Biochemical and Immunological Characterization of Two Distinct Variants of Histone H2A in Friend Leukemia†

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ABSTRACT: Changes in the relative amount of two histone H2A subfractions have been observed in cells at different proliferative stages of Friend leukemia. Biochemical analyses of the purified H2A subfractions reveal them to be different in primary structure, and not the result of postsynthetic modifications of the same parent protein. Antibodies raised against the purified H2A.2 subfraction cross react with H2A.1 and H2A.2, but show high specificity for the immunizing subfraction at higher sera dilutions. Only H2A.2 contains a methionine which appears critical to an antigenic difference that immunologically distinguishes H2A.2 from H2A.1. The observed change in the relative amounts of two nonallelic variants of a histone coincident with changes in the physiologic states of the cell may indicate a correlation between genome structure and function.

We have previously reported that the progressive change in physiologic properties of Friend virus induced leukemia cells is paralleled by a change in the relative amounts of two subfractions of histone 2A, resolved by polyacrylamide gel electrophoresis in the presence of a nonionic detergent (Blankstein and Levy, 1976). Thus, at early stages of the leukemic disease, while the proliferating erythroid cells still are capable of differentiation, the ratio of the subfractions H2A.1/H2A.2 is greater than 3:1. At later stages, when the transformed erythroid cells can be established in tissue culture and lose most of their capacity to differentiate, the H2A.1/H2A.2 ratio is 2:1. After extended passage in tissue culture, when the cells grow to higher density in vivo in Millipore diffusion chambers (manuscript in preparation) and respond to chemical induction of hemoglobin synthesis (Friend et al., 1971; Ross et al., 1972; Gusella and Houssman, 1976; Rueben et al., 1976), the H2A.1/H2A.2 ratio is reduced to 1:1.

The histones are basic protein components of eukaryotic chromatin. They have been resolved into five major classes by chemical fractionation, column chromatography as well as polyacrylamide gel electrophoresis. They have been found to be universally present in eukaryotes with a remarkable degree of conservation of their primary structure (Elgin and Weintraub, 1975). The histones appear to form a core around which the DNA is wound to form a nucleoprotein complex called "nucleosome" (Olins and Olins, 1974; Kornberg, 1974; Kornberg and Thomas, 1974; Noll, 1974; Axel, 1975). Since the histones seem to be the major structural components of the nucleosome, any change in their composition may produce a change in the overall structure of chromatin.

Subfractions of the major histones were first described for the very lysine-rich histone 1 (Kinkade and Cole, 1966a,b). The H1 variants occur in different relative amounts in different tissues (Kinkade, 1969; Bustin and Cole, 1968) and are synthesized at different rates in mammary glands at different hormone-induced physiologic states (Hohmann and Cole, 1971). More recently subfractions were also found in the histone classes 2A, 2B, and 3 (Franklin and Zweidler, 1975; Cohen et al., 1975). The subfractions of these histones also show tissue-specific variations in their relative amounts (Zweidler, 1977). The primary structure differences between the H2A, H2B, and H3 variants of calf thymus and some other mammalian tissues have now been established (Franklin and Zweidler, 1977).

We describe here the biochemical and immunological properties of the H2A variants in Friend leukemia cells, with the demonstration that they are different polypeptides.

Materials and Methods

Friend Leukemia Cells. Tissue culture cell lines have been established from a tumor arising in a DBA/2J mouse at the site where a piece of Friend leukemic spleen had been placed subcutaneously. Pieces of the tumor were placed on 2 × 2 cm grids and the grids were passed in vitro until a cell line was established (Jensen et al., 1964). Cells were grown in Basal Medium, Earle's salts (Gibco) containing penicillin (50 units/mL), streptomycin (150 μg/mL), and 15% fetal calf serum. For these experiments one of our cloned Me_2SO-inducible cell lines, C7D, was used.

Growth and Collection of Cells. To obtain enough cells for the purification of the two H2A subfractions, DBA/2J mice were injected subcutaneously at multiple sites with 10^6 cells. Large tumors developed within 3 weeks. The cells of the tumors were always identical with the injected cells and the histone composition was the same. This procedure allowed us to collect easily 30 to 50 g of tissue with a H2A.1/H2A.2 ratio of 0.8–1.0.

Preparation of Nuclei. The procedure used was as previously described (Blankstein and Levy, 1976), except that phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$)[1] at 0.1 mM was used in all buffers.

Extraction of Total Histones from Nuclei. Purified nuclei were extracted three times for 1 h each with 0.4 N H_2SO_4 in

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[1] Abbreviations used: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.

the presence of 0.1 mM $PhCH_2SO_2F$ at 4 °C. The histones were precipitated from the combined supernatants by the addition of 6 volumes of 95% ethanol and placed at −20 °C overnight. This procedure removed greater than 95% of the histones as demonstrated by analysis of the acid-extracted pellets in sodium dodecyl sulfate–polyacrylamide gels.

Chemical Extraction of H2A in Histones from Nuclei. Purified nuclei, washed three times with 80% ethanol, were extracted overnight at 4 °C with 10% guanidine hydrochloride–80% ethanol (pH 7.0) as described (Johns, 1967). This extraction procedure was repeated twice for 2 h. The H2A and H4 histones in the combined supernatants were precipitated by the addition of 4 volumes of acetone. Under these conditions, analytical gels revealed the presence of H2A and H4 histones with some H3 contamination.

The H2A fractions were further purified on large diameter acid urea–Triton X-100 gels (see below) and the individual histones retrieved from the gels (see below).

Separation of Histones by Polyacrylamide Gel Electrophoresis. Histones were identified by separation in 12% polyacrylamide gels containing 5% acetic acid, 8 M urea, and 6 mM Triton X-100 (Zweidler, 1977). Purification of the individual histones was achieved by electrophoresis in large diameter (1.2 × 14 cm) gels with 7.5% acrylamide, 8 M urea, 6 mM Triton X-100. Separation of the individual histone H2A subfractions from 1 mg of histones was easily obtained. After electrophoresis the gel was frozen and a small longitudinal section was sliced for staining to locate the individual histones. After corrections were made for the increase in length of the stained and destained gel, the areas of the unstained gels corresponding to the histone subfractions were cut out and placed above a 3-cm, large diameter, 7.5% acid urea–Triton gel. A dialysis sac containing 3 mL of 5% acetic acid was placed around the bottom of the large gel tube. Buffer (5% acetic acid) was then added to both upper and lower gel chambers. The gels were run for 2.5 h at 150 V. By this procedure the individual subfractions were obtained in a homogeneous preparation and the preparations were greater than 95% free of contamination with other histones (Figure 1).

Minimal contamination with other histones could be removed by repeating the electrophoresis in a third gel.

Oxidation of Methionine Residues. Oxidation of the methionine residues in the histones was achieved by the hydrogen peroxide procedure described by Neuman (1967). Purified histones (10–50 μg) were dissolved in 50 μL of 10 mM perchloric acid. Fresh hydrogen peroxide was added to a final concentration of 1.5% and the reaction was allowed to continue for 15 min at room temperature. The histones were precipitated by the addition of 70% perchloric acid to a final concentration of 10%. The samples were dissolved either in distilled water for assay in the complement fixation test (below) or in Triton gel loading buffer for analysis by Triton X-100 gel electrophoresis.

Preparation of Antisera. New Zealand white rabbits were immunized with histone–RNA complexes (Stollar and Ward, 1970). A primary dose of 150 μg of histone and 100 μg of yeast RNA (Sigma Chemical Co. type III), emulsified with complete Freund's adjuvant, was injected intradermally into each rabbit; two rabbits were used for each subfraction. A similar dose in incomplete Freund's adjuvant was given intradermally 2 weeks later, followed by an intravenous booster of histone–RNA complexes alone after another week. Sera were obtained a week later and were heated for 30 min at 56 °C to inactivate complement.

Complement Fixation Assay. The complement fixation assay was performed, as described by Stollar and Ward (1970), in a total volume of 1.2 mL of isotonic saline buffer + BSA (0.14 M NaCl, 0.01 M Tris, pH 7.4, 5×10^{-4} M Mg^{2+}, 1.5×10^{-4} M Ca^{2+}, and 0.1% bovine serum albumin). Antigen, antibody, and guinea pig complement were incubated in this buffer at the appropriate dilutions overnight at 4 °C. The extent of antigen–antibody interaction was determined by incubation with sensitized sheep red blood cells at 37 °C. The amount of red cell lysis was measured at 412 nm.

Amino Acid Analysis. Protein samples were hydrolyzed at 110 °C for 48 h in constant boiling HCl. Samples were lyophilized, dissolved in citrate buffer, pH 2.2, and analyzed in a Durrum 500 amino acid analyzer.

Tryptic and Thermolytic Digestion. Histones, 2.5 mg/mL, were digested with trypsin in 0.1 M ammonium bicarbonate pH 8.1 (ratio of 1:50 enzyme to substrate) for 2 h at 37 °C. Another aliquot of trypsin was added and the incubation continued for another 2 h. The sample was lyophilized, suspended in electrophoresis buffer (see below), and centrifuged in a microcentrifuge to recover the tryptic core. The protein concentration for the thermolytic digestion of the core was 0.5 mg/mL. Thermolysis was in 0.1 M ammonium acetate pH 7.0. Incubation was for 2.5 h at 37 °C (ratio enzyme to substrate 1:500). Electrophoresis of both tryptic and thermolytic peptides was in pyridine–glacial acetic acid–1-butanol–water (1:1:2:36) for 1 h at 600 V on Avicel microcrystalline cellulose plates. Ascending chromatography was for 3–4 h with pyridine–glacial acetic acid–1-butanol–water (10:3:15:12). Plates were dried for under 2 h, washed with acetone, sprayed with 10% triethylamine in acetone, and then with 0.01% fluorescamine in acetone.

Results

Preparation and Purification of Individual Histone Subfractions. Histone H2A extracted from Friend leukemic cells gives a single band in acid urea–polyacrylamide gel electrophoresis (Figure 1A). However, it can be separated into two subfractions in acid urea–Triton X-100–polyacrylamide gels (Figure 1B). Since the acid urea–polyacrylamide gels are the classic gel system for identifying and quantitating histones from different tissues, it is not surprising that the variants of H2A have not been previously observed. By the procedures described in Materials and Methods, we have successfully purified both H2A components. Little if any detectable contaminating other histone classes were noted in Triton X-100 gels or sodium dodecyl sulfate gels.

Demonstration of Methionine Residue in H2A.2. The presence of methionine in histones can be demonstrated by the alteration of their mobility in Triton X-100 gels after oxidation with hydrogen peroxide at low pH (Cohen et al., 1975; Zweidler, 1977). The two H2A subfractions of Friend leukemia cells revealed that only the H2A.2 subfraction migrated differently following oxidation (Figure 2). These findings strongly suggested that the H2A components were indeed different polypeptides and that only H2A.2 contained a methionine residue.

Amino Acid Analysis and Tryptic Digestion. As seen in Table I, amino acid analyses of the H2A subfractions from Friend leukemic cells show them to be very similar except: (1) H2A.2 contains a methionine apparently replacing a leucine in H2A.1; (2) there appears to be a serine-threonine substitution between these two variants. The histone variants appeared similar to those for H2A in calf thymus.

Our initial interpretation of the amino acid data concerning the primary structure of H2A.2 was strengthened by tryptic and thermolytic peptide analysis of Friend H2A.2 in comparison with the tryptic and thermolytic peptides of calf thy-

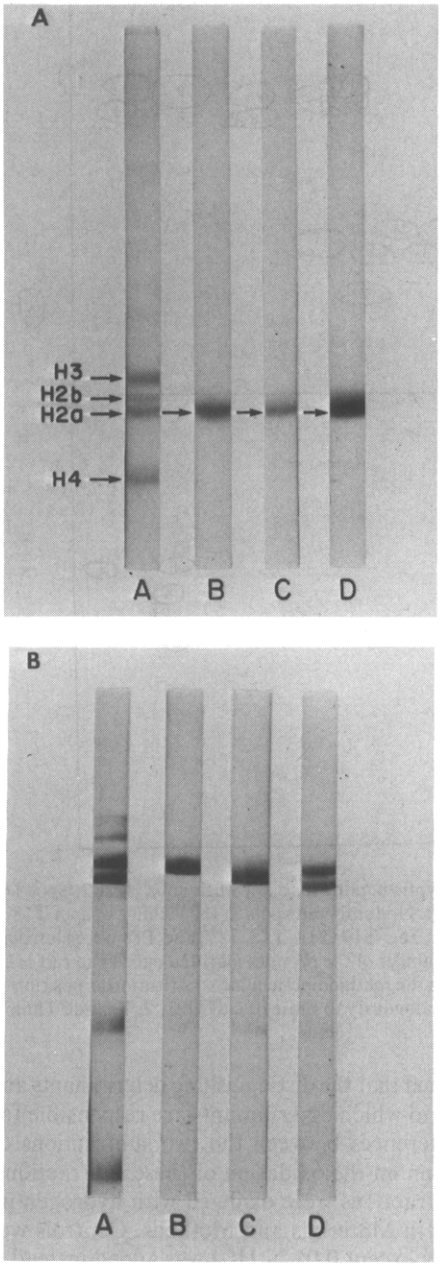

FIGURE 1: (A) Polyacrylamide gels, 15%, containing 2.5 M urea prepared as described by Panyim and Chalkley (1969). Histones were extracted from nuclei purified from C7D tumors. (A) H2A-H4 fraction, 30 μg, by Johns' procedure (Johns, 1967). (B) Gel purified H2A.1, 10 μg. (C) Gel purified H2A.2, 10 μg. (D) Mixture of gel purified H2A.1 and H2A.2, 10 μg of each. (B) Acid urea–Triton X-100–polyacrylamide gels of histones extracted from C7D tumors A–D are identical with the above samples.

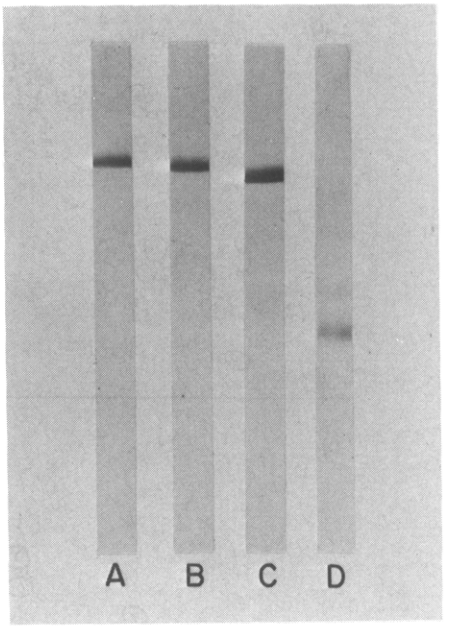

FIGURE 2: Acid urea–Triton X-100–polyacrylamide gels of untreated and oxidized H2A.1 and H2A.2 fractions. (A) H2A.1 untreated (10 μg). (B) H2A.1 oxidized (10 μg). (C) H2A.2 untreated (10 μg). (D) H2A.2 oxidized (10 μg).

TABLE I: Amino Acid Analysis of H2A.1 and H2A.2 from Calf Thymus, Friend Leukemia Cells, and Mouse Testes.[a]

	H2A.1			H2A.2		
	Calf thymus	Mouse testes	Friend leukemia	Calf thymus	Mouse testes	Friend leukemia
Cys	0	0	0	0	0	0
Asp	8	8.1	8.2	8	7.9	8.0
Thr	5	5.0	5.2	4	4.1	4.6
Ser	4	4.9	4.7	5	4.5	5.6
Glu	12	12.1	12.3	12	12.0	12.4
Pro	5	5.1	5.2	5	5.0	5.1
Gly	14	13.9	13.8	14	14.0	14.3
Ala	17	16.7	16.1	17	17.4	16.8
Val	8	7.6	7.9	8	8.0	7.6
Met	0	0	0	1	1.0	1.1
Ile	6	6.0	6.0	6	6.0	6.0
Leu	16	16.8	15.5	15	15.0	14.3
Tyr	3	3.0	3.0	3	3.0	2.9
Phe	1	1.1	1.3	1	1.0	1.4
His	4	3.9	3.8	4	4.0	3.8
Lys	14	13.6	13.1	14	14.6	13.7
Arg	12	12.6	12.4	12	11.9	11.7

[a] Calf thymus is according to Franklin and Zweidler (1977) and Yoeman et al. (1972). Mouse testes is according to Zweidler et al. (in preparation). Residues calculated relative to the calf thymus standard which was hydrolyzed and analyzed in parallel.

mus H2A.2. The complete digest map of H2A subfractions 1 and 2 from calf thymus has been recently determined and the amino acid sequence of each H2A variant polypeptide from calf thymus has been deduced (Franklin and Zweidler, 1977).

From the pattern of peptides after digestion (Figure 3), and the amino acid analyses (Table I) compared with that of calf thymus (Yoeman et al., 1972), it appeared that the H2A.2 variant from Friend cells was identical with H2A.2 variant from calf thymus cells having a serine at position 16 and a methionine at position 51. Using calf thymus sequence of H2A (Yoeman et al.) and our amino acid analysis and tryptic data from Friend cell and calf thymus H2A.2, the amino acid sequence of Friend cell H2A.2 was deduced (Tables II and III).

Immunochemical Comparisons of the H2A Subfractions. Both immunized rabbits produced antibodies to H2A.2. Anti-H2A.2 sera did not react with any of the other histones. This finding confirmed the purity of the histone preparation used as immunogen.

The H2A.2 serum was tested at several dilutions with each subfraction in quantitative microcomplement fixation assays. At low serum dilutions, the similarity of the two proteins was noted in their strong cross-reaction. At high sera dilution,

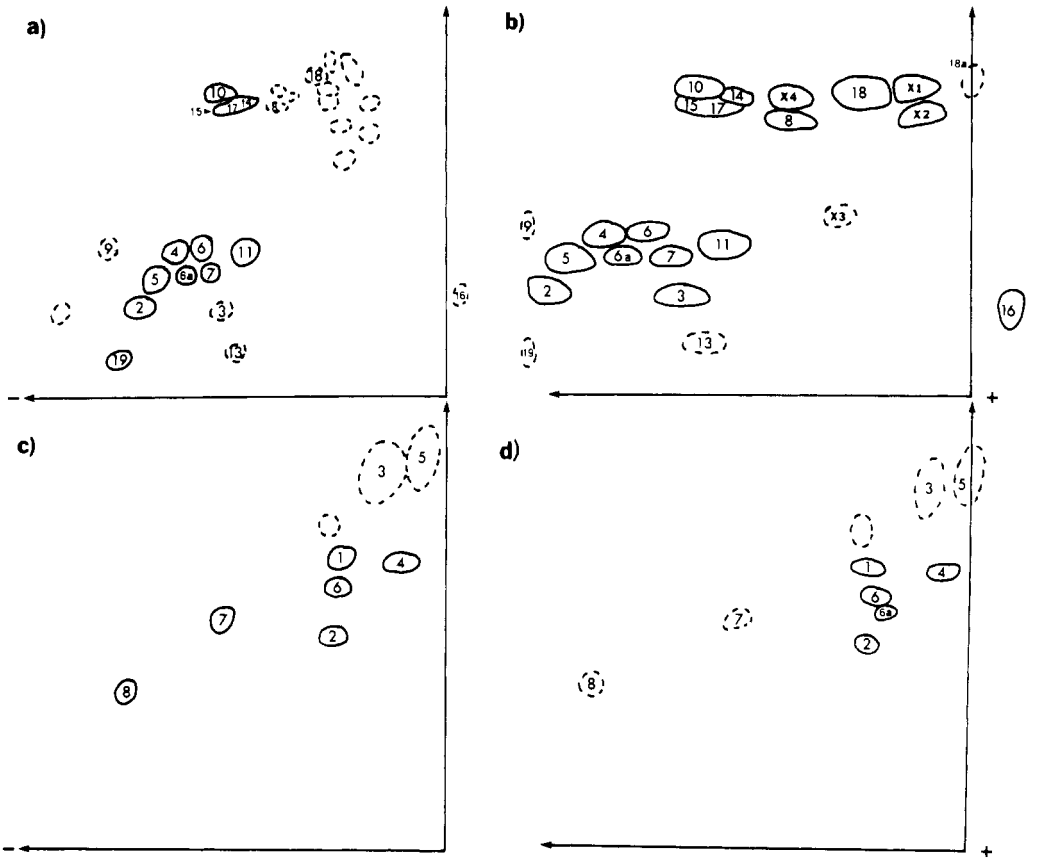

FIGURE 3: Comparison of calf and Friend cell histones 2A.2 by peptide mapping. (a) Tryptic peptide map of calf thymus H2A.2. Spot T6a is Ser-Arg arising from a serine-threonine substitution at position 16 (Franklin and Zweidler, 1977). Unnumbered spots in the vicinity of spot T18 are probably different forms of the same peptide. (b) Tryptic peptide map of Friend cell H2A.2. Peptides T6a, T10, T14, T15, T17, and T18 were eluted and identified by amino acid analysis. Peptides T18a and X1–X4 appear to be derived from T18. The remainder of the peptides map analogously to calf H2A.2 peptides. (c) Thermolytic peptide map of the tryptic core of calf thymus H2A.2. Peptide Th1 shows the methionine-leucine substitution at position 51. (d) Thermolytic peptide map of the tryptic core of Friend cell H2A.2. All of the peptides map analogously to those of calf H2A.2. Peptide Th6a is glycine.

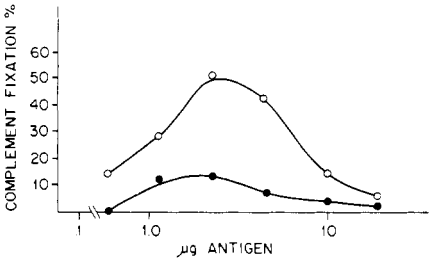

FIGURE 4: Complement fixation analysis of anti-H2A.2 against H2A.1 (●—●) and H2A.2 (○—○) (serum dilution $1/9600$).

however, the two proteins were distinguishable. Anti-H2A.2 (final dilution 1:9600) showed a stronger complement fixation reaction with H2A.2 then with H2A.1 (Figure 4). The dilution (titer) required to give a curve with a peak of 50% complement fixation was determined for the anti-H2A.2 sera. The ratio of the titer with homologous antigen to the titer with heterologous antigen was 1.3 for the anti-H2A.2 sera. This ratio, or indice of dissimilarity (Prager and Wilson, 1971a,b), corresponded to a difference of less than 1% of the amino acid residue positions in other protein antigen systems with proteins of similar size (Prager and Wilson, 1971a,b), suggesting that H2A.1 and H2A.2 differ in a small number of residues.

Methionine Influences Antigenicity of H2A.2. Although there is immunologic evidence that the two subfractions are similar, there is also measurable antigenic difference between them as described above. At higher dilutions of antisera it can be presumed that the distinguishing determinants are in excess. A hint as to which determinants are responsible for the antigenic differences between the two subfractions came from information on the oxidation of these subfractions. Purified H2A subfractions were oxidized with hydrogen peroxide as described in Materials and Methods. Controls were treated identically except 0.01 N HCl was added instead of the peroxide. After incubation, each sample was divided into aliquots. One aliquot was prepared for electrophoresis on acid urea–Triton X-100–polyacrylamide gels, while the other half of the sample was tested in a complement fixation assay. The polyacrylamide gel (Figure 2) revealed that only the H2A.2 histone had been affected by the oxidation. The complement fixation assay revealed no difference between the control and oxidized H2A.1 (Figure 5). Oxidation of H2A.2 significantly altered its immunologic reactivity (Figure 5). The shift in the complement fixation curve produced by the oxidation of H2A.2 suggested that a dramatic change in the structure of the protein had occurred. Considering the fact that no change in the complement fixation of H2A.1 was observed after oxidation suggests strongly that the methionine residue of the molecule is responsible in part for the antigenic differences observed between H2A.1 and H2A.2.

Discussion

We have previously described a change in the ratio of subfractions of histone H2A between Friend leukemic cells in vivo (H2A.1/H2A.2, 3:1) and Friend leukemic Me_2SO-inducible cells in vitro (H2A.1/H2A.2, 1:1) (Blankstein and Levy,

TABLE II: The Tryptic Peptides of H2A.2 from Friend Leukemia Cell C7D.

```
        1
T1  Ac-Ser-Gly-Arg
          5
T2  Gly-Lys
T3  Glu-Gly-Gly-Lys
    10
T4  Ala-Arg
T5  Ala-Lys
          15
T5  Ala-Lys
T6a Ser-Arg
              20
T7  Ser-Ser-Arg
                    25
T8  Ala-Gly-Leu-Gln-Phe-Pro-Val-Gly-Arg
    30
T9  Val-His-Arg
                35
T10 Leu-Leu-Arg
                      40
T11 Lys-Gly-Asn-Tyr-Ala-Glu-Arg
                                  50                      55
T12 Val-Gly-Ala-Gly-Ala-Pro-Val-Tyr-Met-Ala-Ala-Val-Leu-
                        60                    65
      Glu-Tyr-Leu-Thr-Ala-Glu-Ile-Leu-Glu-Leu-Ala-Gly-Asn-
          70
      Ala-Ala-Arg
                   75
T13 Asp-Asn-Lys-Lys
T6  Thr-Arg
              80
T14 Ile-Ile-Pro-Arg
                   85
T15 His-Leu-Gln-Leu-Ala-Ile-Arg
          90                  95
T16 Asn-Asp-Glu-Glu-Leu-Asn-Lys
T17 Leu-Leu-Gly-Lys
    100                   105                 110
T18 Val-Thr-Ile-Ala-Glu-Gly-Gly-Val-Leu-Pro-Asn-Ile-Glu-Ala-
            115
      Val-Leu-Leu-Pro-Lys
        120                 125
T19 Lys-Thr-Glu-Ser-His-His-Lys
T5  Ala-Lys
          129
T2  Gly-Lys-COOH
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TABLE III: Thermolytic Peptides of H2A.2 from Friend Leukemia Cell C7D.

H2A.2 core thermolytic peptides	
	43 45 51
Th1	Val-Gly-Ala-Gly-Ala-Pro-Val-Tyr-Met
	52 53
Th2	Ala-Ala
	54 58
Th3	Val-Leu-Glu-Tyr-Leu
	58 61
Th4	Leu-Thr-Ala-Glu
	59 65
Th5	Thr-Ala-Glu-Ile-Leu-Glu-Leu
	65 69
Th6	Leu-Ala-Gly-Asn-Ala
	65 71
Th7	Leu-Ala-Gly-Asn-Ala-Ala-Arg
	70 71
Th8	Ala-Arg

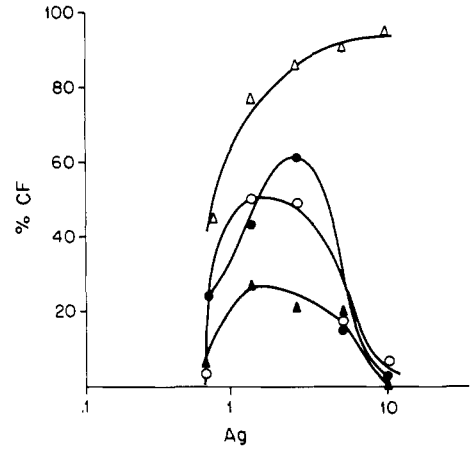

FIGURE 5: Complement fixation assay of anti-H2A.2 against untreated and oxidized H2A.1 and H2A.2 serum dilution $1/4800$. H2A.1 untreated (○—○), H2A.1 oxidized (●—●) H2A.2 untreated (△—△), H2A.2 oxidized (▲—▲).

1976). We have now determined that the subfractions are not due to a chemical modification of a parent protein but represent two different polypeptides. Amino acid composition and peptide analysis of the isolated H2A subfractions of Friend leukemia cells indicate that with high probability they are identical with the H2A subfractions which have been reported for calf thymus (Franklin and Zweidler, 1977) and differ in a threonine-serine substitution located outside the hydrophobic region (position 16) and a leucine-methionine substitution located in the hydrophobic region (position 51). Both these substitutions do not greatly affect the overall structure of these proteins since they are of the same helix promoting ability (Scheraga, 1974). The leucine-methionine substitution, however, seems to have an effect on the hydrophobic properties of H2A.2 since it has a lower affinity for nonionic detergents as indicated by its different electrophoretic mobility in the presence of Triton X-100 (Zweidler, 1977).

Reactions of the different H2A polypeptides with the H2A.2 antisera show cross-reaction at low serum dilution indicating that both H2A subfractions have antigenic sites in common. This cross-reactivity is not unexpected since the proteins are so similar. At higher dilutions, however, the anti-H2A.2 sera can distinguish the two proteins. The fact that oxidation of the methionine in the H2A.2 destroys the specific immunologic reaction strongly implicates the methionine residue as a determinant of H2A.2 antigenicity, participating either directly or through an effect on the protein conformation. Our H2A.2 antisera, therefore, contain two populations of antibodies, one population recognizes sites common between both H2A.1 and H2A.2 and a second population appears uniquely directed against the region of H2A.2 containing the methionine residue. Our antisera appear able to detect a single change in amino acid composition between two proteins that are otherwise nearly identical. Single amino acid differences have also been detected immunologically among closely related cytochrome *c* proteins (Nisonoff et al., 1970). The nonallelic variants of histone 2A have been preserved in parallel, with a similarly high degree of conservation, throughout the evolution of at least the mammals (Franklin and Zweidler, 1977). Similar variants have been observed in chicken, trout, and sea urchin (Zweidler et al., in preparation; Cohen et al., 1975). This indicates that the two H2A variants have independent essential functions. Both variants appear to combine in similar fashion with other histones to form histone complexes which in association with DNA form well-defined nucleoprotein structures

called nucleosomes. However, the histone complexes containing different variants have slightly different properties (Zweidler et al., 1977), suggesting different properties for genome regions containing the different variants.

The relative amounts of the mammalian H2A variants vary from tissue to tissue (Zweidler, 1976; Franklin and Zweidler, 1977; Blankstein and Levy, 1976). In HeLa cells they are synthesized simultaneously from distinguishable messenger RNAs (Borun et al., 1977). In sea urchin, different H2A variants are synthesized at different stages of embryogenesis (Cohen et al., 1975). In Friend erythroleukemia the appearance of erythroid Friend cells at different physiologic states is accompanied by concomitant changes in H2A subfraction ratio. The primary leukemic and tumor cells have a H2A.1/H2A.2 ratio of 3:1, similar to mouse thymus and spleen. This ratio changes to 2:1 when these cells can proliferate in tissue culture and to 1:1 when established culture cell lines are responsive to the differentiating effects of Me_2SO.

The change in the relative amounts of the H2A variants in Friend leukemia occurs in parallel with drastic changes in both cell proliferation and cell differentiation. It is therefore tempting to speculate that the two parallel changes are correlated. However, since neither the mechanism of cell differentiation nor the function of the histones is well understood at this time, such a correlation has to be established by further experimentation.

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