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Reversible dissociation of linker histone from chromatin with preservation of internucleosomal repeat

(histone H5/DNA-cellulose chromatography/chromatin structure/reconstitution)

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ABSTRACT **Procedures are described for the dissociation of histones H1 and H5 from chicken reticulocyte chromatin without disruption of the native core histone–DNA complex. The comparative properties of native and depleted chromatin with respect to sedimentation, thermal denaturation, and sensitivity to nuclease digestion have been studied. The changes in these properties resulting from removal of the linker histones are fully reversed when histone H5 is added back to the depleted chromatin.**

Chromatin contains two functionally distinct groups of histones—namely, the "core" histones (H2A, H2B, H3, and H4) and the linker histones (H1, largely replaced by H5 in chicken reticulocytes). The core histones are responsible for the primary level of DNA folding, with formation of nucleosomes and a consequent diminution of the contour length by a factor of 7 (1). In the nucleosome, the core histones form an octameric complex around which 145 base pairs of DNA are wound. In native chromatin these nucleosome cores are separated by a uniform length of DNA, characteristic of the tissue, which in chicken reticulocytes is about 70 base pairs (2). Histones H1 and H5 are associated with the linker regions between the nucleosomes (3–6), as well as with the core histone complex (7). Moreover, it is thought that H1 and H5 direct the next higher level of DNA folding, apparently helping to bend the chain of nucleosome beads into the form of a solenoid (8) and reducing the contour length by a further factor of 6 (8, 9).

The reconstitution of chromatin from separated components provides the most direct approach to the clarification of the structural and functional roles of these constituents in the complex. Thus, in studies of the reconstitution of core histones with DNA, histones H3 and H4 have been identified as the agents of supercoiling of the DNA in the nucleosome (10–14). Upon reconstitution of core histones with DNA, nucleosomes are formed (10, 15–19), but the unique spacing is not regained (19–21). The resulting irregularity evidently vitiates the reconstitution of the native chromatin structure when the linker histones are introduced (20–23).

Histones H1 and H5 can be selectively removed from chromatin under a variety of conditions (21, 24–29), some of which, however, also lead to a redistribution of the histone octamers on the DNA (21, 24, 30). In the present study, we have developed procedures for the preparation of H1- and H5-depleted chicken reticulocyte chromatin that do not disturb the characteristic spacing of the residual core protein–DNA complex. We show that by all the criteria available to us, replacement of histone H5 on the depleted chromatin results in substantial regain of the native chromatin structure.

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MATERIAL AND METHODS

Preparation of "Native" Chromatin. Nuclei were prepared from chicken reticulocytes as described (31). The nuclei were washed twice with 0.2 M sucrose/1 mM $CaCl_2$/5 mM Tris·HCl, pH 7.5/80 mM NaCl/0.2 mM phenylmethylsulfonyl fluoride (PMSF), and resuspended in the same buffer at a DNA concentration of 6 mg/ml. Micrococcal nuclease (10^5 units/mg) was added to a final concentration of 2000 units/ml and the nuclei were digested for 20 min on ice. The reaction was arrested, and the nuclei were lysed, by the addition of an equal volume of 5 mM Tris·HCl, pH 7.5/5 mM EDTA/80 mM NaCl/0.2 mM PMSF. After 10 min on ice, the nuclear debris were removed by centrifugation for 5 min at 4000 × *g*. Recovery of chromatin in the soluble fraction was usually 50–75%. Chromatin oligomers produced by digestion were fractionated by zonal sedimentation in a 12–45% convex sucrose gradient containing 5 mM Tris·HCl, pH 7.5/80 mM NaCl/0.2 mM EDTA/0.1 mM PMSF. After centrifugation for 3 hr at 39,000 rpm in a Beckman TiX1V rotor at 4°C, fractions of native chromatin containing 20–30 nucleosomes were collected, dialyzed against TEP buffer (10 mM Tris·HCl, pH 7.5/0.1 mM EDTA/0.1 mM PMSF) containing 10 mM NaCl and stored at −20°C.

Preparation of "Stripped" Chromatin. Histones H1 and H5 and the nonhistone proteins were removed from native chromatin by passage over a DNA-cellulose column, prepared by the method of Litman (32) and containing 10 mg of DNA per g of cellulose (CF11). Chromatin (0.5 mg), with absorbance (260 nm) of 1.5, in 80 mM NaCl/TEP buffer, was applied to a 5-ml column (1 g of cellulose) equilibrated with the same buffer, and eluted at a flow rate of 8 ml/hr. The system was then arranged to recycle the chromatin through the column for 17–20 hr at 4°C. After collection of the depleted or stripped chromatin, the column could be regenerated by washing with 2 M NaCl/TEP buffer to remove the bound proteins.

Preparation of Histone H5. Purified histone H5 was prepared from total chromosomal proteins extracted from chicken reticulocytes as described (31). The protein mixture was dialyzed against 2 M guanidinium chloride/5 M urea/1 mM phosphate buffer, pH 7/0.2 mM PMSF and applied to a hydroxyapatite column equilibrated with the same buffer. Under these conditions all proteins bind to the column except the core histones. The column was washed with equilibration buffer; then histones H1 and H5 were eluted with 2 M NaCl/5 M urea/1 mM phosphate buffer/0.2 mM PMSF, pH 7. Histone H5 was separated from H1 by chromatography on Amberlite CG 50 (33). The purified fractions were dialyzed against 2 M NaCl/5 M urea/TEP buffer, and stored at −20°C. Protein

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TEP buffer, 10 mM Tris·HCl, pH 7.5/0.1 mM EDTA/0.1 mM PMSF.

concentrations were determined by the methods of Lowry *et al.* (34) and McKnight (35), with purified erythrocyte histones as standard.

Reconstitution of Histone H5 with Stripped Chromatin. Complexes were formed between histone H5 and stripped chromatin by direct mixing. To purified H5 (2.3 mg/ml) in a siliconized glass tube on ice, stripped chromatin, absorbance (260 nm) of 1.0, was rapidly added with thorough mixing. The samples were then dialyzed for 17–20 hr against 80 mM NaCl/TEP buffer at 4°C. The molar ratio of H5 in reconstituted mixtures was determined by taking 1 mole of H5 as 21,500 g and 1 mole of nucleosomal DNA (215 base pairs) as 144,695 g.

Sedimentation Analysis. Chromatins were analyzed by centrifugation in 11-ml, 6–40% convex gradients, containing 80 mM NaCl/TEP buffer for 3–4 hr at 40,000 rpm in a Beckman SW40.1 rotor.

Protein Analysis. Protein analysis was carried out by electrophoresis in 15% $NaDodSO_4$/polyacrylamide gels (36). The gels were stained with Coomassie blue and evaluated by densitometry.

Nuclease Digestion and DNA Electrophoresis. Chromatin samples (absorbance of 1.0 at 260 nm) were digested at 37°C in 80 mM NaCl/0.6 mM $CaCl_2$/10 mM Tris·HCl, pH 7.5/0.1 mM PMSF, with a micrococcal nuclease concentration of 2 units/ml. Aliquots were taken at different times, and digestion was terminated by the addition of EDTA. Samples were made 0.3 M in NaCl and 0.2% in $NaDodSO_4$ and were extracted with phenol/chloroform. DNA was precipitated from the aqueous phase. Digests were analyzed by electrophoresis in 1% agarose or 6% acrylamide gels containing 30 mM Tris/36 mM NaH_2PO_4/0.1 mM EDTA, pH 7.5. After electrophoresis, the gels were stained with ethidium bromide and photographed under UV illumination. The sizes of the DNA fragments were determined by comparison with restriction enzyme fragments generated by digesting λ DNA with *Eco*RI and simian virus 40 DNA with *Hae* III nucleases.

Thermal Denaturation. Melting profiles of chromatin were measured in a Perkin–Elmer, Coleman 575 spectrophotometer. The solvent was 0.25 mM sodium EDTA prepared by titration of disodium EDTA with NaOH to pH 7.6. The initial absorbance was ≈0.5 at 260 nm. The temperature was raised at 1°C/min. Differentiation was performed graphically for intervals of 4°C.

RESULTS

Preparation and Characterization of Depleted Chromatin. Native chromatin was homogeneous when analyzed in sucrose gradients (Fig. 1). Stripped chromatin, on the other hand, displayed a bimodal distribution due to the appearance of a minor, heterogeneous, more slowly sedimenting component. DNA, isolated from native chromatin and analyzed in 1% agarose gels, migrated as a sharp peak with a broad shoulder of smaller fragments (see Fig. 3). The size of the major component was 4300–5600 base pairs, corresponding to 20–26 nucleosomes.

Histones H1 and H5 and the nonhistone proteins were eliminated from native chromatin by DNA-cellulose chromatography. Recovery in terms of DNA was consistently 90–95%. Stripped chromatin contained only the core histones, H2A, H2B, H3, and H4, as judged by electrophoresis of the extracted proteins in $NaDodSO_4$ gels (Fig. 2). Digestion of stripped chromatin with micrococcal nuclease gave rise to a repeating pattern of DNA fragments, well resolved in 1% agarose gels (Fig. 3*B*). After 1 min of digestion, a progression of fragments was generated in which at least eight components, corresponding to the same number of nucleosomes, could be discerned. The repeat length was 211 ± 8 base pairs, which compares well with the value of 215 ± 7 base pairs obtained with native chromatin.

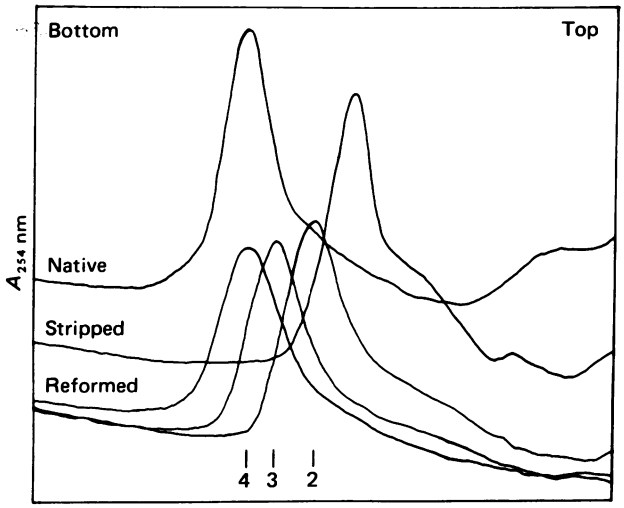

FIG. 1. Sedimentation profiles of native and stripped chromatins compared with chromatin reconstituted in the presence of 2-, 3-, and 4-fold molar excesses of H5.

Reconstitution. Optimal conditions for reconstitution were established from the results of sucrose gradient sedimentation analyses. The sedimentation velocity of reconstituted chromatin increased with the proportion of H5 in the reaction mixture, approaching that of native chromatin only when H5 was in molar excess (Fig. 1). In 5–20% linear sucrose gradients (data not shown), chromatin reconstituted with a 4-fold molar excess of H5 sedimented at 73 S, compared with 79 and 44 S for the native and stripped chromatins, respectively.

Reconstituted chromatins were isolated from sucrose gradients (Fig. 1), dialyzed, and lyophilized and their protein

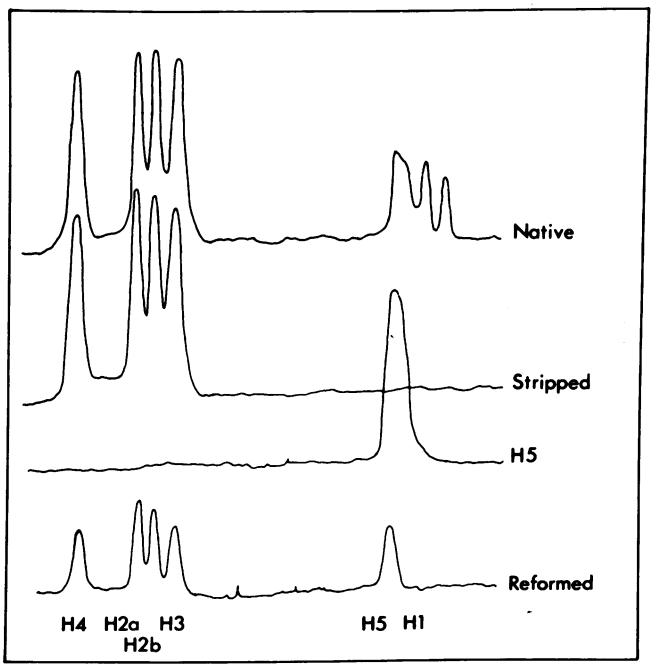

FIG. 2. Protein analyses of the chromatin samples and purified histone H5 by $NaDodSO_4$/polyacrylamide gel electrophoresis. Reconstituted chromatin was prepared with a 4-fold excess of H5 and was isolated from a sucrose gradient (Fig. 1). Migration is from right to left.

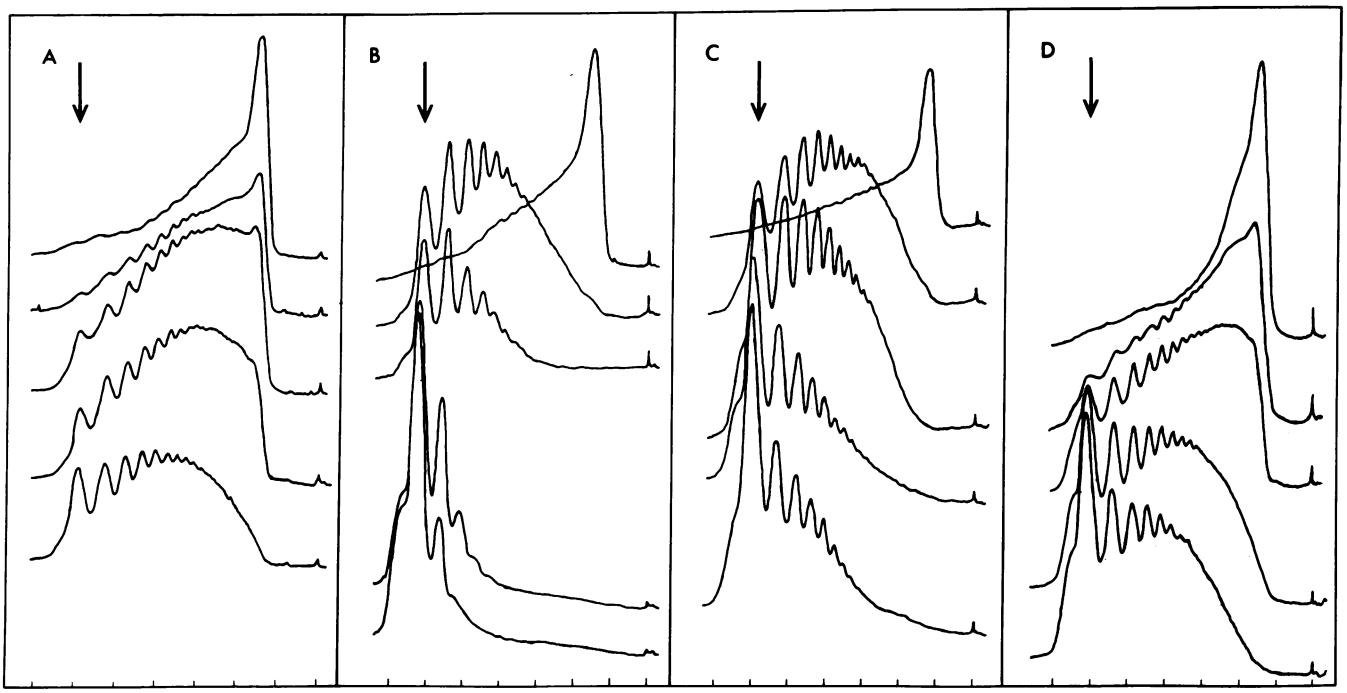

FIG. 3. Gel electrophoresis in 1% agarose of DNA isolated from micrococcal nuclease digests of chromatin samples. Chromatins were digested as described in the text. The figure shows patterns obtained from native chromatin (*A*), stripped chromatin (*B*), and chromatins reconstituted with 2-fold (*C*) and 4-fold (*D*) molar excess of H5. Increasing times of digestion (0, 1, 2, 5, and 10 min) are from top to bottom. Migration is from right to left. Arrow marks the position of monomer DNA.

composition was determined by electrophoresis in Na-DodSO$_4$/acrylamide gels. From densitometer traces of the gels (Fig. 2) stoichiometry was determined by comparing the area under the H5 peak to the area under the core histone peaks. The products recovered from mixtures in which the H5 was present in 2-, 3-, and 4-fold molar excess contained, respectively, 14, 20, and 22% of H5 compared with 26% for the native state.

Nuclease Digestion. Native chromatin preparations contained a considerable residue of micrococcal nuclease, amounting to 20–30 units of enzyme per absorbance unit (260 nm) of chromatin. This is 10–15 times the amount of enzyme added in a standard digest. This contaminating nuclease is completely removed during stripping.

Reconstituted chromatin fractions, isolated from sucrose gradients (Fig. 1), were compared with stripped and native chromatins by digestion with micrococcal nuclease. Digestion was performed under standard conditions for all samples except native chromatin, which was digested under the action of the contaminating nuclease alone at 2°C. At this temperature the rate of internucleosomal scission is reduced to about 1/12th the rate at 37°C (6), which compensates for the higher enzyme concentration in this sample. The products of digestion were analyzed by electrophoresis in 1% agarose gels, and the results of a complete analysis are shown in Fig. 3.

After 10 min of digestion with micrococcal nuclease, stripped chromatin yielded almost entirely mononucleosomal DNA fragments. Native chromatin was much more resistant. An estimate of the difference in the rates of internucleosomal cleavage in these two samples was provided by the nearly identical patterns generated after digestion times of 1 and 10 min, respectively. Reconstituted chromatins prepared with 2- and 4-fold excess of H5 showed restored resistance to internucleosomal cleavage, to an extent depending on the amount of H5 incorporated. The rates of internucleosomal cleavage of the sample prepared with a 4-fold excess of H5 and stripped chromatin again differed by a factor of 10. The relatively prominent monomer peak in the latter reconstituted chromatin digest reflects the presence of some unprotected linker DNA that has not bound H5.

When stripped chromatin is dialyzed against 0.65 M NaCl, the nucleosomes "slide" along the DNA and the native internucleosomal periodicity is largely eliminated. This is reflected in the micrococcal nuclease digestion patterns, both of the stripped chromatin and of the same material after re-addition of H5 (results not shown). In the latter case, H5, as expected, caused some protection of the DNA, as shown by the digestion kinetics, but in neither digest could more than a trace of the original 210-base-pair ladder be distinguished against the high background of DNA fragments produced.

We have also analyzed the monomer and submonomer fragments produced by micrococcal nuclease digestion of native, stripped, and reconstituted chromatins. To achieve comparable degrees of fragmentation, we digested native chromatin at 37°C with no addition of further nuclease and reconstituted chromatin at 37°C with 10 times the standard nuclease concentration. For stripped chromatin the standard conditions were used. DNA extracted from these digests was analyzed in 6% acrylamide gels; typical results are shown in Fig. 4.

The digests obtained from the native and reconstituted chromatin gave essentially identical patterns of monomer and submonomer DNA fragments. In all, seven fragments smaller than monomer were discerned and contained 168, 146, 127, 107, 95, 70, and 50 base pairs. Because stripped chromatin was digested with 1/10th the amount of enzyme used for native and reconstituted chromatins, the rate of production of submonomer bands was slower in this sample. However, these digestion conditions were chosen to illustrate the clear difference between native and stripped chromatins with respect to the production of a 170-base-pair DNA fragment. At no stage during the digestion of stripped chromatin was there a recognizable 170-base-pair fragment, monomer DNA being progressively degraded to a single fragment of 145 base pairs. This is in marked

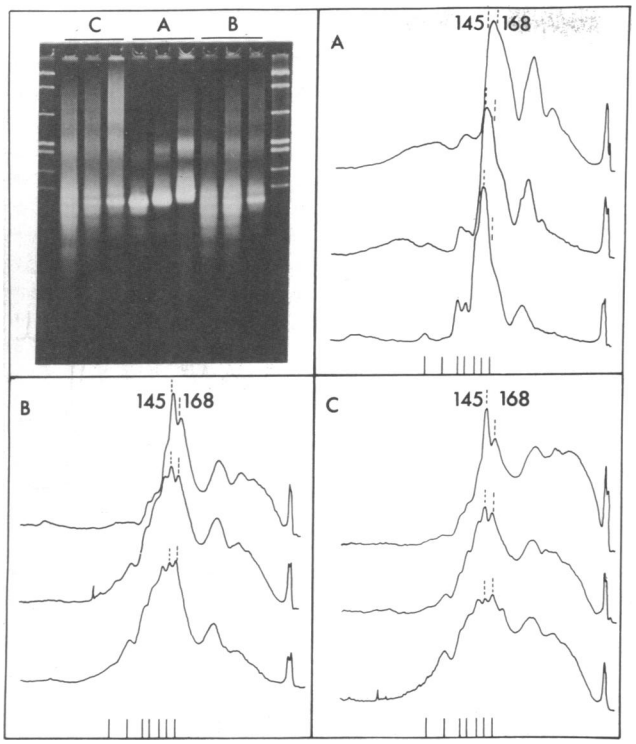

FIG. 4. Gel electrophoresis in 6% polyacrylamide of DNA isolated from micrococcal nuclease digests of chromatin samples. The figure and *Inset* show patterns obtained from native chromatin (*B*), stripped chromatin (*A*), and chromatin reconstituted in the presence of a 4-fold molar excess of H5 (*C*). Digestion conditions were as in the text. Increasing times of digestion (2, 5, and 10 min) are shown, from right to left (*Inset*) and from top to bottom (traces). Migration is from right to left. Broken lines on the traces mark the positions of zones of 145 and 168 base pairs. Vertical lines on the abscissa mark the positions of all clearly resolved submonomer bands. The two outer lanes in the gel (*Inset*) carry an *Hae* III restriction digest of simian virus 40 DNA.

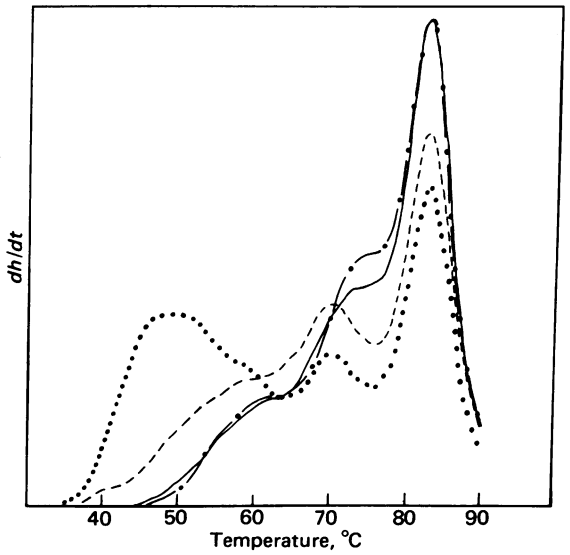

FIG. 5. Derivative thermal denaturation profiles obtained from native (—), stripped (·····), and reconstituted chromatins prepared by addition of 2-fold (– –) and 4-fold (–·–) molar excess of H5.

contrast to the results obtained with native and reconstituted chromatins, where a 170-base-pair fragment is well resolved and, indeed, emerges as the most prominent fragment in these digests.

Thermal Denaturation. The thermal denaturation profiles of reconstituted chromatins, isolated from sucrose gradients (Fig. 1), were compared with those of native and stripped samples; the results are shown in Fig. 5. When H1 and H5 were removed from chromatin, a characteristic change in the melting profile ensued, with the onset of a prominent new transition at 40–60°C and an accompanying reduction in the components melting at higher temperatures. Only when sufficient H5 had been added to yield a reconstitution product of composition similar to that in the native state was the melting profile characteristic of that state completely regained. The correct addition of H5 to stripped chromatin required the presence of salt. Reconstitution carried out in 0.25 mM EDTA produced a nucleoprotein complex that melted in a range intermediate between that of the free DNA and the native complex (results not shown).

DISCUSSION

We have demonstrated that chromatin complexes formed by the reconstitution of purified histone H5 with stripped chromatin have, by a series of criteria, the structural characteristics of native chromatin. Histone H1 (together with H5 in chicken reticulocytes) is responsible for a salt-dependent condensation of the chromatin fiber (37–40). This can be recognized by electron microscopy (8, 9, 39–41) and is due to either the folding or supercoiling of the basic 100-Å nucleofilament (8) to form a thicker fiber, some 200–300 Å in diameter (8, 9, 41, 42). In solution this structural change is accompanied by a sharp increase in sedimentation coefficient (40, 41). Under appropriate conditions, native and stripped chromatins accordingly differ grossly in sedimentation coefficient, for the latter cannot undergo condensation. In the present study native and stripped samples displayed sedimentation coefficients of 79 and 44 S, respectively. Chromatin reconstituted in the presence of a 4-fold molar excess of H5 sedimented at 73 S. Because this material contained 85% of the original amount of the linker histone (H5), the extent of the transformation to the native structure agrees with expectation.

The linker histones determine the pattern of attack of micrococcal nuclease on chromatin. If these proteins are absent, internucleosomal cleavage proceeds at a greatly enhanced rate (6). Histone H1 was also surmised to be responsible for transiently protecting a DNA fragment of about 170 base pairs during micrococcal nuclease digestion (5, 6). Both of these observations are confirmed by our results. The capacity of histones H1 and H5 to protect the linker DNA against scission and trimming is restored when histone H5 is reconstituted with stripped chromatin. Samples recovered from mixtures containing the largest excess of H5 are 10 times more resistant to internucleosomal cleavage than stripped chromatin and exhibit a prominent 170-base-pair DNA fragment after micrococcal nuclease digestion.

The thermal denaturation profile of stripped chromatin is similar to those of H1-depleted chromatins from other tissues (43, 44) obtained without nuclease digestion, but the melting begins at lower temperature and resolution into separate transitions is poorer, possibly because of end effects upon denaturation. Four components can be resolved in these profiles: the two of lowest melting temperature (47 and 58°C) can be interpreted as arising from the melting of uncomplexed DNA located both between and within the core particles. The two higher melting components (71 and 83°C) in the profile of stripped chromatin reflect the denaturation of DNA complexed with the core histones (43). In the presence of histones H1 and H5 in native chromatin, the internucleosomal spacers are

complexed by these proteins and their melting temperature is therefore elevated. Thus, in native chromatin the component melting below 60°C is absent. All the other features resolved in the melting profile of stripped chromatin persist in the native structure. These results affirm the unique function of histone H5 (and H1) in organizing the internucleosomal DNA. Chromatin reconstituted in the presence of excess H5 gave rise to a thermal denaturation profile essentially identical to that of native chromatin, indicating again the specificity of attachment of H5 and the native character of the resulting complex.

It may be inferred from our results that the native nucleosome core spacing is critical for the structurally specific uptake of the linker histone. If this spacing is disturbed, as when the stripped chromatin is exposed to high salt concentrations, the criteria for structurally faithful reattachment of the linker histones are no longer fulfilled. The implication is that in its native environment H5 (or H1) interacts with a nucleosome, which must be separated from its neighbor(s) by a segment of DNA of constant length within narrow limits of tolerance. The addition of H5 to a randomly spaced assemblage of core particles on the DNA chain can evidently not of itself catalyze the regain of the interparticle periodicity. In this light, the failure of experiments involving the recombination of histone mixtures with DNA (20–23) to produce anything resembling native chromatin is unsurprising. The critical effect of the ionic conditions on the nature of the product when H5 is added to stripped chromatin may be regarded as a further reflection of the specificity of the reconstitution process.

The results presented in this paper suggest the feasibility of preparing homogeneous samples of chromatin reconstituted with specific subfractions or derivatives of the linker histones. The availability of such material for biochemical and structural analysis should allow access to new information regarding the structural constraints that the linker histones impose on chromatin and thus lead to a better understanding of the function of these proteins in genetic expression.

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