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A Comparison of the Proteins of Condensed and Extended Chromatin Fractions of Rabbit Liver and Calf Thymus†

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ABSTRACT: Chromatography of sonicated chromatin on Ectham-cellulose allows the resolution of a spectrum of nucleoprotein species differing in melting properties (Reeck, G. R., Simpson, R. T., and Sober, H. A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2317). The early eluting, high melting fractions are condensed in structure, while the late eluting, low melting fractions are in an extended DNA-like conformation. In contrast to early eluting rabbit liver chromatin fractions, late eluted chromatin segments are depleted of the lysine-rich histone and contain a markedly increased amount of nonhistone protein, amounting to 2.5 times the mass of the DNA. While calf thymus chromatin can also be fractionated into low and high melting segments with altered histone contents, the total nonhistone protein content of all the fraction is low and constant. For both tissues the relative concentrations of the detected microheterogeneous variants of the histones are unaltered. Superimposed on a background of overall similarity, the nonhistone proteins of early and late eluted rabbit liver chromatin nevertheless show both qualitative and quantitative differences.

Chromatography of sonicated chromatin on Ectham-cellulose allows the partial resolution of a spectrum of nucleoprotein species which differ in their melting properties (Reeck *et al.*, 1972). Further studies have indicated that the early eluted, high melting chromatin fraction exist in a highly condensed or supercoiled conformation while the late eluted, low melting fractions have an extended DNA-like conformation in solution (Polacow and Simpson, 1973a,b). We now report a comparison of the protein contents and distributions of condensed and extended chromatin segments isolated by preparative level fractionation on Ectham-cellulose more detailed than previously reported (Reeck *et al.*, 1972). Further, we compare rabbit liver and calf thymus chromatins which differ markedly in their content of nonhistone proteins. Our data indicate that extended chromatin is characterized by a decreased content of histone, primarily due to a decrease in the lysine-rich histones. In rabbit liver, the nonhistone protein content of the extended chromatin fractions is nearly three times that of the condensed material. The types of nonhistone proteins present in the two chromatin fractions differ significantly but yet maintain a surprising overall similarity. In contrast to these differences, both condensed and extended chromatins appear to contain identical proportions of the various naturally occurring chemically modified histones which can be detected by high-resolution polyacrylamide gel electrophoresis.

Experimental Section

Chromatin was isolated from livers of mature male New Zealand White rabbits or from frozen calf thymus by previously detailed methods (Simpson, 1971). After isolation, shearing, and centrifugation to remove unsheared materials all chromatin samples were sonicated for 2 min at 0–2° and 70 W using a microtip and the Branson Model W185 sonifier (Reeck *et al.*, 1972). Chromatin was utilized for chromatog-

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raphy within 8 hr of the beginning of the preparation unless otherwise specified. In some cases, calf thymus chromatin samples were treated with diisopropyl phosphofluoridate (iPr_2PF)[1] prior to chromatography. The sonicated sample, at a DNA concentration of 0.5 mg/ml, was adjusted to contain 0.01 M Tris-Cl (pH 6.5) and 0.5 mM Tris base. It was then made 0.25 mM in iPr_2PF and allowed to stand at 0° for 15 min and then the iPr_2PF treatment was repeated. After 15 min of additional incubation the sample was applied directly to the chromatographic column. The pH values quoted are those measured at 20°.

Histones were isolated from chromatin fractions by incubation of chromatin equivalent to 1 mg of DNA in 12.5 ml of 0.4 N sulfuric acid at 0° for 1 hr. After removal of the precipitated nucleic acids and nonhistone proteins by centrifugation at 10,000*g* for 30 min, the supernatants were dialyzed three times for 1.5–2 hr each against 100-fold volume excesses of cold deionized water and lyophilized. Nonhistone proteins were solubilized from the pellet by overnight incubation at room temperature in 1% sodium dodecyl sulfate–1% mercaptoethanol–0.01 M sodium phosphate (pH 6.8).

Chromatography of chromatin was performed on Ectham-cellulose prepared by the method of Peterson and Kuff (1969).

For analytical fractionation, chromatin equivalent to 3.5 mg of DNA was adjusted to contain 0.01 M Tris-Cl (pH 6.5) and absorbed to a 2-g Ectham-cellulose column equilibrated with the same buffer. After washing with the starting buffer, elution was performed with 0.01 M Tris–0.01 M NaCl at a flow rate of 3 ml/hr. For preparative level fractionation, a 40-g Ectham-cellulose column (2.5 × 50 cm) was utilized. The sample, chromatin equivalent to 55–65 mg of DNA, was applied at a flow rate of 75 ml/hr. After washing with the starting buffer, elution was carried out with the Tris base–salt solution at 40 ml/hr. All chromatographic procedures were carried out at cold room temperatures. The properties of fractions obtained at the preparative level were comparable to those previously reported for the analytical level of fractionation (Reeck *et al.*, 1972).

Electrophoretic analyses were performed for histones in both detergent and urea-containing systems for nonhistone proteins in the detergent system. Samples for electrophoresis in sodium dodecyl sulfate gels were dissolved in 1% sodium dodecyl sulfate–1% merceptoethanol–0.01 M sodium phosphate (pH 6.8) incubated at room temperature for 16 hr and then dialyzed against a tenfold dilution of the above buffer. Gels were prepared, stained, and analyzed as previously described (Levy *et al.*, 1972; Reeck *et al.*, 1972). High resolution electrophoresis in acidic, urea-containing gels was performed essentially as described by Panyim and Chalkley (1969a,b). Gels (6 × 240 mm) containing 2.5 M urea, 0.9 N acetic acid, 15% acrylamide, 0.1% bisacrylamide, and 0.5% Temed were poured in the cold, kept at 4° for 1 hr, and then allowed to polymerize for 2 hr at room temperature. Preelectrophoresis was carried out at 2 mA/gel for 18 hr at 4°. Samples, usually containing about 0.075 mg of protein dissolved in 0.9 N acetic acid–10 M urea, were applied to the gels and electrophoresis performed at 4° for 24 hr at a constant voltage of 300 V. Gels were removed by cracking the tubes and stained for 4 hr in either 1% Amido Schwarz or 0.5% Fast Green in 7% acetic acid–40% ethanol. Destaining was carried out in the acetic acid–ethanol solution containing a small amount of Dowex I-X2 resin. Stained gels were scanned with an E-C Apparatus Co. densitometer.

Determinations of DNA were made by the measurement of absorbance at 260-nm of chromatin fractions in 0.2% sodium dodecyl sulfate–0.066 M sodium phosphate (pH 6.8) using 21 as the absorbance of 1 mg of DNA/ml. The total protein content was determined by the Lowry method as detailed by Layne (1957). Histone concentrations were determined by a modification of the Reinecke salt method of Lindh and Brantmark (1966). Chromatin fractions were made 0.3 N in HCl and extracted overnight in the cold. After centrifugation of the extract, an aliquot of the supernatant was diluted to 1 ml with 0.3 N HCl and an equal volume of a saturated solution of recrystallized Reinecke salt in water at 0° was added. The solution was incubated at 0° for 30 min and the precipitated histone–Reineckate isolated by centrifugation for 30 min at 2500*g*. The precipitate was washed once with 2 ml of ice-cold distilled water and extracted once with 2.5 ml of acetone–1 N HCl (98:2). The extracted Reinecke acid was taken to dryness *in vacuo* and redissolved in 5 ml of water and the content of histone determined by measurement of Reinecke acid absorbance at 302 nm. Under these conditions, 0.1 mg of histone leads to an absorbance of 0.48. The method gives essentially the same results for any histone fraction.

Thermal denaturation analyses of chromatin were performed in 0.25 mM EDTA (pH 7.0) using an automated melting system as previously described (Reeck *et al.*, 1972).

Results

Protein Content. Rabbit liver chromatin, containing DNA, histone, and nonhistone proteins in approximately equal masses, was chromatographed on Ectham-cellulose at a preparative level using a direct scale-up from the previously reported analytical column (Reeck *et al.*, 1972). The thermal denaturation profiles of the material obtained at the larger level of fractionation are essentially identical with those previously presented. The early eluting fractions, those which lack any low melting sequences, are significantly enriched in total histones when compared to unfractionated chromatin (Figure 1). Coincident with the appearance of an enrichment for low melting chromatin sequences in the eluate (fractions 38–45), there is a decrease in the histone content from 1.0 g/g of DNA to about 0.75 g/g of DNA. Even more striking is a slightly later increase in the content of nonhistone proteins in this late eluting, extended chromatin. At the tail of the peak, the content of nonhistone proteins approaches 2.5 times the mass of the nucleic acid (Figure 1).

In our hands, calf thymus chromatin contains less than 0.2 times its DNA mass as nonhistone proteins, in marked contrast to the content of these acidic proteins in rabbit liver and most other chromatins (*cf.* Bonner *et al.*, 1968). In spite of this fact, calf thymus chromatin exhibits a melting profile that includes more low melting material than rabbit liver (*cf.* Figure 2 and Figure 2 of Reeck *et al.*, 1972). Fractionation of calf thymus chromatin on columns of Ectham-cellulose produces results analogous to those obtained with rabbit liver chromatin. Early eluted fractions are depleted of low melting components and melt at higher temperatures than bulk chromatin (Figure 2). In contrast, late eluted fractions contain up to 50% of their DNA as low melting nucleoprotein sequences (Figure 2). Due to the larger amount of low melting sequences in calf thymus (*vs.* rabbit liver), a greater proportion of the column eluate is enriched in low melting chromatin for thymus. Thus, the eluted material has melting properties

[1] Abbreviation used is: iPr_2PF, diisopropyl phosphofluoridate.

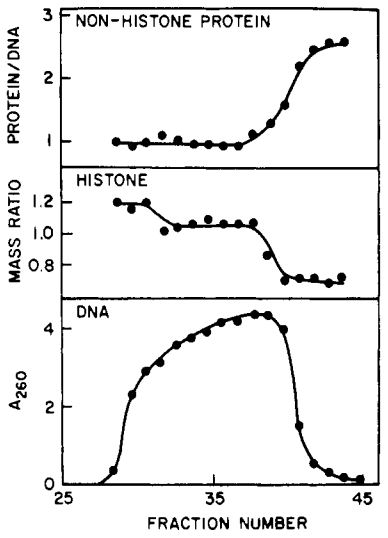

FIGURE 1: Chromatography of rabbit liver chromatin on Ectham-cellulose. Fractionation was performed on a preparative level as indicated in the Experimental Section. Histone was determined by the Reinecke salt method, total protein by the Lowry procedure, and nonhistone by difference. The contents of protein are plotted as the mass ratio of protein to DNA. The histone content of unfractionated rabbit liver chromatin is 1.0 g/g of DNA.

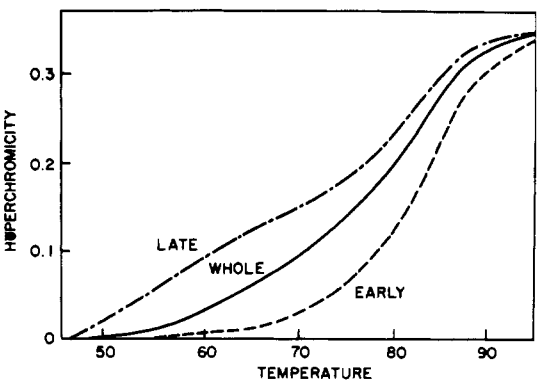

FIGURE 2: Thermal denaturation profiles of calf thymus chromatin (—) and early (- - -) and late (—·—) eluted fractions from an Ectham-cellulose column. Data were obtained with 0.25 mM EDTA (pH 7.0) as solvent.

like those of unfractionated chromatin at about the middle of the column for calf thymus and contains more low melting material thereafter. For rabbit liver this transition point is not reached until roughly 80% of the way through the elution profile.

This distribution of histones in the fractionated calf thymus parallels that of rabbit liver. The initial fractions have a somewhat higher histone content than bulk chromatin (Figure 3). The drop in histone content again occurs coincident with the appearance of chromatin fractions which are enriched in low melting sequences (fractions 28–42). The histone content of the latter portion of the eluted peak is 0.8 g/g of DNA (Figure 3). In marked contrast to the rabbit liver fractionation, there is no apparent alteration in the content of the nonhistone proteins between the early and late eluted fractions of calf thymus chromatin (*cf.* Figures 1 and 3).

Histones. Acid-extracted histones from early and late regions of the column eluate were examined using the high resolution gel electrophoresis system developed by Panyim and Chalkley (1969a,b). Such electrophoresis allows the resolution of all five of the main classes of histones. The marks at the bottom of Figure 4 indicate, from left to right, the positions of migration of histones. Additionally, partial resolution of the heterogeneity within some of the histone classes is achieved. Thus, f1 is fractionated into two of its primary sequence variants, acetylated and intramolecularly disulfide linked modifications of f3 are detected, and the acetylated form of f2a1 is separated from the parent molecule.

Figure 4 details the comparison of the histones of unfractionated rabbit liver chromatin with those of early and late eluted fractions. Early eluted chromatin fractions contain more f1 than unfractionated chromatin, while the late eluted fractions are depleted of their content of this histone, consistent with the earlier results obtained by sodium dodecyl sulfate electrophoresis of total chromatin proteins from Ectham-cellulose fractions (Reeck *et al.*, 1972). In contrast to this difference, the relative concentration of all the other histones remains invariant throughout the elution profile as judged by quantitative densitometry (Figure 4). Particularly, the relative amounts of the detectable microheterogeneous variants of histones f1, f3, and f2a1 are unaltered from the beginning of the eluted peak to the end.

The distribution of individual histones in fractionated calf thymus chromatin parallels that observed for rabbit liver (Figure 5). Again, the sole detectable alteration between early eluted, high melting chromatin, and late eluted, low melting chromatin is the decreased content of f1 histone in the late eluted material. The total content of the other four histones is apparently the same in all the column fractions. In some cases alterations in the microheterogeneity of histone f3 have been observed, but the more general observation is again in variance of the proportions of the detected variants of histones f1, f3, and f2a1 throughout the column elution profile.

On occasion, we see a significant proportion of the intermolecularly disulfide bonded dimer of histone f3 in preparations of calf thymus chromatin. The origins of this dimeric histone are currently unclear and have been a topic of debate previously (see Hnilica, 1972). Whatever its source, it is of interest that in those preparations in which we do observe f3 dimer, it is confined to the early eluted fractions of chromatin from the Ectham-cellulose column (Figure 6). Chromatin which is depleted of low melting material has all of the f3 histone in the dimeric form. When a maximal enrichment in low melting chromatin is achieved in the chromatographic

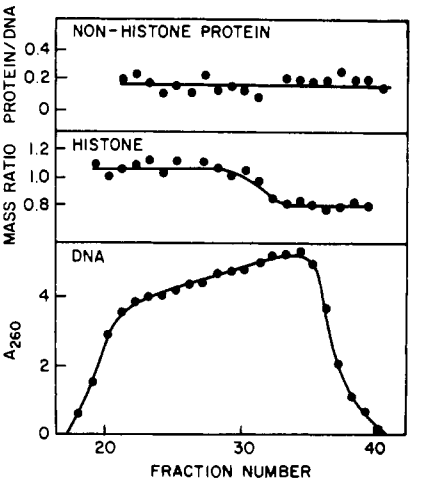

FIGURE 3: Chromatography of calf thymus chromatin on Ectham-cellulose, performed as in the legend to Figure 1. The histone content of unfractionated calf thymus chromatin is 1.0 g/g of DNA.

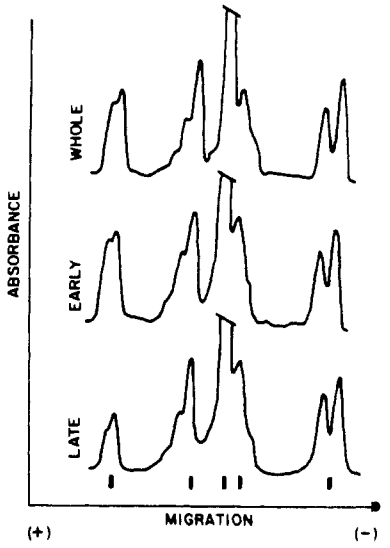

FIGURE 4: Acidic urea–polyacrylamide gel electrophoresis of histones extracted from whole rabbit liver chromatin, an early eluted fraction, and a late eluted fraction, as indicated. The data are direct tracings of densitometric scans of gel stained with Fast Green and are linear with optical density on the ordinate. The direction of migration is from left to right and only about the bottom 30% of the gel is shown. From left to right the marks at the bottom indicate the positions of migration of histones f1, f3, f2b, f2a2, and f2a1.

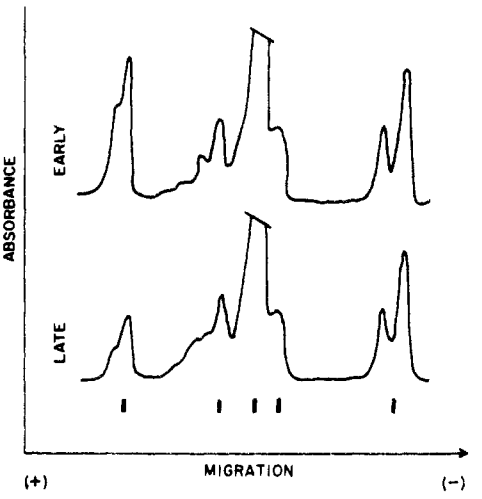

FIGURE 5: Acidic urea–polyacrylamide gel electrophoresis of histones extracted from early and late eluting fractions of calf thymus chromatin, as indicated. Conditions are as in legend to Figure 4.

system, all of the f3 histone is found in the monomeric form (Figure 6). The total amount of f3 present in the two regions of the elution profile does not appear to differ, consistent with the results of chromatography of chromatin from rabbit liver (Figure 4) or calf thymus chromatin lacking f3 dimer (Figure 5).

Nonhistone Proteins. Most of the available methodology for the separation of total chromatin proteins from DNA has been developed with sheared, but not sonicated, chromatin. The smaller size of the DNA in our chromatin, resulting from sonication and required for the chromatographic procedures, has thus far precluded the successful separation of large quantities of protein to allow chromatographic separation of the nonhistones. By prior extraction of histones, we have been able to subject larger loads of nonhistone proteins to sodium dodecyl sulfate gel electrophoresis, thereby noting more subtle differences in nonhistone protein composition between early and late eluted chromatin fractions than previously (Reeck *et al.*, 1972). Densitometric scans of the nonhistone proteins from early and late eluted fractions of rabbit liver chromatin are shown in Figure 7. It is apparent that there is a general similarity in the size distribution of the nonhistones from these two fractions of chromatin which differ so strikingly in their physical properties. There are, however, both quantitative and qualitative differences between the two samples of acidic proteins. The marked augmentation in the content of the high molecular weight nonhistone with a mobility of 0.16 has been noted previously (Reeck *et al.*, 1972). The proteins with mobilities of 0.21 and 0.31 appear to be present in significantly larger amounts in the late eluted chromatin fragments. In contrast, the early eluted chromatin appears to contain a group of lower molecular weight nonhistone proteins, mobility 0.35–0.50, which are absent from the late eluted material (Figure 7). In contrast to the results of others (Elgin and Bonner, 1970; Mac Gillivrey *et al.*, 1972), we do not see nonhistone protein components with molecular weights which are approximately those of the smaller histones in any column fractions, nor have we in previous studies (Levy *et al.*, 1972; Reeck *et al.*, 1972).

Evidence against a Chromatin Protease Artifact. Many chromatins are known to contain protease activity (Furlan and Jericijo, 1967; Stellwagen *et al.*, 1968; Panyim *et al.*, 1968; Bartley and Chalkley, 1970; Kurecki *et al.*, 1971; Garrels *et al.*, 1972). The optimal conditions for the activity of this protease are similar to those existing at the end of the elution of chromatin from the Ectham-cellulose column. Further, the histone preferentially attacked in whole chromatin by the protease is f1. Hence, it would seem necessary to establish that the decrease in f1 in the late eluted fractions and the attendant melting changes do not arise from protease activity after elution of the chromatin fractions.

We present here several experiments that seem to preclude this possibility. Incubation of unfractionated chromatin from rabbit liver under conditions similar to those experienced by the last eluted fractions (0.01 M Tris-Cl–0.01 M NaCl, pH 8.1, 0°, 8 hr) is without effect on the thermal denaturation or circular dichroic properties of the chromatin. Neither treatment of calf thymus chromatin with iPr_2PF, reported to inhibit its protease (Panyim *et al.*, 1968), nor storage of calf thymus chromatin for 24 hr at 4° affects the histone distribution observed in early and late chromatin fractions. In displacement chromatography of rabbit liver chromatin, material with low melting character and depleted of f1 histone is eluted well before the pH change (Reeck *et al.*, 1972). Finally, when chromatin from the central region of the elution profile is *rechromatographed*, the last eluted fractions have protein contents and melting properties similar to the first eluted fractions. Taken together, these observations make it quite unlikely that the conditions of chromatography produce the altered protein contents which we see in fractionated chromatin. They do not, of course, preclude the generation of f1 depleted regions as a physiological *in vivo* effect of the chromatin protease.

Discussion

A number of methods for the fractionation of chromatin into species with differing physicochemical and biological properties have been reported (Frenster *et al.*, 1963; Chalkley and Jensen, 1968; Duerksen and McCarthy, 1971; Janowski *et al.*, 1972). All these methods are based on separation of

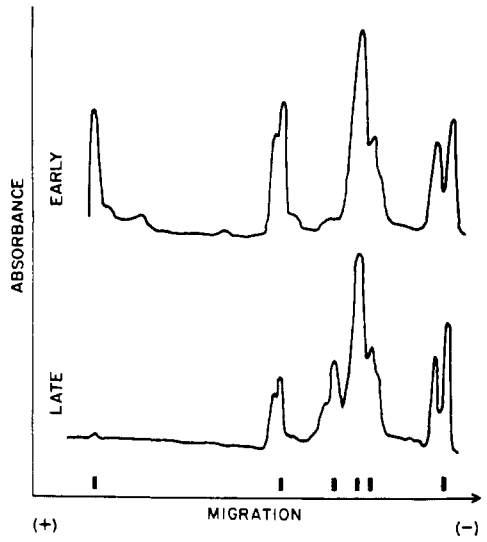

FIGURE 6: Acidic urea–polyacrylamide gel electrophoresis of histones extracted from early and late eluting fractions of calf thymus chromatin which contained the intermolecular disulfide linked dimer of histone f3. Conditions are as indicated in the legend to Figure 4 except that a greater portion of the gel is shown and the leftmost index mark indicates the position of migration of the f3 dimer.

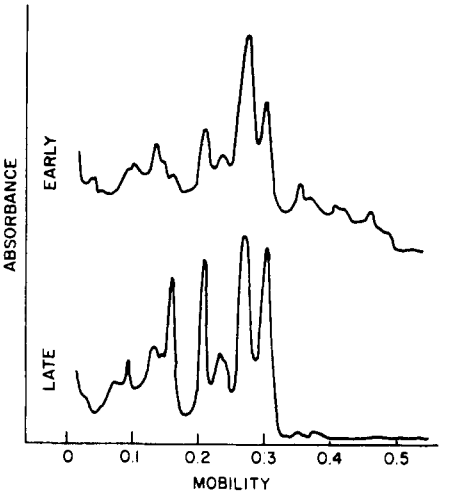

FIGURE 7: Sodium dodecyl sulfate gel electrophoresis of early and late eluting rabbit liver chromatin nonhistone proteins. Nonhistone proteins were obtained as described in the Experimental Section. Electrophoresis was performed on 0.6 × 15 cm gels for 8 hr at 5 mA/gel. Mobilities are relative to the migration velocity of Bromophenol Blue.

chromatin fragments according to size or shape, either by sedimentation or gel filtration, and hence are sensitive to small variations in the techniques used for shearing the chromatin. The procedure we have reported, ion-exchange chromatography on Ectham-cellulose (Reeck *et al.*, 1972), differs in kind from previously available methods. It might be expected to be less sensitive to minor size variations. Further, it allows the display of nucleoprotein types, a conceptual feature we feel to be of extreme importance in such separations. The magnitude of the physicochemical differences between the earliest and the latest eluted materials from the Ectham-cellulose column is equal to or greater than those reported in any other fractionation scheme. In our continuing investigation of the spectrum of molecular types in chromatin, we have reported here a study of the distribution of proteins in the condensed and extended chromatin regions.

In addition to utilizing a totally different fractionation method we feel that our current studies cover aspects of the proteins of fractionated chromatin which have been neglected previously. Thus, previous studies have generally agreed that the chromatin fragments thought to be transcribable *in vivo* had a decreased content of histones and an increased content of nonhistone proteins, when compared to repressed segments (Frenster, 1965; Chalkley and Jensen, 1968; Marushige and Bonner, 1971). However, quantitation of changes in individual histone fractions has either not been done or not commented upon. Further, the distribution of modified histones, thought by some to be of import in gene regulation (for reviews see Comings, 1972; Simpson, 1973), has not been evaluated in fractionated chromatin. Additionally, we have compared the fractionated chromatin of two tissues, rabbit liver and calf thymus. These two tissues differ strikingly in their content of nonhistone chromatin proteins, being about 1.0 g/g of DNA for liver and less than 0.2 g/g of DNA for thymus. Detailed comparison of two disparate types of chromatin in terms of their physical properties and distribution of histones and nonhistone proteins might be expected to aid in elucidation of the mechanisms of repression of transcription in eucaryotic cells.

In terms of the melting properties of chromatin fractions, fractionation of both liver and thymus chromatins on Ectham-cellulose leads to similar results. Early eluted fractions are depleted of low melting material and the last eluted fractions are enriched to about 50% of the total DNA existing as low melting chromatin regions. The differences between the two tissue systems are the result of more low melting chromatin in thymus than in liver. The total histone content of the chromatographic fractions has a direct inverse correlation with the degree of enrichment of low melting regions. The initial portions of the eluted chromatin have a relatively constant histone content of 1.0–1.1 g/g of DNA. Coincident with the appearance of low melting regions in the eluate there is a decrease in histone content to values of 0.75–0.8 g/g of DNA. Consistent with the earlier appearance of low melting segments for calf thymus, the transition in histone content occurs at an earlier point in the elution pattern for thymus than for rabbit liver.

The greatest portion of this change in histone content from early to late eluted chromatin appears to be due to a loss of f1, the lysine-rich histone. Quantitative densitometry of stained gels shows that the last eluted fractions (later than those shown in Figures 4–6), contain only about 25% as much f1 as the early eluted material. In contrast, densitometry of the histone electrophorograms does not demonstrate any significant changes in the relative amounts of the other four histone classes. Since f1 constitutes only about 20% of the total histone (Panyim and Chalkley, 1969a) and the decrease in histone at the end of the eluted peak is of the order of 30%, it is apparent that there must also be a depletion of other histones as well in the extended chromatin, although not to nearly the degree shown for f1.

In physicochemical studies of histone removal from chromatin, removal of histone f1 has only been shown to lead to a decrease in melting temperature (Ohlenbusch *et al.*, 1967; Henson and Walker, 1970) and lessening of the degree of condensation of chromatin as viewed by electron microscopy (Mirsky *et al.*, 1968). Additionally, in nuclear magnetic resonance studies of complexes of f1 and DNA, Bradbury and collaborators (1973) have provided evidence suggesting that f1 might function to provide cross-links to condense chromatin DNA into a more compact particle, an idea consistent with some of our earlier postulates concerning the role of

histones in the maintenance of DNA conformation in chromatin (Simpson, 1972). We now find that fractions of *native* chromatin which are deficient in f1 content exist in an extended conformation, agreeing with these other studies in suggesting a role for lysine-rich histones in condensing the DNA of repressed chromatin into a supercoiled or otherwise more compact particle.

While the decrease in histone correlates with the appearance of low melting chromatin sequences, the increase in nonhistone protein at the end of the column pattern does not. First, in calf thymus, there is not any significant increase in histone protein content at the end of the elution pattern (Figure 3). Secondly, in rabbit liver, the increase is displaced to a later position than the histone decrease (Figure 1). Due to their complexity and lack of knowledge concerning function, the details of the alterations in these nonhistone proteins must currently remain a matter for speculation.

Acknowledgment

The authors are indebted to Dr. Herbert A. Sober for continuing encouragement and to Linda Propst for valuable technical assistance.

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