Regulation of the Higher-order Structure of Chromatin by Histones H1 and H5

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ABSTRACT Chicken erythrocyte chromatins containing a single species of linker histone, H1 or H5, have been prepared, using reassembly techniques developed previously. The reconstituted complexes possess the conformation of native chicken erythrocyte chromatin, as judged by chemical and structural criteria; saturation is reached when two molecules of linker histone are bound per nucleosome, as in native erythrocyte chromatin, which the resulting material resembles in its appearance in the electron microscope and quantitatively in its linear condensation factor relative to free DNA. The periodicity of micrococcal nuclease-sensitive sites in the linker regions associated with histone H1 or H5 is 10.4 base pairs, suggesting that the spatial organization of the linker region in the higher-order structure of chromatin is similar to that in isolated nucleosomes. The susceptible sites are cut at differing frequencies, as previously found for the nucleosome cores, leading to a characteristic distribution of intensities in the digests. The scission frequency of sites in the linker DNA depends additionally on the identity of the linker histone, suggesting that the higher-order structure is subject to secondary modulation by the associated histones.

The repeating unit of chromatin, the nucleosome, comprises two domains, a central "core" and a "linker" region. The core contains 146 base pairs of DNA and an octamer of core histones, H2A, H2B, H3, and H4, whereas the linker region contains various amounts of DNA depending on the provenance of the chromatin and up to two molecules of linker histone. The length of the linker DNA may differ among genes in the same tissue, as well as between tissues and species, from a minimum of zero base pairs in yeast to a maximum of some 100 base pairs in sea urchin sperm (16).

The known range of linker histone content lies between zero in yeast (19), through one molecule per nucleosome in most cell types examined, and two in chicken erythrocytes (26, 34, 35). The amino acid sequence of the linker histone found in different tissues and different species is at the same time less conserved than the sequences of the four core histones (12). In a given tissue the amount of linker histone appears to be correlated with the rate of cell division (27, 35), whereas the nature of the linker histone seems to be a function of cell differentiation (12). The addition of H5 to the existing H1 during the maturation of chicken erythrocytes represents a dramatic change in the overall character of the linker histone content, because the two histones differ grossly in sequence and indeed in size (35). The biological significance in the variability of the linker histone is unknown, but it is reasonable to suppose that this is related in some manner to gene expression.

On the working hypothesis that chromatin structure will provide the key to understanding gene expression, we have focused on the role of the linker histones in determining the higher-order structure. Previous workers (25) have found that histone H1 protects the linker DNA and especially the 10 base pairs adjacent to the core. The linker histone evidently lies close to the surface of the nucleosome core very near histones H2A and H3, as shown by cross-linking studies (5, 6). The central globular region of H1 serves to locate the histone on the nucleosome core, allowing the basic amino- and carboxy-terminal tails to effect the condensation of the chromatin fiber in the physiological range of ionic strength (1). This H1-dependent conformational transition has been observed in the electron microscope by comparing native and H1-depleted chromatin at various ionic strengths (33). H1-depleted chromatin appears as a disordered array of nucleosomes, regardless of ionic environment. Native chromatin is partially ordered at low ionic strength and, as the ionic strength is raised, folds up into a structure variously described as a solenoid (10, 33) or a superbead (13, 30). Transitions between the loose and disordered polynucleosome chain and the compact and regular

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higher-order structure can be expected to exert a major effect on the template activity of the chromatin.

This study is concerned with the effects of systematic variation in the nature and amount of linker histone on chromatin structure. We have taken advantage of a reconstitution procedure developed earlier (2), whereby the linker histones may be dissociated from monodisperse chromatin fragments, without apparent serious disturbance of the nucleosomes or of the internucleosomal spacing, and then replaced at will. For the mixture of histone H1 and H5 normally present in native erythrocyte chromatin to the extent of about two molecules in all per nucleosome (26, 34, 35), we have substituted purified chicken erythrocyte H1 or H5 or calf thymus H1 in systematically varying amounts and examined the resulting physical and chemical properties of the chromatin. We show that H1 and H5 direct the refolding of the polynucleosome chain to give similar higher-order structures that closely resemble that of native chicken erythrocyte chromatin.

MATERIALS AND METHODS

Chromatin

"Native" chromatin from chicken erythrocyte nuclei was prepared and fractionated into discrete size classes as previously described (2) except that the concentration of micrococcal nuclease was reduced to 500 U/ml. H1- and H5-depleted chromatin (stripped chromatin) was prepared from native chromatin by passage over a DNA cellulose column (2).

Linker Histone Preparation

Purified H1 and H5 were prepared from chicken erythrocyte chromatin by chromatography on hydroxyapatite and Amberlite CG50 (2). Calf thymus H1, prepared by acid extraction, was a gift from Dr. Peter Hartman (Portsmouth Polytechnic, Portsmouth, England), and was further purified by hydroxyapatite chromatography. Each of the linker histone preparations was exhaustively dialyzed against 10 mM HCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), before precipitation with acetone. Weight samples of each histone were dissolved in 10 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM PMSF, pH 7.5 (TEP buffer), containing either 80 mM or 1 M NaCl, and were stored frozen at −20°C. Protein concentrations were routinely determined by the method of McKnight (23), using linker histone standards. Concentrations of the latter were determined by nitrogen analysis (14), taking the amino acid analysis into account.

Radioactive Labeling of Linker Histones

Chicken erythrocyte H5 (0.23 mg/100 μl) was dialyzed against 2% $NaHCO_3$ and dansylated by incubation with [^{3}H]dansyl chloride (50 μCi, 18 Ci/mmol, dissolved in 150 μl of acetone; Amersham Radiochemical Centre, Buckinghamshire, England) for 3 h at 20°C in the dark. The reaction was terminated by precipitation of the histone with 1 ml of acetone and storage overnight at −20°C. Labeled protein was collected by centrifugation, washed with actone, and dried in vacuo.

Calf thymus H1 (50 μg) was labeled with *N*-succinimidyl (2,3-^{3}H)propionate (100 μCi, 43 μCi/mmol; Amersham Radiochemical Centre) in 80 mM NaCl-TEP buffer for 30 min at 4°C, and was recovered by repeated acetone precipitation.

Labelled H1 and H5 had specific activities of 19,600 and 18,000 dpm/μg, correspondinbg to 0.4% (H1) and 0.9% (H5) modification of amino groups.

Reconstitution of Chromatin

Complexes were formed between linker histones and stripped chromatin by direct mixing. Purified histone was diluted, when necessary, to 80 mM NaCl with TEP buffer, and stripped chromatin (absorbance, 1.0 at 260 nm) in the same buffer was rapidly added with thorough mixing. The samples were then dialyzed for 17–20 h against 80 mM NaCl-TEP buffer at 4°C. The stoichiometry of the linker histones in reconstituted mixtures was calculated on the basis of molecular weights of 20,160 and 21,500 for H5 and H1 histones, respectively, and 145,000 for 215 base pairs of DNA.

Solubility of Chromatin

Reconstituted chromatins were centrifuged for 2 min at 10,000 rpm in a bench-top microcentrifuge. The supernate was removed and its absorbance at 260 nm was determined. Stripped chromatin treated in the same manner served as a control for 100% solubility.

Sedimentation Analysis

Chromatins were analyzed by centrifugation in linear 5–20% or isokinetic 5–27% sucrose gradients. Except where indicated, centrifugation was carried out in 80 mM NaCl/TEP buffer for 100–120 min at 40,000 rpm in a Beckman SW40.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C. Sedimentation coefficients were determined in the Beckman model E ultracentrifuge by use of ultraviolet absorption optics and were corrected to 20°C. Analyses were carried out on chromatin samples of absorbance (260 nm) 0.8 in 80 nM NaCl/TEP buffer.

Protein Analysis

Protein analysis was carried out by electrophoresis in 15% SDS polyacrylamide gels (17). The gels were stained with Coomassie Blue and evaluated by densitometry.

Nuclease Digestion and Electrophoresis

Chromatin samples (absorbance, 1.0 at 260 nm) were digested at 37°C in 80 mM NaCl/TEP buffer containing 0.6 mM $CaCl_2$ at a micrococcal nuclease concentration of either 2 or 20 U/ml. Aliquots were removed at different times, and digestion was terminated by the addition of EDTA. DNA was extracted as previously described (2). Digests were analyzed by electrophoresis in 1% agarose or 7% polyacrylamide (acrylamide:bisacrylamide = 20:1) gels as previously described (2).

The sizes of the DNA fragments were determined by comparison with restriction enzyme fragments generated by digesting lambda DNA with EcoRI or HindIII and PM2 DNA with HaeIII nuclease.

Thermal Denaturation

Absorbance melting profiles were carried out as previously described (2).

Electron Microscopy

Chromatin samples were fixed in 80 mM NaCl/TEP buffer containing 0.1% glutaraldehyde for 15 h at 4°C. The fixed samples were diluted at room temperature to an absorbance (at 260 nm) of ~0.04, and benzyldimethylalkylammonium chloride was added to a final concentration of 2×10^{-4}% wt/vol. After 30 min, 5-μl drops were placed in indentations on a Teflon block, and carbon-coated grids were floated on the drops. After 5 min for adsorption, the grids were washed for 10 min in distilled, deionized water and then dried in 90% ethanol. The grids were rotary shadowed with platinum at an angle of 7°. Samples were examined in a Siemens Elmiskop 1A electron microscope operating at a 100 kV and photographed at × 20,000 magnification. The microscope magnification was calibrated by means of carbon grating replica grid from Agar Aids (Stanstead, England).

Dissociation of Histones

After reconstitution, polynucleosomes were titrated to increasing salt concentrations by diluting samples fivefold into buffers to give the final salt concentrations indicated. TEP buffer was supplemented for these experiments with 10 mM phosphate, pH 7.0. After 30 min at 4°C, the samples were added to Eppendorf microcentrifuge tubes (Brinkmann Instruments Inc., Westbury, N.Y.) containing 0.4 ml of hydroxyapatite with the appropriate salt concentration as described above. The samples were then continuously rotated for 30 min. The hydroxyapatite was pelleted by centrifugation and the supernate was removed. After addition of 50 μg of BSA, the eluted proteins were precipitated by the addition of an equal volume of cold 40% TCA. The precipitates were collected on GF/C (Whatman) filters and counted.

RESULTS

Complex Formation

Increasing amounts of the three linker histones (chicken erythrocyte H1 (ceH1) and H5 and calf thymus H1 (ctH1) were added to stripped chromatin in 80 mM NaCl-TEP buffer, and the amount of DNA precipitated was determined. The results (Fig. 1) show that with none of these histones was there appreciable precipitation at ratios of less than two histone molecules per nucleosome. Above this ratio, rapid precipitation

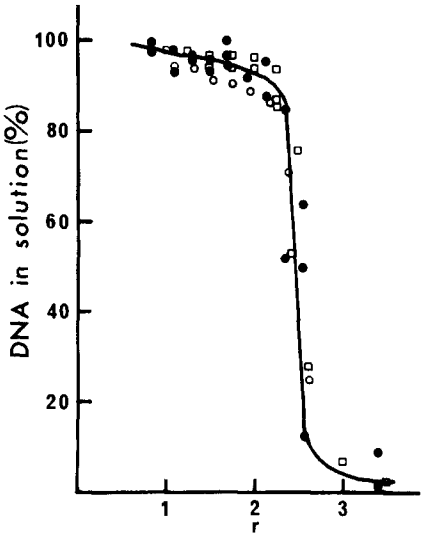


FIGURE 1 Solubility range of reconstituted chromatins. The solubility of reconstituted chromatin is shown as a function of the amount of ceH1 (○), H5 (●) and ctH1 (□) added. Molar ratio of H1: nucleosome = r.

ensued, all the DNA being carried down when the ratio reached about 2.5. Neither the length of the chromatin (25–75 nucleosomes) nor the time of reconstitution (45 min or 20 h) was found to influence the profiles.

Sedimentation

The sedimentation properties of reconstituted chromatins were studied in the range of linker histone to nucleosome ratios (r) in which soluble complexes were formed ($0 < r < 2$). The mean sedimentation rate of the complexes increases linearly with H1 or H5 content between the limiting values for the depleted and native chromatins (Fig. 2*b*). Two points emerge from the data in Fig. 2*a* and *b*: firstly, the sedimentation profiles of the reconstituted chromatins are symmetrical, with the same zone half-width as the native or depleted material. Taken together with the linear increase of sedimenation coefficient with linker histone content, this suggests that the linker histones do not bind cooperatively in the conditions of our experiments. Secondly, with both H1 and H5 it was necessary to introduce two linker histone molecules per nucleosome to generate a complex that sedimented at the velocity characteristic of native chicken erythrocyte chromatin.

Protein Binding

To confirm that all of the linker histone in the mixture indeed bound to the stripped chromatin, two experiments were carried out. First, aliquots of complexes with H1 and H5 prepared at a linker histone:nucleosome ratio of 2 were pelleted by sedimentation through a 10% sucrose column containing TEP buffer, 80 mM NaCl, and the pellet was recovered for analysis. Additional aliquots were exhaustively dialyzed against 0.1 mM PMSF and lyophilized. All were then analyzed for protein composition by electrophoresis in SDS polyacrylamide gels, followed by staining and densitometry. The results are shown in Fig. 3*A*. The proportion of linker to core histone was found to be the same in the pelleted and dialyzed samples.

The second type of experiment consisted in reconstituting chromatin with radioactively labeled linker histone. Complexes prepared with [^{3}H]dansylated H5 at a ratio of 2 were analyzed

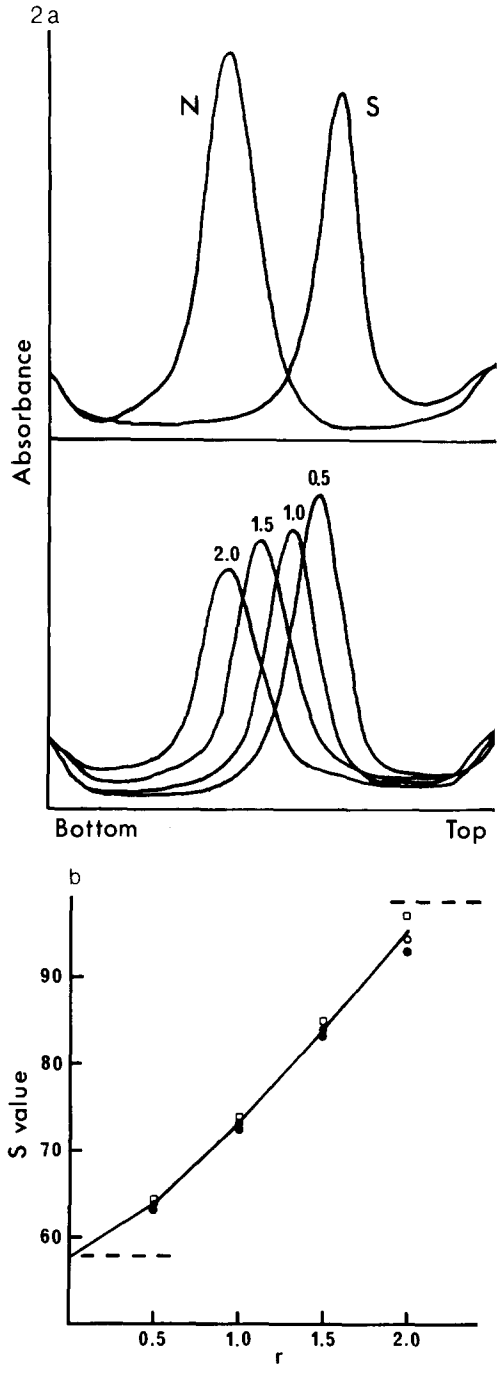


FIGURE 2 (*a*) Sedimentation analysis of reconstituted chromatins. Sedimentation profiles of native (*N*) and stripped (*S*) chromatins are shown in the upper panel and those of chromatins reconstituted with ctH1 at the indicated ratios of linker histone to nucleosome are shown in the lower panel. (*b*) Dependence of sedimentation coefficient on the linker histone ratio. The relative rates of sedimentation of the polynucleosomes shown in *a* (□), and of similar reconstitutes containing ceH1 (○) and H5 (●), are plotted against the linker histone to nucleosome ratio. Dashed lines mark the position of depleted ($s^0_{20,w}$ = 58S) and native ($s^0_{20,w}$ = 99S) polynucleosomes for reference.

by sucrose gradient sedimentation. Fig. 3*B* shows that essentially all the labeled H5 sedimented with the zone of absorbing material. Furthermore, 92% of the total label was recovered from the gradient.

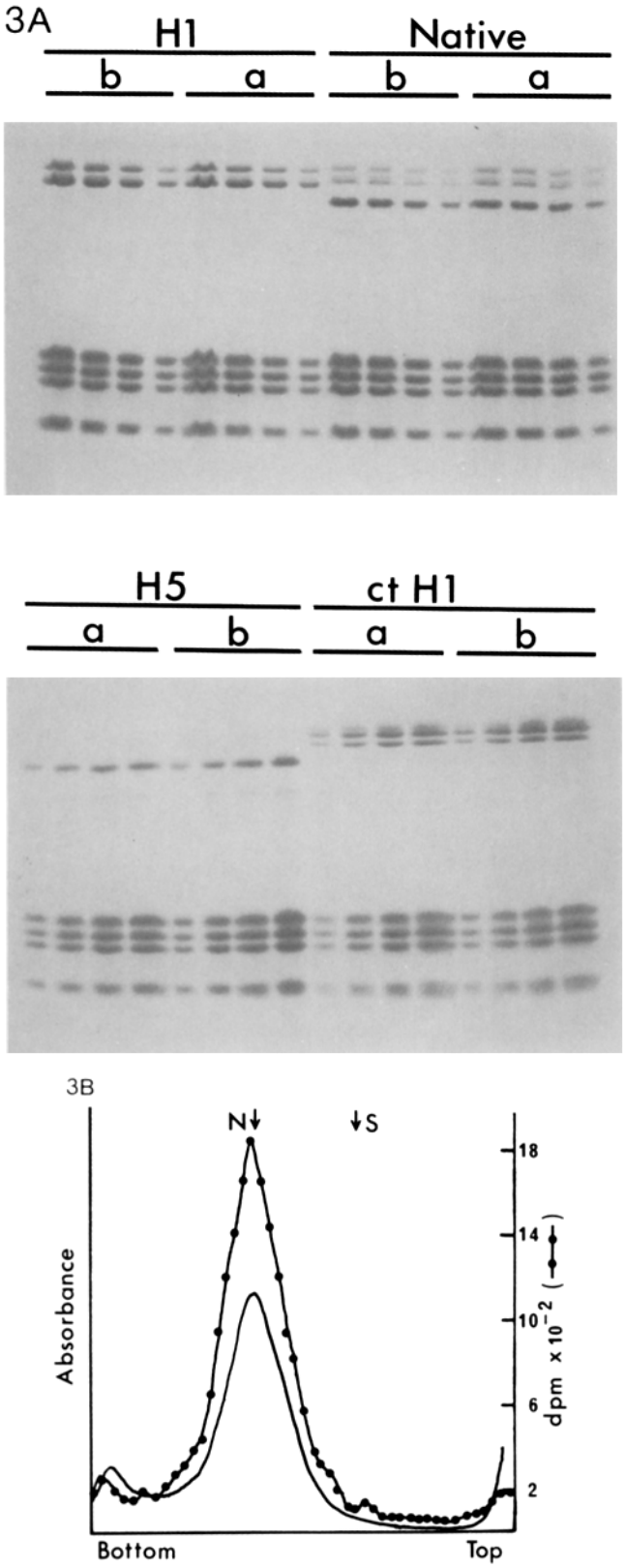


FIGURE 3 (*A*) Electrophoretic analysis of reconstituted chromatins. Histones from chromatins reconstituted with two linker histone per nucleosome were analysed by gel electrophoresis (*a*) after dialysis and (*b*) after centrifugation. Analyses were performed at four histone loadings (4, 8, 12, and 16 μg) increasing from right to left (*top*) and from left to right (*bottom*) to obtain quantitative results. Results for native chromatin are also shown. (*B*) Sedimentation analysis of labeled complexes. Chromatin was reconstituted at a linker histone to nucleosome ratio of 2 with ^{3}H-H5. The position of native (*N*) and stripped (*S*) chromatins and the distribution of label and absorbance of the complex is shown.

Micrococcal Nuclease Digestion

The degradation of chromatin by micrococcal nuclease occurs primarily within the spacer region and is perturbed in two ways by the presence of linker histone. Firstly, the spacer DNA is protected against the enzyme, as shown by the reduction in the rate of degradation of the DNA to fragments corresponding to single nucleosomes (2, 25). Secondly, the nature of the fragments is changed by the linker histones, for only in their presence is a partially resistant 168 base pair produce formed (2, 25) in addition to the 146 base pair fragment derived from the core particle itself (21).

We have followed the time-course of digestion of the stripped and reconstituted chromatins with micrococcal nuclease and analyzed the DNA products by electrophoresis in 1% agarose gels. The results, shown in Fig. 4*A*, demonstrate that each of the reconstituted chromatins has acquired a degree of resistance to internucleosomal cleavage similar to that shown by native chicken erythrocyte chromatin. Chromatins containing either H5 or ctH1 are about 5–10 times more resistant than stripped chromatin. The ceH1 chromatin is less resistant than the other reconstituted complexes, this effect being most marked in the early stages of the digestion.

To analyze the mononucleosomal DNA fragments produced during digestion, the enzyme concentration was increased by a factor of 10 and the products were fractionated on 7% polyacrylamide gels. The results are shown in Fig. 4*B*. As digestion proceeds, a prominent component containing 168 base pairs of DNA appears in each sample in addition to 146 base pair fragments. The digest of stripped chromatin contains only a trace of 168 base pair species.

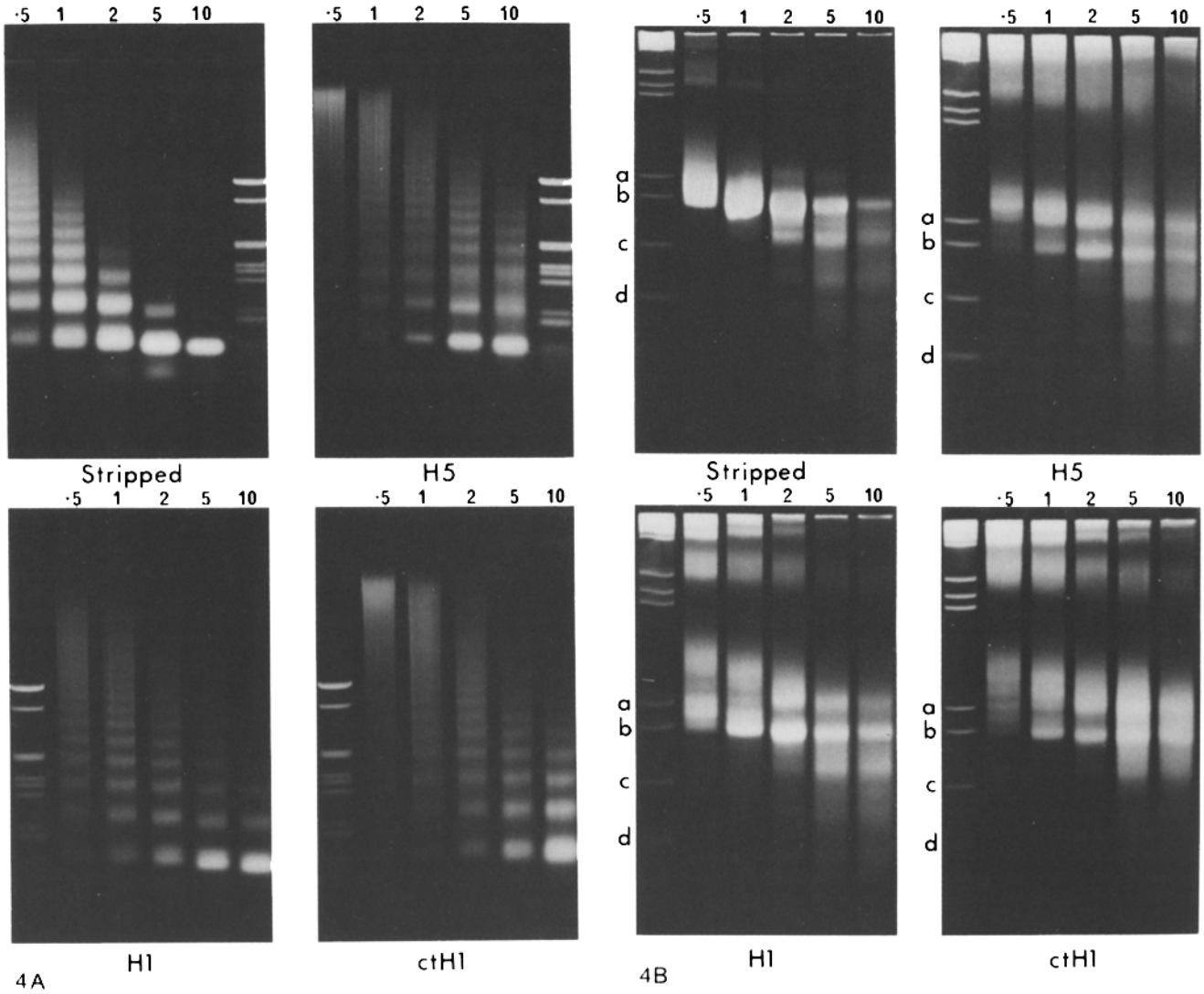
Two other features of the digests shown in Fig. 4*B* are conspicuous. In the early stages of digestion (0.5–1 min), the mononucleosomal products have the form of a discrete series of fragments corresponding to 146, 157, 168, 178, 189, and 200 base pairs. This progression is not so apparent in stripped chromatin. The bands are clearly multiples of the 10.4 base pair repeat occurring within the core particle (21).

In the early stages of digestion, the specificity of micrococcal nuclease digestion of the linker DNA was influenced by the identity of the linker histone. Thus, in the digest of H5 chromatin the 178 base pair fragment preponderated, whereas in the ceH1-containing analogue fragments of 157 and 168 base pairs were most prominent and that of the 178 base pair band much less so, although larger fragments could still be discerned. For the ctH1-containing material, all fragments between 146 and 200 base pairs were visible, the largest being the most pronounced. These patterns reflect the accessibility of the linker DNA backbone to the enzyme and the differences between the patterns obtained from the three complexes suggest that the structure of the complex is modulated by the type of linker histone.

Native chromatin (Fig. 4*C*) displayed early digestion products consistent with the content of both ceH1 and H5 linker histones. All bands (146–200) were again observed but the most prominent were those of 157 and 168 base pairs.

Thermal Denaturation

The derivative thermal denaturation profiles of native and stripped chicken erythrocyte chromatins (Fig. 5) are readily



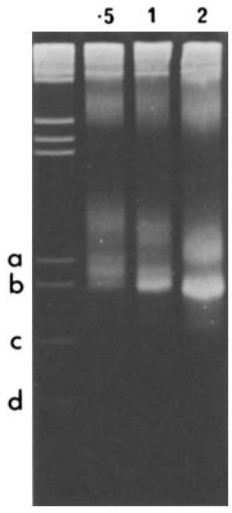


FIGURE 4 (*A*) Mild digestion of reconstituted chromatins with micrococcal nuclease. Chromatin was reconstituted at a ratio of two linker histones per nucleosome and digested with micrococcal nuclease at 20 U/ml. The results of digestion for increasing times, indicated in minutes (top left) are shown for reconstitutes with the three types of linker histones. The restriction marker is a HaeIII digest of PM2 DNA. Electrophoresis was in 1% agarose gels. (*B*) Extensive digestion of reconstituted chromatins with micrococcal nuclease. Chromatins were reconstituted at a ratio of two linker histones per nucleosome and digested with micrococcal nuclease at 20 U/ml. The results of digestion for increasing times, as indicated, are shown for reconstitutes with the three types of linker histones. The restriction marker is a HaeIII digest of PM2 DNA; the sizes of the fragments *a–d* shown are 167, 152, 120, and 95 base pairs. Electrophoresis was in 7% polyacrylamide gels. (*C*) Extensive digestion of native chromatin with micrococcal nuclease. Native chromatin was treated as the reconstituted chromatins described in *B*.

distinguished. Stripped chromatin displays a large component of DNA melting between 40° and 60°C, which is not present in native chromatin. This low-temperature transition reflects the denaturation of spacer DNA (32), which in native chromatin is associated with the linker histones and therefore melts at a higher temperature. Two prominent transitions centered at about 73° and 83°C are common to both native and stripped chromatins and reflect the melting of DNA in the nucleosome core (32).

The melting profiles obtained from reconstituted chromatins

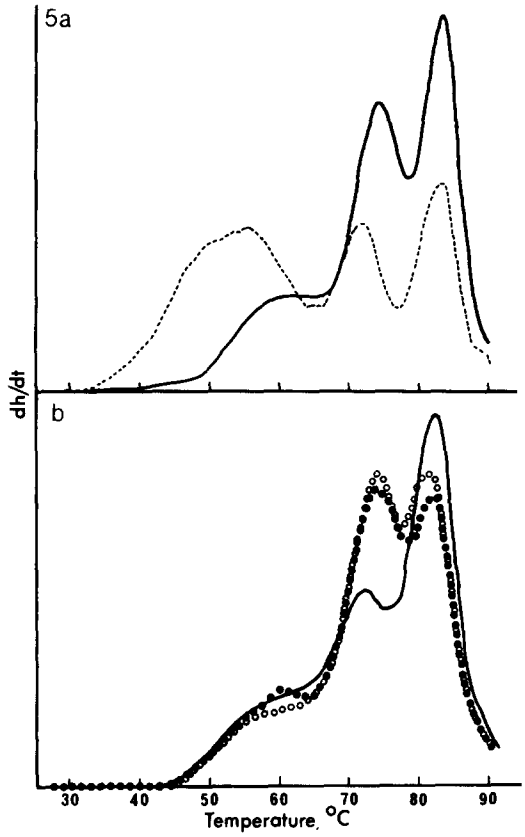


FIGURE 5 Thermal denaturation of DNA in reconstituted chromatins. The derivative melting profiles of (*a*) native (*N*; solid line) and stripped (*S*; dashed line) chromatins and (*b*) chromatins reconstituted with the three types of linker histones (H1, ●; ctH1, ○; H5, solid line) at a ratio of two per nucleosome are shown. The solvent was 0.25 mM EDTA, pH 7.5.

(r = 2) are shown in the lower panels of Fig. 5. In no case was there a transition in the low-temperature range as seen in stripped chromatin. The curves obtained with both H1 chromatins were similar and displayed a like distribution of melting between the two high-temperature maxima (73° and 83°C). In the H5 chromatin, on the other hand, the proportion of DNA melting in the high-temperature transition was greatly increased.

Dependence of Sedimentation Coefficient on Ionic Strength

Native chromatin undergoes a condensation in response to increasing ionic strength of the solution, in the range 0.005–0.1 M. This process, which is enhanced by the presence of linker histone, is reflected by changes in sedimentation coefficient (7, 30) and the contour length in the electron microscope (33).

Fig. 6 shows the ionic strength dependence of the sedimentation coefficients of polynucleosomes ($\bar{N}$ = 43 nucleosomes, where $\bar{N}$ is the average number of nucleosomes per chain of the chromatin fraction) reconstituted with ceH1 and H5 at ratios of one and two molecules per nucleosome, and of the original native and depleted polynucleosomes. All samples displayed a monotonic increase in sedimentation coefficient with increasing ionic strength in the range 5–80 mM NaCl, which in no case attained a plateau. Although the change was proportionately greater for polynucleosomes containing one or two linker histone per nucleosome as compared with none, there was no difference in the range over which the changes occurred. At intermediate ionic strengths (10 and 20 mM NaCl) a small difference was observed between chromatin containing one molecule of H5 per nucleosome and the corresponding material containing ceH1. This difference was diminished at higher ionic strength. There was no difference between the complexes containing two molecules of linker histone per nucleosome, and the response of these chromatins to ionic strength closely paralleled that of native polynucleosomes. The results obtained for native and stripped polynucleosomes are comparable with those recently presented by Butler and Thomas (7), although the sedimentation coefficients we have obtained with stripped chicken erythrocyte chromatin are significantly higher than those obtained with stripped polynucleosomes from rat liver chromatin of similar size.

Electron Microscopy

Electron microscopy was used to examine the state of extension of the reconstituted polynucleosomes. This study was carried out on a polynucleosome fraction with a narrow DNA size distribution (Fig. 7*c*). All samples were fixed with glutaraldehyde in 80 mM NaCl before spreading for electron microscopy. Fig. 7*a* shows micrographs of complexes formed at a series of ceH1 ratios, together with native and depleted polynucleosomes.

Depleted polynucleosomes were irregular in structure, exhibiting within chains both heterogeneous aggregates and isolated nucleosomes. At a ratio of one H1 per nucleosome,

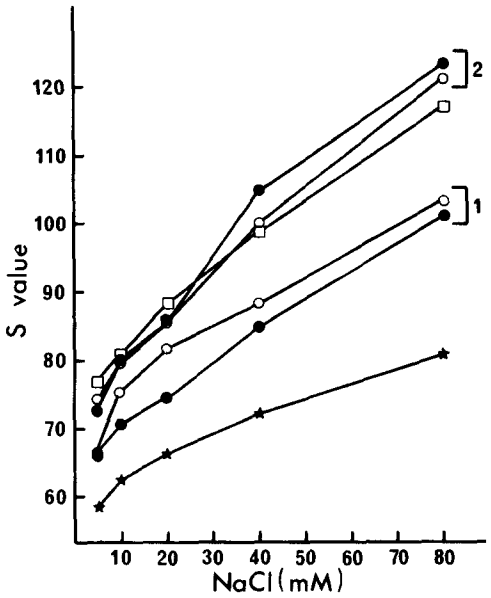


FIGURE 6 Dependence of sedimentation coefficient on ionic strength. Chromatins were reconstituted with one or two linker histones per nucleosome and dialyzed against TEP buffer with NaCl concentrations varying between 5 and 80 mM. The complexes were then sedimented in isokinetic sucrose gradients at the same salt concentrations, all those containing a given type of linker histone being analyzed in parallel, together with a sample of depleted chromatin at 80 mM NaCl as a marker. Sedimentation coefficients corresponding to the distances of sedimentation were established by analytical centrifugation using ultraviolet absorption optics on reference samples spanning the range of values observed. The results for native (□), depleted (★), ceH1 reconstituted (●), and H5 reconstituted (○) chromatins are shown, at the stoichiometries of one (*1*) or two (*2*) indicated in brackets.

reconstituted chromatin was largely in the form of thick fibers of diameters ranging up to ~350 Å. The thick fibers had a distinctly beaded appearance and were occasionally interrupted by unraveled sections displaying isolated nucleosomes. As the ratio of linker histones per nucleosome approached the limiting value of two, the chromatin fibers became more homogeneous in diameter along their length and less beaded in appearance, and unraveled stretches of nucleosomes no longer occurred. These changes were accompanied by a reduction in the mean length of fibers; the distributions of fiber lengths in typical fields are shown in Fig. 7*b*. Although the individual fiber diameters were variable at low H1 ratios, the average diameter in the condensed regions was always ~340 Å, which is also the average value for the more homogeneous fibers formed at the high H1: nucleosome ratios. The reduction in length brought about by increasing proportions of H1 was thus unaccompanied by any appreciable change in fiber diameter.

From the data of Fig. 7*b* and *c*, the packing ratios of DNA in the reconstituted and native chromatins were extracted and are expressed in Fig. 7*d* as a function of the linker histone ratio in the complex. The condensation factor of the DNA is seen to be proportional to the H1 content, increasing from 32 at a ratio of one mole per nucleosome to 50 at two. The agreement of the limiting value with that predicted for the solenoidal model of chromatin (10) is excellent. The estimated packing ratio for native chromatin is consistent with an H1 content of about 1.8 per nucleosome.

Linker Histone Dissociation

Because there are two linker histone binding sites per nucleosome, we sought to determine whether these were different in properties. For this purpose, mixtures of labeled and unlabeled ctH1 were used to prepare reconstituted complexes containing one or two molecules of H1 per nucleosome. For the latter, one equivalent each of labeled and unlabeled H1 were added either simultaneously or serially. The linker histones were eluted from the reconstituted chromatins, which were bound to hydroxyapatite, by raising the NaCl concentration of the eluant in steps up to 0.7 M. The ionic strength required to elute the H1 is a quantitative measure of the binding affinity. In each case, regardless of the stoichiometry or sequence of labeled histone binding, all of the histone was dissociated within the same narrow range (0.4–0.6 M NaCl) of ionic strength (Fig. 8).

DISCUSSION

The starting point of this investigation was our earlier observation (2) that addition of a saturating amount of histone H5, derived from chicken erythrocyte chromatin, to the denuded chromatin residue led to full recovery of the native structure. We have here presented an analysis of the mechanism and stoichiometry of the interaction of the DNA-core histone complex with the linker histones H1 and H5 from chicken erythrocytes and H1 from calf thymus, and have shown that each individually induces the formation of the higher-order structure characteristic of the native chromatin.

Either linker species is taken up by depleted chromatin to a limit of two molecules per nucleosome, and by the criteria of micrococcal nuclease fragmentation, thermal denaturation of nucleosome DNA, ionic strength dependence of sedimentation coefficient, and electron microscopic image of the chromatin, the regaining of the native structure is then complete. The capacity of chromatin to bind two molecules of linker histone is not unique to erythrocyte chromatin, for it has recently been shown to apply equally to calf thymus chromatin (24). As the linker histones are directly associated with the core histones (5, 6), the ability of chromatin to accommodate two molecules of H1 per nucleosome suggests that the twofold symmetry of the latter structure (11, 36) may be extended to include two H1 binding sites. However, as the primary H1 site of interaction with the nucleosome is contained in the globular domain of H1 (1), and because the experiment we have carried out to assess the equivalence of binding of the two H1 molecules (Fig. 8) is primarily a reflection of the basic tails of the H1 molecule (3), we are not able at present to say whether the nucleosome has equivalent binding sites for two H1 globular domains.

Although chicken erythrocyte chromatin is exceptional in that it is the only preparation known to contain two linker histones per nucleosome (26, 34, 35), it represents only one extreme of the range of linker histone to nucleosome ratios that have been found in chromatins of different origins. Yeast chromatin contains no H1 (19), whereas the proportion of linker histone in pea chromatin depends on the source of the tissue, increasing by as much as a factor of three during maturation (9). Finally, Pehrson and Cole (27) have recently demonstrated that, when the growth of HeLa cells is arrested in culture, histone $H1^0$ accumulates in the chromatin with no diminution in the amount of the normal H1. It would appear, therefore, that a wide variation in linker histone to nucleosome ratios between the limiting values of zero and two is an intrinsic feature of chromatin, and that increases in this ratio are invariably associated with decrease in the template activity of the chromatin, both in replication and in transcription.

The capacity of a nucleosome to accommodate two molecules of linker histone raises the possibility that, even in those tissues containing only one molecule of H1 per nucleosome, the distribution of that H1 is not random (one per nucleosome). Renz et al. (30) have shown that H1 displays a preference for binding to chromatin that is long enough to form higher-order structures such as would be found in the solenoid model (10, 22, 33). The ability of chromatin to form higher-order structure is therefore a feature that H1 can recognize. In the event that a substantial portion of the genome must be maintained in an unfolded, extended conformation, such as might be envisaged to occur for transcription or replication to take place, then this chromatin, being unable to form higher-order structure, may be a poor substrate for H1 binding. Such a situation would lead to an asymmetric distribution of H1 and, as a result, a partitioning of condensed and extended chromatin. Nonhistone proteins could influence the distribution of H1 either by competing with H1 for DNA binding sites or by helping to maintain regions of the chromatin in an extended (or condensed) conformation. In addition, the preference that H1 displays for DNA sequences enriched in adenosine + thymidine content (28, 29) could add a degree of specificity to the process.

The nature of the higher-order chromatin structure is dependent not only upon the presence of linker histone but also upon on its relative amount for, as the linker histone to nucleosome ratio is increased from one to two, an additional contraction of the fibers ensues, resulting in a 50% further increase in the overall packing ratio of the DNA (Fig. 7 *d*). This, however, need not signify a qualitative change in structural organization, because the observed changes can be readily accommodated within the framework of the solenoid model (10, 33). The average length corresponding to the maximal

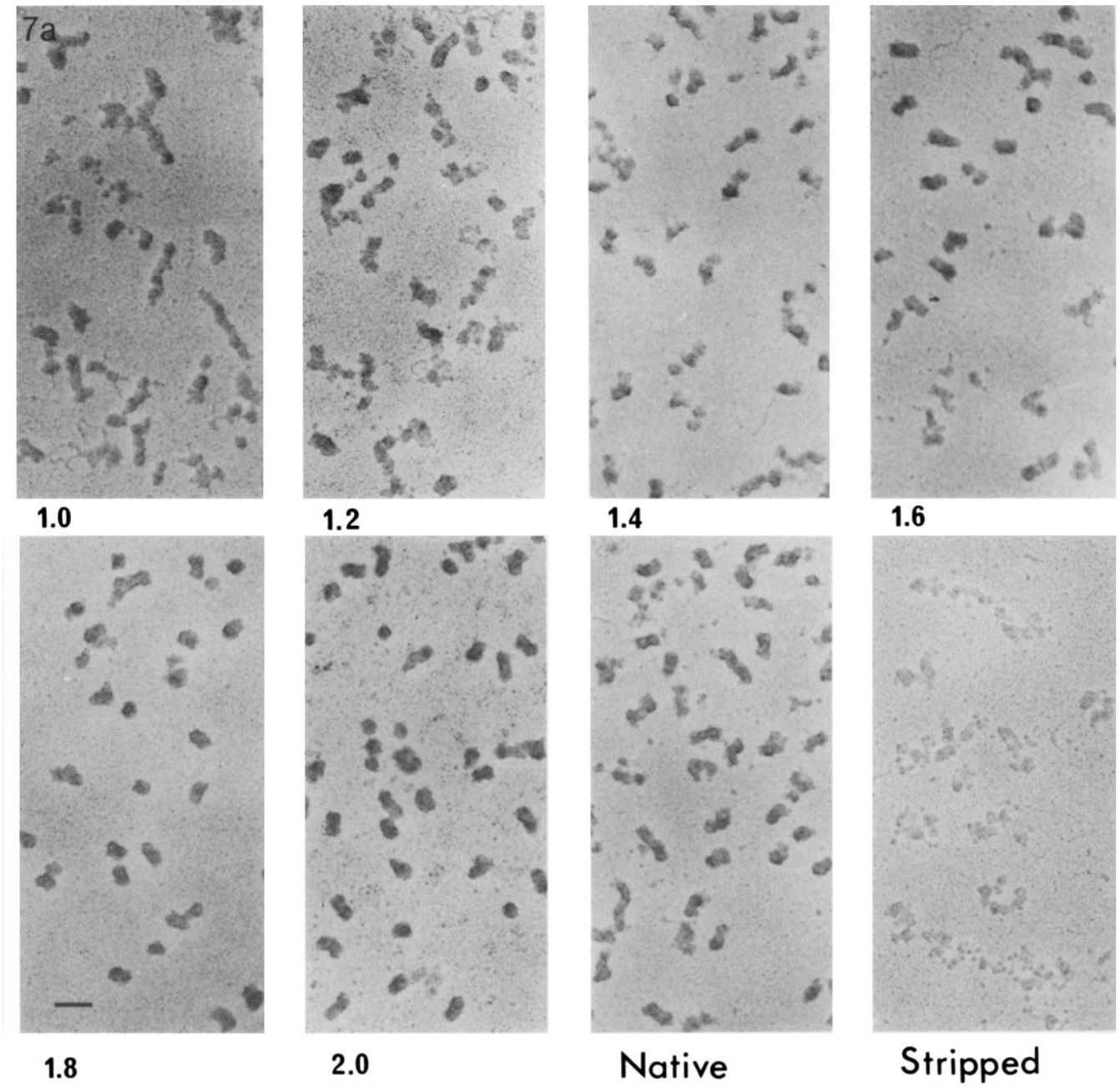
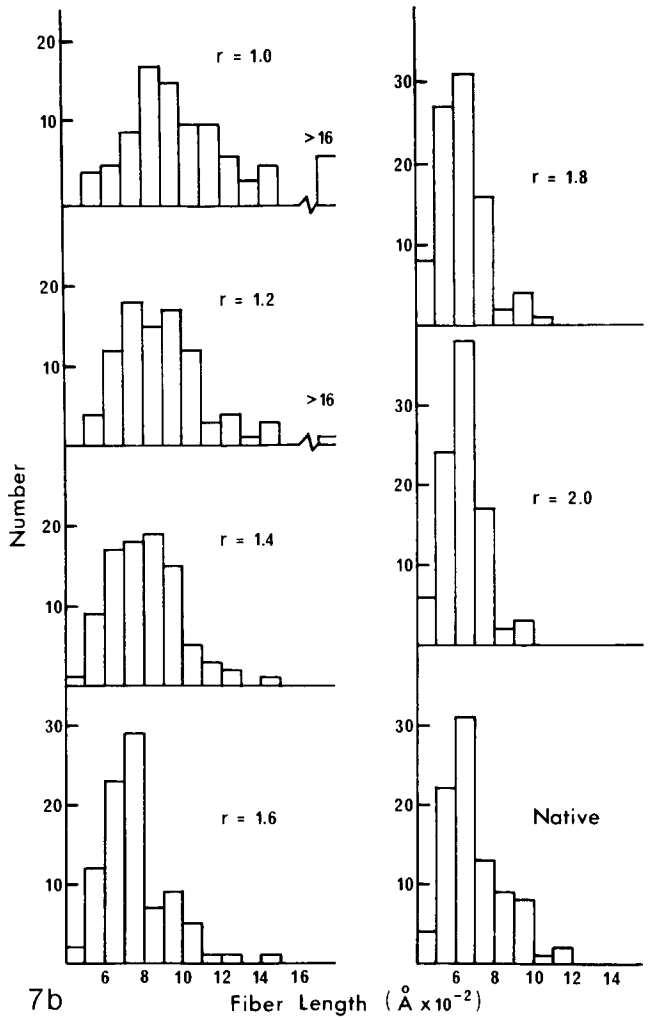
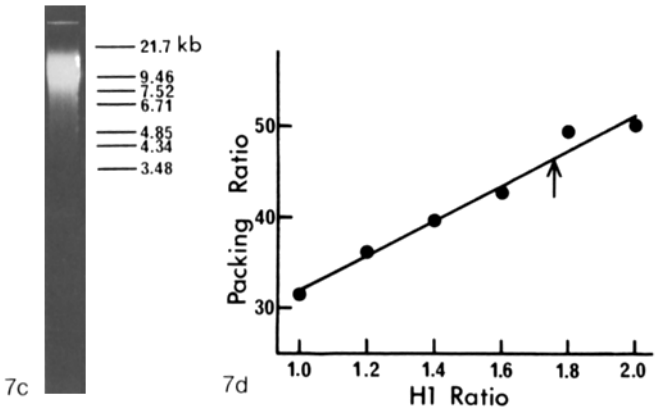


FIGURE 7 (*a*) Electron micrographs of reconstituted chromatins. Polynucleosomes of narrowly defined size (*c*) were complexed with various amounts of ceH1, fixed with glutaraldehyde in 80 mM NaCl and spread for microscopy. Reconstituted chromatins prepared at the indicated ratios of H1 relative to nucleosome cores are compared with native and stripped chromatin used for the reconstitution. (*b*) Analysis of the length distribution of reconstituted chromatin. The histogram shows the results of measurements on 90 molecules in a typical field. (*c*) Molecular weight distribution of polynucleosome DNA. The molecular weight distribution of the DNA ion the chromatin used for microscopy (see *a*) was determined by electrophoresis in an 0.8% agarose gel, here shown stained with ethidium bromide and photographed under ultraviolet illumination. Densitometry of the negative revealed that 60% of the DNA was between 8,200 and 12,000 base pairs (38–56 nucleosomes), a further 15% being larger than 5,750 base pairs (27 nucleosomes). The average DNA size was found to be 9,175 base pairs (43 nucleosomes). (*d*) Linear condensation of DNA as a function of linker histone content. The mean chromatin fiber lengths (derived from *b*) and the mean DNA length base pairs (as described in *c*) were used to determine the packing ratio of the chromatins, shown as a function of the linker histone to nucleosome ratio.

condensation attained in our system (Fig. 7 *b*, r = 2) is consistent with a regular solenoid of pitch 100 Å containing about seven nucleosomes per turn. The lower packing ratio observed with chromatin reconstituted with a single linker histone per nucleosome could be accounted for by an increase in the pitch of the solenoid to ~150 Å or a reduction in the number of nucleosomes per turn to about five, or a combination of both effects. Thoma and co-workers (33) have measured the pitch of solenoids obtained from native calf thymus chromatin under conditions similar to those used here. They estimated that each turn contains approximately six nucleosomes and observed a pitch varying between 100 and 150 Å. In the light





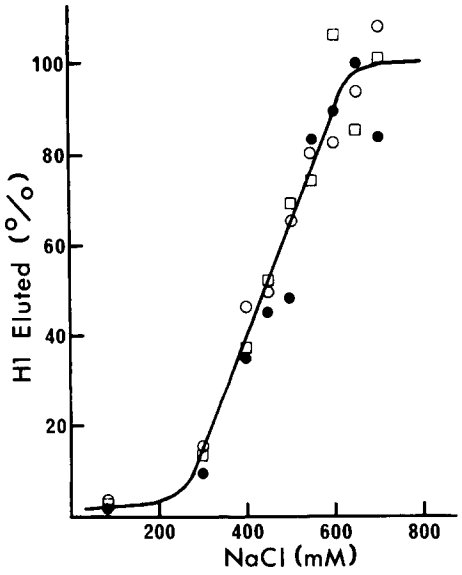


FIGURE 8 Salt-dependent dissociation of linker histones from reconstituted chromatins. Reconstituted chromatins were prepared with either one or two molecules of ^{3}H-labeled ctH1. The labeled proteins were then eluted from the chromatin by increasing the ionic strength. Free histones were separated from the nucleoprotein complex on hydroxyapatite. The fraction of H1 eluted is expressed as a percentage of the total H1 eluted by 2 M NaCl. Dissociation experiments were performed with chromatin containing one H1 per nucleosome (○) or two H1 molecules per nucleosome, added simultaneously (●), or with one labeled equivalent added after reconstitution with one unlabeled equivalent of H1 (□).

of our data, these values are expected for a solenoid containing about one molecule of linker histone per nucleosome, as is indeed found for this chromatin (15).

Regular cleavage sites extending beyond 146 base pairs of DNA have been observed previously on digestion of yeast chromatin with DNase I (18, 20) and of chicken erythrocyte chromatin with exonuclease III (31). These results suggested that the length of the spacer DNA in these chromatins was quantized and, as a result, regular bands larger than 146 base pairs were generated by cleavage within adjacent core particles. A similar interpretation for our results would require extensive sliding of nucleosomes before digestion as well as double-stranded cleavage within adjacent core particles. The results presented in Fig. 4 *A* suggest that sliding does not occur during stripping or reconstitution, and it seems unlikely that extensive sliding would occur during the brief period of digestion (30 s) required to generate the specific bands observed (Fig. 4 *B*). Furthermore, native chromatin, which has not been subjected to treatment that could induce sliding, displays a banding pattern similar to that of the reconstitutes (Fig. 4 *c*). Although micrococcal nuclease does make double-strand cuts within the core particle (8), these are not apparent, under our conditions, until late in digestion, when DNA fragments shorter than 146 base pairs begin to accumulate (Fig. 4 *B* and *C*).

We believe, therefore, that the discrete monomer-sized DNA products that are produced by micrococcal nuclease arise not from quantization of the spacer DNA and cleavage within adjacent core particles, but rather by cleavage at regularly spaced cutting sites within the spacer DNA itself. The spacer DNA must, therefore, be organized in such a way as to make it accessible to nuclease at 10.4-base-pair intervals that are in phase with the cutting sites in the core particle (21). The simplest interpretation of this observation would involve an extension of the supercoiled path of the DNA in the core particle into the spacer DNA and on to the adjacent nucleosome, a proposal that has been made in previous studies (22, 37).

It is evident from Fig. 4 that access of micrococcal nuclease to regularly spaced cutting sites in the spacer DNA is under the influence of the linker histone. Distinct, characteristic monomer DNA patterns were obtained for reconstitutes with the two species (calf and chicken) of H1 and with histone H5. Variation in the nature of monomer DNA patterns obtained after digestion of chromatins from various tissues has been

observed previously (4, 18). The results presented in this work suggest that this variation may be, at least in part, a reflection of the types of linker histones present in the chromatin of these tissues.

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