic and thermolytic peptides was in pyridine–acetic acid–1-butanol–water (1:1:2:36), pH 4.7, for 1 h at 600 V on Avicell microcrystalline cellulose plates. Ascending chromatography was for 3–4 h in pyridine–acetic acid–1-butanol–water (10:3:15:12). Visualization was with fluorescamine. Peptides were eluted with constant-boiling 6 N HCl and hydrolyzed for amino acid analysis as described above.

Results

The electrophoretic resolution of the chicken histones can be significantly improved over the acid–urea system of Panyim & Chalkley (1969) by the addition of Triton X-100 to polyacrylamide gels (Figure 1). By using the optimum concentration of urea and Triton X-100, two subfractions each of chicken erythrocyte histones 2A, 2B, and 3 can be resolved. Since some of the histone components occur in very small relative amounts, it was necessary to eliminate cytoplasmic contaminants as much as possible. For this purpose, the nuclei were not isolated by the standard methods of cell lysis with detergents but rather by shearing off the cell membranes and cytoplasmic layer in a modified Waring blender, allowing the complete exclusion of air (A. Zweidler, M. Urban, and P. Goldman, unpublished experiments), followed by low-speed centrifugation through a denser underlayer to separate the nuclei from cytoplasmic debris. If globin is used as a marker for cytoplasmic contamination, this method is the most efficient in obtaining "clean" nuclei (Figure 2). In order to prevent complications due to degradation and partial oxidation of methionine residues (Zweidler, 1978), it was essential to isolate

Table I: Comparison of the Amino Acid Composition of Chicken and Mammalian Histone Variants[a]

	amino acid composition (mol %)										
	2A.1		2A.2		2B.1		2B.2				
	calf	ch	calf	ch	calf	ch	mouse	ch	3.1, calf	3.2, ch	3.3, ch
Asx	6.2	7.0	6.2	6.3	4.8	4.7	4.8	4.7	3.7	3.6	3.8
Thr	3.9	3.1	3.1	3.3	6.4	7.4	6.4	7.5	7.4	7.5	7.6
Ser	3.1	3.4	3.9	3.9	11.2	11.3	12.0	11.1	3.7	4.2	4.7
Glx	9.3	8.6	9.3	9.6	8.0	8.0	8.0	8.0	11.1	10.6	11.2
Pro	3.9	3.9	3.9	4.0	4.8	4.8	4.8	4.8	4.4	4.8	4.4
Gly	10.8	10.4	10.8	11.1	5.6	5.7	4.8	4.8	5.2	4.9	5.8
Ala	13.2	14.3	13.2	13.1	10.4	9.8	10.4	10.4	13.3	13.1	13.1
Cys	0	0	0	0	0	0	0	0	1.5	0.7	0.7
Val	6.2	6.2	6.2	6.4	7.2	6.1	7.2	6.4	4.4	4.3	4.5
Met	0	0	0.8	0.8	1.6	1.6	1.6	1.5	1.5	1.5	1.0
Ile	4.7	4.7	4.7	4.5	4.8	5.3	4.8	5.3	5.2	5.4	5.3
Leu	12.4	12.5	11.6	11.4	4.8	4.7	4.8	4.8	8.9	9.0	8.6
Tyr	2.3	2.2	2.3	2.3	4.0	3.8	4.0	4.0	2.2	2.2	1.9
Phe	0.8	0.8	0.8	0.8	1.6	1.5	1.6	1.6	3.0	3.0	2.8
His	3.1	2.3	3.1	2.3	2.4	2.4	2.4	2.4	1.5	1.5	1.6
Lys	10.9	11.2	10.9	10.7	16.0	16.7	16.0	16.4	9.6	9.7	10.0
Arg	9.3	9.3	9.3	9.3	6.4	6.2	6.4	6.1	13.3	13.9	13.0

[a] Analysis was performed in triplicate and normalized to cohydrolyzed calf histone standards (De Lange et al., 1972; Hayashi & Iwai, 1971; Hnilica et al., 1970; Ishikawa et al., 1972; Sautière et al., 1975) as a correction for hydrolytic losses and is expressed as mole percent.

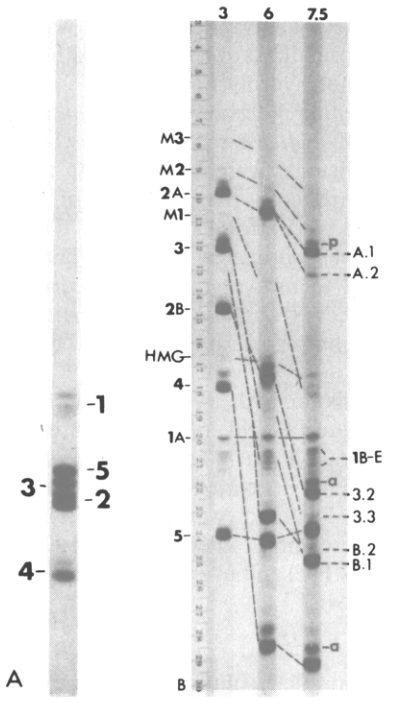

FIGURE 1: Polyacrylamide gels (A) prepared with 6 M urea as described by Panyim & Chalkley (1969) and (B) prepared with 6 mM Triton X-100 and 3, 6, and 7.5 M urea of chicken erythrocyte histones. Nomenclatures: for histone classes and HMG, high mobility group proteins, Bradbury (1974); for minor (M) histone components and histone variants, Franklin & Zweidler (1977), Zweidler (1976, 1978), and this text; for internal modifications, p = phosphorylation and a = acetylation.

the histones in the presence of proteolytic inhibitors and an efficient scavenger (thiodiglycol) of oxidizing agents.

The chicken histone components resolved by Triton X-100–polyacrylamide gel electrophoresis behave similarly to those described for mammals (Zweidler, 1976, 1978). However, in order to identify and compare the different histone components of the chicken with those of the calf and mouse, we isolated and analyzed them for primary structure differences. Chicken erythrocyte histones depleted in H1 and H5 were first enriched in individual components by ion-exchange chromatography on CM-cellulose (Figure 3A,B). Individual histone species were then isolated from CM-cellulose fractions by preparative gel electrophoresis in the presence of 6 mM Trition X-100 and the following concentrations of urea: 8 M for the isolation of M2, H2A.1, and H2A.2 from CM-cellulose fractions 2–4 (Figure 3C,D); 5 M urea for the isolation of M1, H2B.1, and H2B.2 from CM-cellulose fractions 5–6 (Figure 3E,F); 6 M urea for the isolation of H3.2, H3.3, and H4 from CM-cellulose fractions 10–12 (Figure 3G,H). Despite our precaution to prevent oxidation during histone isolation, some of the isolated H2B.2 contained oxidized methionine residues (Figure 3F). That these additional electrophoretic bands do not represent proteolytic degradation products of H2B is evident from electrophoresis of fraction 6 (Figure 3F) are acid–urea–polyacrylamide gels lacking Triton X-100 (Figure 4). M3 was isolated from CM-cellulose fractions enriched in H2A-like components by a two-step procedure: preparative 6 M urea–5% acetic acid–polyacrylamide gel electrophoresis, followed by 7.5 M urea–6 mM Triton X-100–polyacrylamide gel electrophoresis of the fractions migrating slower than H1 (Figure 5).

Amino Acid Composition of Histone Variants. The amino acid compositions of the chicken histone variants are compared to the corresponding components of mammals in Table I. Although the amino acid compositions of the corresponding fractions from the two species are very similar, several differences are apparent. Chicken H2A.1 appears to have one more aspartic acid and alanine residue than calf and one less threonine, glutamic acid, and histidine. H2A.2 of chicken differs from that of calf by having one less histidine residue and differs from calf H2A.1 by having an additional methionine and serine and one less threonine and leucine. Chicken H2B.1 differs from calf H2B.1 in having one more threonine and isoleucine residue and one less alanine and valine residue. Chicken H2B.2 appears to have one more alanine residue than chicken H2B.1 and contains the one-residue decrease in glycine as does mouse H2B.2. Chicken H3.2 and H3.3 both have one less cysteine and one more serine than calf H3.1. In addition, chicken H3.3 may have one more glycine and one less methionine than calf H3.1. These differences from calf H3.1 are also found in calf H3.2 and H3.3 (Franklin & Zweidler, 1977).

Peptide Maps of Chicken Histone Variants. In order to confirm and localize the amino acid substitutions between

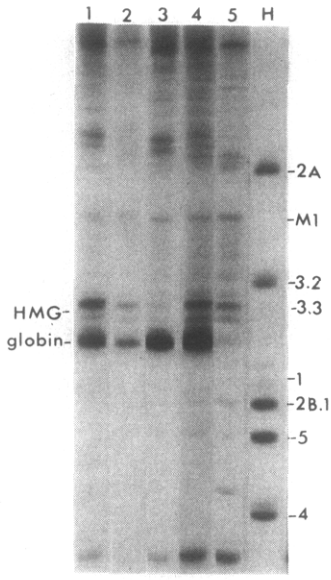

FIGURE 2: Triton X-100 (6 mM)–urea (5.75 M)–polyacrylamide gels of 0.27 M NaCl–10 mM EDTA nuclear extracts prepared from erythrocytes lysed in (1) water, (2) 0.05% saponin in buffer A, (3) 1% Triton X-100 in buffer A, (4) buffer B, and (5) direct homogenization of −70 °C frozen cells in buffer A. All preparations involved homogenization at 3300 rpm for 1 min in a Waring blender. (H) NaCl (1 M)–HCl (0.25 M) extract of nuclei prepared by method 5.

variants, we analyzed their tryptic and thermolytic peptides. As an example, Figure 6 shows the tryptic peptide maps of calf and chicken H2A's. The peptides were eluted from the plates and subjected to amino acid analysis; the position within the protein of the peptide was determined by homology in amino acid composition with the amino acid sequences for the major components of these histones in calf thymus (De Lange et al., 1972; Hayashi & Iwai, 1971; Hnilica et al., 1970; Ishikawa et al., 1972; Sautière et al., 1975). Only those peptides containing amino acid substitution are discussed below.

Laine et al. (1978) previously reported the primary sequence of chicken H2A, without prior separation into histone variants. This analysis agrees with our analysis for a threonine → serine substitution at position 16, glutamic acid → aspartic acid substitution at position 121, a histidine deletion at position 123 or 124, and a glycine → alanine substitution at position 128. Peptide T6a (Ser-Arg), which is found in chicken H2A.1 and chicken and calf H2A.2 but not in calf H2A.1, contains the threonine → serine substitution at position 16. Chicken H2A.1 peptide T19a moves considerably slower than its homologue in calf H2A.1 (Figure 6). Amino acid analysis of this peptide revealed a composition depleted in 1 mol of histidine and an aspartic acid residue in place of glutamic acid compared to calf H2A.1 peptide T19 representing residues 119–125 (Lys-Thr-Glu-Ser-His-His-Lys). Peptide T19b of chicken H2A.2 maps identically with T19a of H2A.1 because of the histidine deletion, but by composition it lacks the glutamic acid → aspartic acid substitution. Two additional spots (T4a and T2a) are found in the tryptic map of chicken H2A.2. T4a (Val-Arg) probably arises from an alanine → valine substitution at position 10. Peptide T2a (Ser-Lys) could arise by the substitution of a serine for glycine at position 4 or 128 or for alanine at position 12, 14, or 126. This substitution probably involves a replacement of glycine, since an alanine replacement would have reduced the content of alanine for the whole molecule by three residues, which would have been detected by amino acid analysis. Substitution of glycine at position 128 by valine, alanine, and serine has been reported for the H2A's of respectively trout testis (Bailey & Dixon, 1973), chicken (Laine et al., 1978), and sea urchin (P. Sautière, personal communication). Peptide Th1a, obtained by digestion of the insoluble tryptic core of chicken H2A.2 with thermolysin (map not shown), contains a methionine residue not present in H2A.1. The depletion of one leucine residue by amino acid composition of Th1a and the analysis of overlapping peptides place the methionine substitution at position 51. This substitution also occurs in calf H2A.2 and possibly in sea urchin H2Aα (Cohen et al., 1975). The presence of methionine in chicken H2A.2 has been confirmed by its cleavability with cyanogen bromide (data not shown). Except for the gain of one valine residue in chicken H2A.2, these substitutions are supported by the amino acid composition of the entire protein (Table I).

The tryptic peptides of chicken H2B.1 and H2B.2 show no significant differences in mobility from those of calf H2B.1 (not shown). However, peptide T5a of both chicken H2B variants contains Thr-Gln-Lys instead of Ala-Gln-Lys recovered from calf H2B, which probably results from an alanine → threonine substitution at position 21. Contaminating chymotryptic activity produced a small amount of peptide T8c-1a (Glu-Ser-Tyr-Ser-Ile-Tyr) by cleavage of tyrosine-40. This peptide contains a valine → isoleucine change at position 39. This substitution was confirmed by the recovery of two peptides containing residues 35–38 (Glu-Ser-Tyr-Ser) and 39–40 (Ile-Tyr) after thermolytic digestion of the tryptic core. The alanine → threonine substitution at position 21 and the valine → isoleucine substitution at position 39 were previously reported by Van Helden et al. (1978). In addition, the predominant H2B of chicken, H2B.1, has been reported to contain an inversion of residues 26 and 27 (Van Helden et al., 1978). Chicken H2B.2 has two substitutions not found in chicken H2B.1 or calf H2B: tryptic peptide T7a (Ala-Arg) probably results from a serine → alanine substitution at position 32 and core peptide Th5a (Ala-Met-Ser) contains a glycine → serine change at position 60.

Chicken H3.2 and H3.3 tryptic maps are indistinguishable from that of calf H3.1 (not shown). Two species of peptide T21, residues 123–128 (Asp-Ile-Gln-Leu-Ala-Arg and Asp-Ile-Gln-Leu-Thr-Arg), are present in all H3 variants of both chicken and calf, indicating that each actually comprises two variants differing at position 127. Thus, it appears that there are at least four H3 proteins in chicken erythrocytes and six H3 proteins in calf thymus. Peptide Th6a (Leu-Gln-Glu-Ala-Ser-Glu-Ala-Tyr), isolated from the thermolytic digest of the tryptic cores of chicken H3.2 and H3.3, contains the same cysteine → serine substitution at position 96 found in calf H3.2 and H3.3 (Franklin & Zweidler, 1977) and previously reported for chicken erythrocyte H3 (Brandt & Von Holt, 1974). Chicken and calf H3.3 also have two additional substitutions. The thermolytic peptide Th4 recovered from H3.2 and homologous to calf H3.1 residues 88–93 (Ala-Val-Met-Ala-Leu) is not present in the thermolytic digest of the H3.3 tryptic core. Instead, we recover the tripeptides Th4a (Ala-Ile-Gly) and Th5 (Ala-Leu-Gln), which are most likely derived from the substitution of isoleucine for alanine and glycine for methionine at positions 89 and 90. The order of these substitutions awaits confirmation by sequencing. The lack of one methionine in chicken H3.3 was confirmed by cyanogen bromide treatment of each H3 variant and polyacrylamide gel electrophoresis of the products (Figure 7).

Minor Histones. Three minor components were consistently coextracted from erythrocyte chromatin. These proteins occur

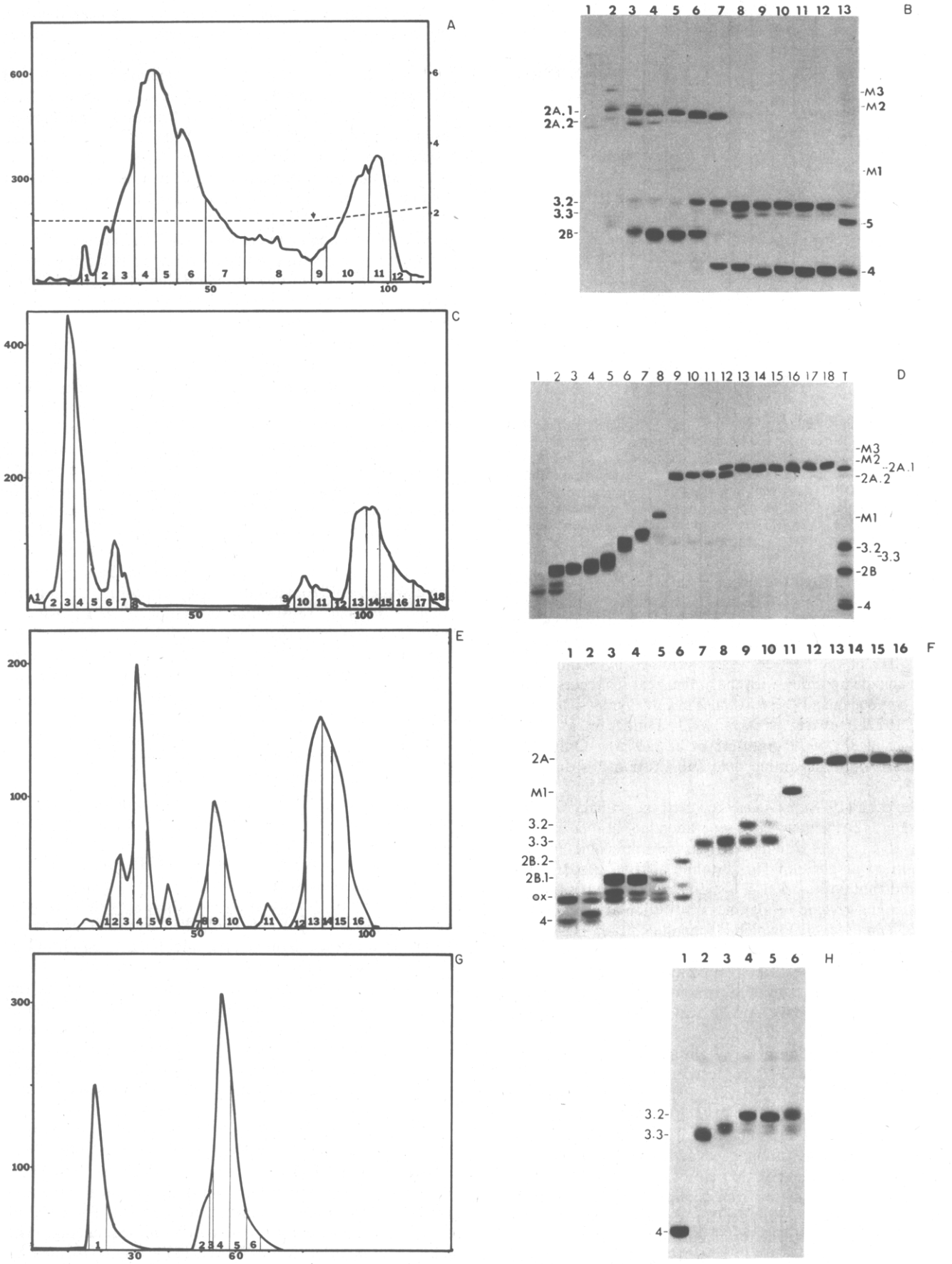

FIGURE 3: Isolation of chicken erythrocyte histone variants. (A) Chromatography of histones depleted in H1 and H5 on a carboxymethylcellulose column. Elution was with 1.8%, a gradient of 1.8–2.5%, and 5% guanidinium chloride (---) in 4 M urea and 0.1 M sodium acetate buffer, pH 4.85. Column fractions were monitored for turbidity in 18% Cl_3AcOH at 400 nm (ordinate; micrograms per milliliter). (B) Urea (7.5 M)–Triton X-100–polyacrylamide gels of the fractions pooled as indicated in Figure 3A. Preparative Triton X-100–polyacrylamide gel electrophoresis of the pooled CM-cellulose fractions: (C) 2–4, at 8 M urea; (E) 5–6, at 5 M urea; (G) 10–12, at 6 M urea. Fractions (abscissa) were monitored for turbidity as described in Figure 3A (ordinate; micrograms per milliliter). Polyacrylamide gel electrophoresis of pooled protein fractions from (D) Figure 3C, (F) Figure 3E, and (H) Figure 3G. The polyacrylamide gels contained 6 mM Triton X-100 and (D) 7.5 M urea, (F) 6 M urea, and (H) 5 M urea.

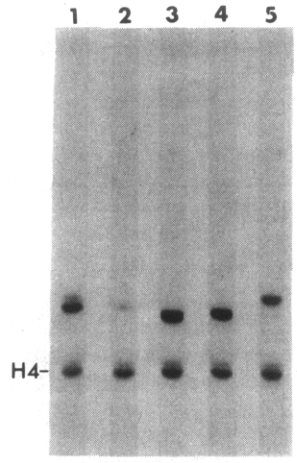

FIGURE 4: Urea (6 M)–polyacrylamide gels (Panyim & Chalkley, 1969) of isolated histone fractions. (1) H2A.1, Figure 3D, fraction 15; (2) H2A.2, Figure 3D, fraction 10; (3) H2B.1, Figure 3F, fraction 4; (4) H2B.2, Figure 3F, fraction 6; (5) H3.2 and H3.3, Figure 3H, fractions 2 and 4. Each gel contained H4 as an electrophoretic migration marker.

FIGURE 5: Urea (7.5 M)–Triton X-100 (6 mM)–polyacrylamide gels of (1) H2A.1 and (2) M3 isolated from chicken erythrocyte nuclei (see Materials and Methods). Each gel contained H4 as an electrophoretic migration marker.

Table II: Amino Acid Composition of the Minor Histones of Chicken Erythrocytes[a]

	amino acid composition (mol %)				
	2A.1	M1	M2	M3	A-24[b]
Asx	7.0	4.3	7.0	9.1	7.3
Thr	3.1	6.3	3.5	5.0	6.5
Ser	3.4	5.9	5.0	6.6	4.5
Glx	8.6	8.1	9.4	13.2	12.3
Pro	3.9	2.5	4.4	3.2	5.6
Gly	10.4	12.3	10.0	11.2	9.2
Ala	14.3	13.9	13.5	11.2	9.6
Cys	0	0	0	NT[c]	NT
Val	6.2	5.8	5.7	3.5	4.9
Met	0	0	tr[d]	0	0.3
Ile	4.7	6.9	4.7	6.2	5.8
Leu	12.5	9.4	11.6	10.4	10.9
Tyr	2.2	1.6	2.3	1.1	1.3
Phe	0.8	0.9	1.2	1.0	0.9
His	2.3	4.6	2.3	2.6	2.4
Lys	11.2	10.7	9.8	11.0	11.3
Arg	9.3	6.9	9.1	6.5	7.4
M_r	13.8	13.2	14.4	20.4	27.0

[a] Analysis was performed in triplicate (except for M3 which was analyzed once) by using cohydrolyzed calf H2A (Sautière et al., 1975) as a correction for hydrolytic losses and is expressed as mole percent. Molecular weight ($\times 10^3$) was estimated by $NaDodSO_4$-polyacrylamide gel electrophoresis according to Laemmli (1970) by using H2A, H2B, and H3 monomer and dimer as standards. [b] Goldknopf & Bush (1975). [c] NT, not tested. [d] tr, trace.

in variable amounts in many different tissues and species and appear to be conserved throughout the vertebrates (Zweidler, 1976; A. Zweidler, M. Urban, and P. Goldman, unpublished experiments). The amino acid composition of chicken M1 (Table II) is different from any of the major histones, but tryptic peptide analysis of the predicted analogous protein in calf thymus suggests a histone-like distribution of basic residues (S. Franklin and A. Zweidler, unpublished experiments). Component M2 appears to be an H2A-like variant by electrophoresis on Triton gels (Figure 1), amino acid composition (Table II), and tryptic peptide analysis (not shown). The amino acid composition and molecular weight of M3 differ considerably from the major histones (Table II). However, tryptic peptide analysis of an analogous fraction isolated from mouse liver corresponds to that of H2A (A. Zweidler, unpublished experiments). Furthermore, its composition and molecular weight, if estimated by nonhistone markers in $NaDodSO_4$–polyacrylamide gels (~27 000), are similar to mammalian protein A24 (Goldknopf & Bush, 1975), a

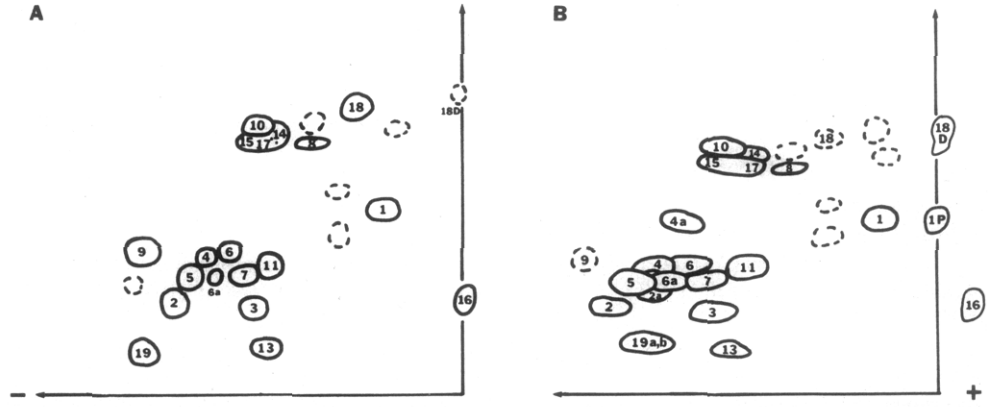

FIGURE 6: Composite tryptic peptide maps of calf and chicken H2A's. (A) Calf H2A. Peptide T6a is the only tryptic peptide unique to calf H2A.2. Peptide T18D probably arises from deamidation of T18. (B) Chicken H2A. Peptides T2a and T4a are unique tryptic peptides of chicken H2A.2. In chicken H2A.1 peptide T4 is absent. Peptide T19a is found in chicken H2A.1 and T19b is found in chicken H2A.2. Peptide T1P is probably phosphorylated T1.

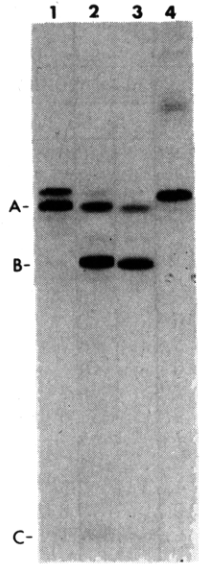

FIGURE 7: Separation of H3 cyanogen bromide cleavage fragments on 6 M urea–polyacrylamide gels. (1) Chicken H3.3, (2) chicken H3.2, and (3) calf H3.2 (50 μg) were reacted with an excess of cyanogen bromide in 100 μL of 70% (v/v) formic acid at room temperature for 12 h. The reaction mixture was lyophilized and dissolved in electrophoresis sample buffer. (4) Calf H3.2 not reacted with cyanogen bromide. Bands A and C result from cleavage at methionine-120; band B results from cleavage at methionine-90.

branched covalent complex of histone 2A and ubiquitin (Goldknopf & Bush, 1977).

Discussion

Polyacrylamide gel electrophoresis in the presence of Triton X-100 has made it possible to resolve histone variants differing only by the substitution of a single neutral amino acid (Franklin & Zweidler, 1977; Zweidler, 1978). Utilizing this system, we have isolated and characterized two variants each of chicken erythrocyte histones 2A, 2B, and 3. These variants are not, however, erythrocyte specific, since they have also been isolated from chicken liver nuclei and characterized. These variants can also be detected in Triton–polyacrylamide gels from several other chicken tissues (Urban et al., 1978).

Comparison of the primary amino acid sequence of the histone variants from chicken erythrocytes and calf thymus reveals that nucleosomal core histones exhibit a low rate of evolutionary change from birds to mammals. As previously noted (De Lange, 1978; De Lange & Smith, 1975; Elgin & Weintraub, 1975), this conservation is particularly striking for histones 3 and 4. The sequence of chicken H4 (M. Urban, S. Franklin, and A. Zweidler, unpublished experiments) is identical with that of calf H4 (De Lange et al., 1969). All vertebrate H3.2's which have been sequenced are also identical (Brandt & Von Holt, 1972; Brandt et al., 1974; Franklin & Zweidler, 1977; Hooper et al., 1973), containing a serine in place of cysteine at position 96 of calf H3.1 (De Lange et al., 1972). Furthermore, both chicken and calf H3.3 variants contain amino acid substitutions at positions 89 and 90 (Figure 8). A serine substitution at position 90 has also been reported for the H3 of pea seedling (Patthy & Smith, 1973). Using the mutation probability matrix of Barker & Dayhoff (1972) to assign the evolutionary order of the substitutions found in residues 89–90 of H3, we predict that the ancestral H3 contained the sequence reported for pea seedling (Ala-Val-Ser; 88–90) and from which H3.2 (Ala-Val-Met) and H3.3 (Ala-Ile-Gly) independently evolved. In accordance with the calculations of Fasman et al. (1976), both of these substitutions

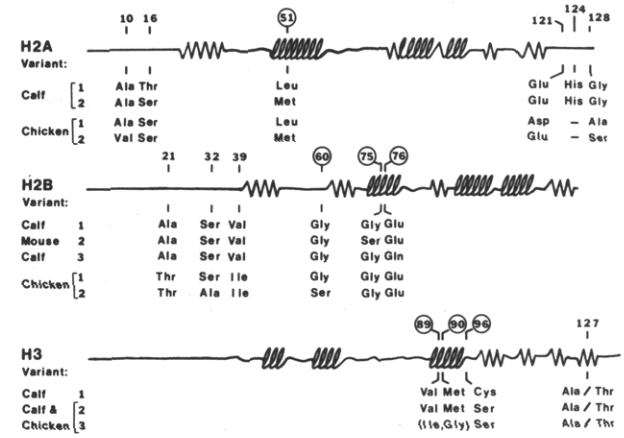

FIGURE 8: Proposed substitution sites in the mammalian and chicken histone variants, based on amino acid composition of tryptic and thermolytic peptides. The degree of high helix and β-sheet probability in different regions of the proteins (Fasman et al., 1976) are symbolically indicated. Substitutions within hydrophobic regions are circled.

have the potential to promote significant conformational changes in H3.

Histones 2A and 2B have evolved at a slightly more rapid rate than H3 and H4 [see reviews by De Lange & Smith (1975), Elgin & Weintraub (1975), and Sautière et al. (1975)]. Both variants of chicken H2B differ from both variants of calf H2B by substitution at positions 21 and 39 (Figure 8). Substitution at position 21 of H2B has also been reported for *Xenopus* and crocodile erythrocytes (Van Helden et al., 1978) and trout testis (Kootstra & Bailey, 1978) and at position 39 for both H2B's from sea urchin sperm (Strickland et al., 1977a,b) and trout testis (Kootstra & Bailey, 1978). The major variant of chicken H2A (H2A.1) has been previously shown to contain three amino acid changes from calf H2A.1 at positions 16, 121, and 128 (Laine et al., 1978) and a histidine deletion at position 123 or 124. In trout and sea urchin H2A's the two adjacent histidines (123 and 124) are deleted (Bailey & Dixon, 1973; Sautière et al., 1975).

A comparison of the amino acid sequence for histones of different species indicates that most evolutionary changes involve conservative amino acid substitutions located preferentially within hydrophilic regions, which interact with DNA (Adler et al., 1975; Bradbury et al., 1975). However, the differences distinguishing the variants of the same histone class always affect the hydrophobic central region (Figure 8) which is involved in histone–histone interactions (Bradbury et al., 1972; D'Anna & Isenberg, 1974; Kootstra & Bailey, 1978). Although most of the amino acid substitutions distinguishing histone variants are also conservative, they produce conformational differences detectable by electrophoresis in the presence of nonionic detergents as well as by chromatography in carboxymethylcellulose (Franklin & Zweidler, 1977; A. Zweidler and S. Franklin, unpublished experiments; Figure 3 above). In the case of the H2A variants of the mouse, the differences have been shown to be immunologically detectable (Blankstein et al., 1977). Differences in the conformation of the hydrophobic region of nucleosome core histones are likely to produce nucleosomes of different structural and possibly functional properties.

Further support for the hypothesized functional significance of histone variants is derived from the observation that in the case of H2A and H3 the same types of variants have been preserved in parallel throughout the evolution of the vertebrates. In trout and carp (A. Zweidler, unpublished experiments), in the chicken [this report and Urban et al.

(1978)], in the boa (A. Zweidler, unpublished experiments), and in 15 species from five orders of mammals (A. Zweidler, unpublished experiments; Zweidler et al., 1978; A. Zweidler, M. Urban, and P. Goldman, unpublished experiments), both types of H2A variants are present simultaneously in adult tissues, although in different relative amounts. The sea urchin also has both types of H2A variants, which are expressed at different stages of embryonic development (Brandt et al., 1979; Cohen et al., 1975). The variants H3.2 and H3.3 occur simultaneously in all examined vertebrate species with the possible exception of *Rana pipiens*.

In the case of H2B the situation is more complex. Most species have only one major form of H2B [the H2B.3 variant described for calf thymus by Franklin & Zweidler (1977) occurs in very low amounts and is usually not detected in electrophoretic analysis]. The sea urchin (Cohen et al., 1975), the chicken (this report), the boa (A. Zweidler, unpublished experiments), and the mouse (Franklin & Zweidler, 1977) all have at least two major somatic H2B variants. Although the primary structure difference between the variants affects different residues in the chicken and the mouse, it involves the same type of amino acid substitution within the same region of the molecule. Furthermore, the same region of H2B (residues 60–77) is the site of the amino acid substitutions which distinguish the predominant H2B's of chicken and trout (alanine → serine, 77; Kootstra & Bailey, 1978) as well as chicken and *Xenopus* (isoleucine → valine, 69; Van Helden et al., 1978). Considerably more differences have been found in the H2B's isolated from sea urchin sperm (Strickland et al., 1977a,b), but these histones may be specific variants with specialized functions for spermatogenesis. Mouse spermatocytes also contain a specific variant of H2B (Zweidler & Franklin, 1979) which differs from calf H2B.1 in at least eight positions.

It has been assumed that the chromatin of eucaryotes contains only five histones, H1-(H5)-H2A-H2B-H3-H4, and the lack of complexity and tissue variability made histones unlikely candidates for a role in the functional organization of chromatin. We now have evidence that eucaryotic chromatin contains several species of both nucleosomal core histones and very lysine-rich histones, plus several histone-like minor components. The existence of several variants within the same cell must create a substantial heterogeneity in the nucleosome population, which may have an effect on the expression of different segments of chromatin. It is very likely that chromatin exists in different conformations, since the highly supercoiled form normally seen in nuclei would be expected to be transformed into a linear form during transcription and replication. Since such conformational changes would probably involve alterations in the histone–histone interactions, they may be influenced by the presence and distribution of specific histone variants and minor histone-like components. It will therefore be important to investigate the properties of histone complexes formed by different variants as well as minor histone-like species and to determine the distribution of these components in chromatin fractions in different functional states.

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Structural Changes of Nucleosomes in Low-Salt Concentrations†

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ABSTRACT: We report transient electric dichroism studies of the unfolding of 140 base pair calf thymus nucleosomes in the salt concentration range from 26 mM down to 0.1 mM. A single unfolding transition was found, occurring within the range 0.3–3 mM, with a midpoint at ~1.3 mM. A concentration of 100 μM Mg^{2+} is sufficient to reverse completely the unfolding, yielding the native structure. Nucleosomes cross-linked with dimethyl suberimidate do not undergo unfolding in low-salt solution. The unfolding transition is characterized by an increase in the negative limiting reduced dichroism from 0.29 to 0.48 and an increase in the field-induced, viscosity-limited, rotational orientation time from 0.8 to 1.9 μs. The results imply a model for the low-salt structure consisting of a 178-Å diameter disk, 60 Å in thickness, containing 140 base pair deoxyribonucleic acid (DNA) wound in 0.9 superhelical turn. The low-salt structure orients by a permanent dipole mechanism, with a dipole moment directed along the C_2 symmetry axis of 2600 D, compared to 1200 D for the native 140 base pair nucleosome. At least two to three counterions are released when the nucleosome unfolds. We propose that electrostatic repulsion between adjacent DNA sections in native nucleosomes is primarily responsible for unfolding. This repulsion is relieved in the structure having only 0.9 superhelical turn since the overlap of 0.4–0.75 turn of DNA is lost. We estimate that the free energy of forming the low-salt unfolded structure under physiological conditions may be as small as 7 kcal mol^{-1}, making it an energetically plausible candidate for an enzyme-induced intermediate in functional unfolding of nucleosomes.

The association of conformationally altered nucleosomal particles with transcriptionally active structural genes (Weintraub & Groudine, 1976; Garel & Axel, 1976) and replicating chromatin (Seale, 1978) attracts current interest in the conformational state and transitions of nucleosomal particles. Recently, several studies have been reported on the effect of low salt on chromatin or nucleosomes using electron microscopy (Tsanev & Petrov, 1976; Oudet et al., 1977), hydrodynamics and light scattering (Gordon et al., 1978), and fluorescence (Zama et al., 1977; Dieterich et al., 1977). Most of these studies showed an alteration or transition of structure induced by low salt, but the detailed structures still remain unknown.

Transient electric dichroism with its dual characteristics of rotational relaxation time and reduced dichroism, and a recently developed theory (Crothers et al., 1978), is a sensitive method to study the size, shape, and orientation of DNA molecules free in solution (Hogan et al., 1978), in nucleoprotein complexes (Klevan et al., 1977; Crothers et al., 1978), and in bacteriophages (Kosturko et al., 1979). We report here electric dichroism measurements on the structural transition of nucleosomes induced by low salt. The origin and mechanism of unfolding of nucleosomes in low salt are also discussed.

Materials and Methods

Preparation of Nucleosomal Core Particles. The 140 base pair nucleosomal core particles were prepared by micrococcal nuclease digestion of H1 depleted chromatin as described by Klevan & Crothers (1977) with some minor modifications. The digestion was continued for 10–15 min, and the products were assayed by DNA gel electrophoresis to obtain more homogeneous 140 base pair nucleosomal core particles. After elution from a Bio-Gel A5m column, nucleosomes were run through 5–20% sucrose gradients in an SW27 rotor at 25 krpm for 30 h to remove any small free DNA fragments.

Dichroism Measurement. Dichroism amplitude and relaxation time were measured on a modified T-jump apparatus as described previously (Hogan et al., 1978). Nucleosome stock solutions were made in 10 mM Tris-HCl and 1 mM Na_2EDTA, pH 8 (TE buffer); 100 × TE buffer or 1/100 TE buffer (pH 7.5) was added to the stock solution to obtain the desired salt concentration. Ionic strengths of the solutions were calculated by assuming complete ionization of Tris-HCl and Na_2EDTA at pH 7.5. The rotational correlation time was determined by the viscosity enhancement method as described previously (Klevan et al., 1977). The reduced dichroism did not show any appreciable temperature dependence from 0 to 25 °C. No measurable changes in dichroism amplitude or

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