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Interactions between Core Histones and Chromatin at Physiological Ionic Strength†

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ABSTRACT: Addition of core histones to chromatin or chromatin core particles at physiological ionic strength results in soluble nucleohistone complexes when polyglutamic acid is included in the sample. The interaction between nucleosomes and added core histones is strong enough to inhibit nucleosome formation on a closed circular DNA in the same solution. Complexes consisting of core particles and core histones run as discrete nucleoprotein particles on polyacrylamide gels. Consistent with the electrophoretic properties of these particles, protein cross-linking with dimethyl suberimidate indicates that added core histones are bound as excess octamers. Histones in the excess octamers do not exchange with nucleosomal core histones at an ionic strength of 0.1 M and can be selectively removed from core particles by incubating the complexes in a solution containing sufficient DNA. Under conditions where added histones are confined to the surface of chromatin, the excess histones are mobile and can migrate onto a contiguous extension of naked DNA and form nucleosomes.

During chromatin replication, newly made histones enter the nucleus and rapidly form nucleosomes on nascent DNA (Seale, 1978; Cremisi, 1979; Laskey & Earnshaw, 1980). Although in vitro studies have shown that, under certain conditions, histones spontaneously fold DNA into nucleosomes (McGhee & Felsenfeld, 1980; Laskey & Earnshaw, 1980), it has also been found that the core histones interact rather strongly with chromatin (Voordouw & Eisenberg, 1978; Stein, 1979). Because there is a large amount of chromatin in the nucleus, relative to nascent DNA, it is of interest to know how nucleosome assembly is influenced by the presence of chromatin. It is obvious that chromatin does not inhibit nucleosome assembly in vivo but it is not really clear why this is the case (Jackson et al., 1976).

Histone–chromatin interactions have been difficult to study because the nucleohistone complexes formed are insoluble at physiological ionic strength. Added histones induce precipi-

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tation (see below) of large chromatin fragments prepared by methods which produce chromatin that is initially quite soluble at physiological ionic strength (Ruizz-Carrillo et al., 1980; Fulmer & Bloomfield, 1981). Similarly, added histones induce precipitation of purified chromatin core particles (Stein, 1979). We have found a way to solubilize such nucleohistone complexes and thus have been able to study them more easily and more thoroughly than has been previously possible. An understanding of these interactions might provide insight into the mechanism of chromatin replication.

Materials and Methods

Materials. Chicken blood was purchased from Pel-Freeze. Micrococcal nuclease (Worthington) was dissolved in water at 10 units/μL and stored frozen in small portions. A 10 mg/mL stock solution of polyglutamic acid of molecular weight 64 000 (Miles) in 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.2, was prepared by neutralization with sodium hydroxide. T4 DNA polymerase, T4 DNA ligase, and restriction enzymes as well as SV40 form I DNA and ϕX174 RF DNA were purchased from Bethesda Research Laboratories. Tritium-labeled deoxynucleoside triphosphates were purchased from Amersham, and unlabeled deoxynucleoside triphosphates were from Boehringer Mannheim. Dithiothreitol and adenosine 5′-triphosphate were from Sigma, and dimethyl suberimidate was from Pierce.

Preparation of DNA Samples. Chicken erythrocyte DNA, 2000–3000 base pairs in length, was prepared by fractionating sonicated high molecular weight DNA, extracted from nuclei as previously described (Künzler & Stein, 1983), on a Bio-Gel A 150-m column in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM disodium ethylenediaminetetraacetate (Na_2EDTA).

Blunt-end ^{3}H-labeled ϕX174 RF DNA was prepared by first incubating 20 μg of purified *Pst*I-cut ϕX174 RF DNA 10 min at 37 °C with 16 units of T4 DNA polymerase in 33 mM magnesium acetate, 5 mM dithiothreitol, and 1 mg of bovine serum albumin/mL (Maniatis et al., 1982) and then filling in using all four ^{3}H-labeled deoxynucleoside triphosphates. A 10-min chase reaction with unlabeled deoxynucleoside triphosphates (80 μM each) ensured the formation of a blunt-end product. Analysis of the relative intensities of *Hinc*II restriction fragments on a gel by fluorography indicated that the radiolabel was distributed throughout the whole molecule, although incorporation occurred to a greater extent closer to the ends, as expected. By counting a small portion of the purified ^{3}H-labeled DNA using a Beckman Model LS-250 liquid scintillation counter, we estimated the specific activity to be approximately 6×10^5 dpm/μg.

*Hae*III restriction fragments of ϕX174 RF DNA, for use as DNA size markers, were end labeled with ^{3}H-labeled deoxynucleoside triphosphates and T4 DNA polymerase (Maniatis et al., 1982).

Preparation of Core Histones and Chromatin. Core histones were prepared by salt extraction of 0.7 M NaCl washed chicken erythrocyte nuclei as previously described (Stein & Bina, 1984). Core histones were radiolabeled by reductive methylation (Rice & Means, 1971; Ruiz-Carrillo et al., 1975). Approximately 600 μg of core histones in 100 μL of 2.0 M NaCl and 10 mM sodium borate, pH 9.0, was mixed with 2.5 μL of freshly dissolved sodium borohydride (25 mg/mL in the same buffer). Five 0.5-μL additions of a 1% solution of [^{3}H]formaldehyde (28 μCi/μL, from New England Nuclear) were made at 1-min intervals at 0 °C. The sample was then dialyzed extensively against the 2.0 M NaCl buffer with several changes of the dialysate using Spectrapore dialysis tubing. All core histone samples were stored frozen in a buffer containing 2.0 or 2.5 M NaCl. Small portions were dialyzed against 0.10 M NaCl and 10 mM Tris-HCl, pH 8.0, just before use by using Millipore VSWP 02500 microdialysis filters.

Chromatin to be ligated onto blunt-end DNA was prepared by digestion of chicken erythrocyte nuclei with the restriction enzyme *Hae*III. Nuclei containing about 1 mg of DNA were gently washed several times with 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 10 mM $MgCl_2$ and then suspended in about 30 μL of this solution. The sample was digested at 37 °C for 2 h with 200 units of the restriction enzyme. Chromatin fragments were liberated from the digested nuclei by addition of 1.0 mL of 50 mM Tris-HCl, pH 7.5, and 1.0 mM Na_2EDTA and equilibration at 4 °C overnight. Insoluble material was removed by centrifugation for 1 min at 15000*g*. Typically, soluble chromatin containing about 500 μg of DNA was obtained. The sample was concentrated by $MgCl_2$ precipitation at 0 °C for 10 min by addition of $^1/_{10}$th volume of 0.1 M $MgCl_2$. The precipitate was collected by centrifugation at 1000*g* for 5 min and dissolved in a small volume of 50 mM Tris-HCl and 2–3 mM Na_2EDTA (the amount required for complete solubilization) at a DNA concentration of 5 mg/mL.

Soluble chromatin to be used in other experiments was prepared by micrococcal nuclease digestion of nuclei using conditions similar to those of Ruiz-Carrillo et al. (1980). Nuclei containing about 5 mg of DNA were gently suspended, after two washes, in 1 mL of 0.10 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM Na_2EDTA. The sample was adjusted to 2 mM $CaCl_2$ after equilibration at 37 °C and digested for 10 min with 5 units of micrococcal nuclease. The reaction was stopped by adjusting the Na_2EDTA concentration to 10 mM, and the digested nuclei were pelleted by low-speed centrifugation. The pellet was suspended in 1 mL of 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM Na_2EDTA, and the suspension was dialyzed overnight against the same solution at 4 °C. After centrifugation to clear the sample, the supernatant generally contained about 1 mg (DNA concentration) of soluble chromatin fragments with an average length of about 30 nucleosomes. Chromatin fragments prepared by either method contained intact histones including H1 and H5.

Nucleosome core particles containing 146 base pair DNA were prepared as described by Simpson (1978).

Histone Transfer and Exchange Assay. A mixture containing nucleosome core particles (200 μg of DNA/mL), core histones (200 μg/mL), and polyglutamic acid (1.0 mg/mL) was prepared from concentrated stock solutions, with each component in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 0.2 mM Na_2 EDTA; histones were added last. After a 1-h incubation at room temperature, portions containing 2 μg of DNA were removed for addition of competing DNA (2000–3000 base pairs long in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 0.2 mM Na_2 EDTA) from a concentrated stock solution. All samples were adjusted to the same volume by using the 0.1 M NaCl buffer. Samples were incubated for 2 h at room temperature with occassional agitation, diluted 2-fold with 2× sample buffer, and loaded directly on a 4% polyacrylamide gel for analysis.

Histone Cross-Linking. Nucleosome core particles containing 100 μg of DNA were mixed with either 50 or 100 μg of core histones in about 0.1 mL of a solution containing 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.2 mM Na_2EDTA, and 400 μg of polyglutamic acid. Control samples in which a component was omitted were also adjusted to these conditions. Samples were incubated 30 min at room temperature and then diluted to 2.0 mL by addition of a freshly prepared solution

containing 5 mg of dimethyl suberimidate/mL, 0.30 mg of polyglutamic acid/mL, 0.037 M NaCl, and 0.037 M sodium borate, pH 9.5 (adjusted with NaOH). After 1 h at room temperature, samples were adjusted to pH 5.5 with sodium acetate to stop the reaction and precipitated with 2.5 volumes of ethanol. The dried residues were dissolved in 1% sodium dodecyl sulfate ($NaDodSO_4$) and heated to 100 °C for 5 min before analysis on 5% polyacrylamide gels containing $NaDodSO_4$ (Weber & Osborn, 1969). Cross-linked products were identified as previously described (Stein et al., 1977).

Ligation of Core Histone–Chromatin Complexes to DNA. Approximately 10 μg of chromatin, obtained by digestion of nuclei with *Hae*III as described above, was preincubated 10 min with 5 μg of core histones in 50 mM Tris–HCl, pH 7.5, 2 mM Na_2EDTA, and 1 mg of polyglutamic acid/mL and then adjusted to contain, in addition, 14 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM ATP, and 3 units of T4 DNA ligase in a final volume of 20 μL. The sample was incubated 2 h at room temperature with occasional agitation. A control sample not containing T4 DNA ligase was incubated at otherwise identical conditions. For micrococcal nuclease digestion, samples were dialyzed against 20 mM Tris-HCl, pH 7.2, and 0.2 mM Na_2EDTA using microdialysis filters (Millipore VSWP 02500). Each filter was rinsed several times with dialysate to fully recover the sample, giving a final volume of 100 μL. Samples were then digested at 37 °C with 20 units of micrococcal nuclease for 3 min, after addition of 0.1 M $CaCl_2$ to a concentration of 1 mM. Digestion was stopped by adjusting samples to 10 mM Na_2EDTA and 1% $NaDodSO_4$. Samples were then phenol extracted and ethanol precipitated, and the DNA was analyzed on a 5% polyacrylamide gel in Tris–borate buffer (TBE) (Peacock & Dingman, 1967). DNA purified from undigested samples was analyzed on a 1% agarose gel (Tris–acetate buffer). Gels were stained with ethidium bromide, photographed under UV illumination, and then soaked for 30 min in 500 mL of Fluoro-Hance (RPI) and dried for fluorography. Fluorograms were prepared by using Kodak XAR X-ray film and an intensifying screen (Du Pont Cronex, Lighting-Plus); exposures were 1 to 3 days at −80 °C.

Other Procedures. The supercoiling assay for nucleosome formation was performed with relaxed SV40 DNA (SV40 form Ir). This DNA (0.5 μg in 0.1 mL) was incubated at 37 °C with core histones and chromatin fragments or nucleosome core particles in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1.0 mM Na_2EDTA, 1.0 mg of polyglutamic acid/mL, and 1 μL of a topisomerase I extract from HeLa cells, prepared according to Germond et al. (1975). Reactions were stopped by adjusting the samples to 1% $NaDodSO_4$. The DNA was purified by extraction with phenol and analyzed on a 1% agarose gel.

Sucrose gradients isokinetic for particle densities of 1.51 g/cm^3 at 4 °C, C_m = 5%, were prepared according to McCarty et al. (1974) in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM Na_2EDTA, and samples were sedimented by using an SW41 rotor. Gradients were tapped, and the absorbance at 258 nm was monitored by using an ISCO density gradient fractionator connected to a Pharmacia UV-I monitor. Histone to DNA ratios were measured spectroscopically as previously described (Stein, 1979). Photographic negatives of gels were scanned on an E-C Apparatus densitometer.

Results

System for Studying Histone–Chromatin Interactions in Solution. When core histones are mixed with chromatin fragments (predominately 20–40 nucleosomes long) that are initially soluble in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1.0 mM Na_2EDTA, precipitation occurs. Figure 1 (solid curve) shows that more than half of the chromatin becomes insoluble at 0.1 equiv (octameric unit of two each of the four core histones) per nucleosome. At greater than 0.2 equiv of histones added per nucleosome, more than 90% of the nucleoprotein becomes insoluble. Thus, the addition of even small amounts of core histones greatly decreases the solubility of a chromatin sample that was initially soluble even at a 10-fold higher concentration than used here (not shown). The nature of the interactions between core histones and chromatin in the aggregated insoluble material is difficult to determine.

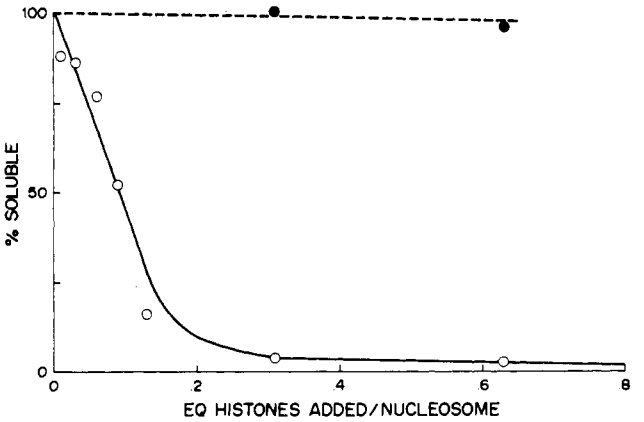

FIGURE 1: Effect of polyglutamic acid on the solubility of core histone–chromatin complexes in 0.1 M salt. Increasing amounts of core histones were added to soluble chromatin fragments (2 A_{260} units/mL) in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1.0 mM Na_2EDTA in the absence (solid curve) or presence (broken curve) of polyglutamic acid (1.0 mg/mL). After a 5-min incubation at room temperature, samples were centrifuged 2 min in a microfuge, and the fraction of soluble chromatin was determined from the absorbance of the supernatant at 260 nm.

During our studies of chromatin assembly using polyglutamic acid (Künzler, & Stein, 1983), we observed that in the presence of this acidic polypeptide chromatin remains soluble upon addition of core histones to the solution, as shown by the broken curve in Figure 1. A trivial explanation for this behavior would be that core histones, under these conditions, no longer interact with the chromatin. We show below, however, that the core histones do interact quite strongly with chromatin, as well as with chromatin core particles.

It is known that in the presence of polyglutamic acid core histones efficiently fold DNA into nucleosomes at physiological ionic strength (Stein et al., 1979). The supercoiling of a relaxed closed circular DNA provides a convenient assay for this reaction. The number of superhelical turns induced in the initially relaxed DNA, after incubation with histones and topoisomerase I and subsequent deproteinization, is an approximate measure of the number of nucleosomes that had formed (Germond et al., 1975). Using this assay, we examined whether premixing histones with chromatin core particles in a buffer containing 0.1 M NaCl an 1 mg of polyglutamic acid/mL would result in inhibition of nucleosome formation on a relaxed closed circular DNA.

Figure 2 shows that premixing histones with chromatin core particles (lane 2) results in a significantly lower extent of nucleosome formation on the circular DNA than in the control sample (lane 1), where the histones were mixed with the DNA *before* addition of chromatin. Final compositions of the two samples were identical. The 146 base pair core particle DNA has a much greater mobility than the DNA topoisomers and does not appear on the gel. Inhibition of assembly even occurs when histones are added to a mixture of chromatin core particles and circular DNA, lanes 3–5, although greater

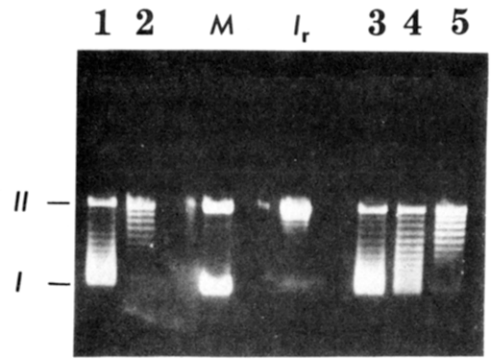

FIGURE 2: Inhibition of nucleosome assembly on relaxed closed circular DNA by chromatin core particles in solution. In lane 1, 0.38 μg of core histones was mixed with 0.50 μg of DNA in a solution containing polyglutamic acid, and then the sample was incubated with 0.75 μg of core particles and topoisomerase I for 45 min. In lane 2, the core histones were premixed with the core particles in a solution containing polyglutamic acid, and the sample was then incubated with DNA under the same conditions as for lane 1. In lanes 3–5, 0.38 μg of core histones was added to mixtures containing 0.50 μg of DNA, polyglutamic acid, and topoisomerase I and 0, 4, or 40 μg (DNA content) of core particles, respectively, and the samples were incubated 45 min. Lane M contained a mixture of DNA forms I (native supercoiled) and II (nicked circles) to serve as markers. Lane I_r (relaxed closed circular) shows the DNA starting material used in this experiment. The labels I and II at the left identify the bands corresponding to DNA forms I and II, respectively, in lane M.

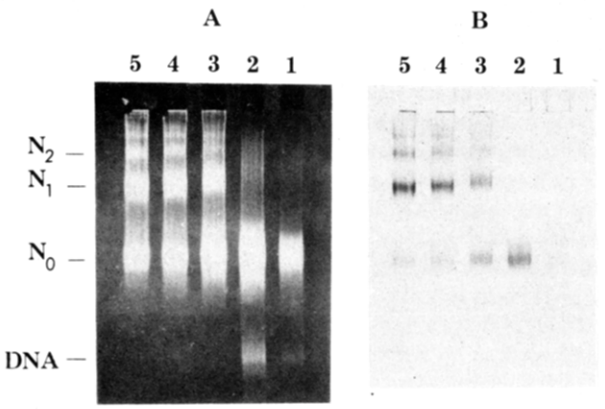

FIGURE 3: Electrophoresis of nucleohistone particles. Core particles were mixed with 0, 1, 2, or 3 equiv of core histones, lanes 2, 3, 4, and 5, respectively, in the presence of polyglutamic acid and analyzed on a 4% polyacrylamide gel as described under Materials and Methods. Lane 1 shows the mobility of core particles loaded in 0.1 × TBE buffer in the absence of polyglutamic acid. Band N_0 denoted core particles; bands N_1 and N_2 denote the other nucleoprotein particles observed, in order of decreasing electrophoretic mobility. The position of naked core particle DNA is also indicated. (A) DNA stained with ethidium bromide. (B) Protein stained with Coomassie blue.

amounts of core particles are required to inhibit the reaction compared to the premixing experiment. Additionally, we observed similar inhibition using soluble chromatin fragments in place of core particles (data not shown). These results suggest that nucleosomes can interact strongly with histones, with all components in solution.

Nature of Core Histone–Nucleosome Interactions in Solutions Containing Polyglutamic Acid. We next examined whether discrete soluble nucleoprotein complexes are formed when core histones are added to nucleosome core particles. Mixtures were prepared containing increasing amounts of core histones per nucleosome in a buffer containing 0.1 M NaCl and 1 mg of polyglutamic acid per mL. The samples were then analyzed on a 4% polyacrylamide gel, shown in Figure 3A (DNA stained wiith ethidium bromide). Lane 1 was loaded with a sample of untreated nucleosome core particles in a low

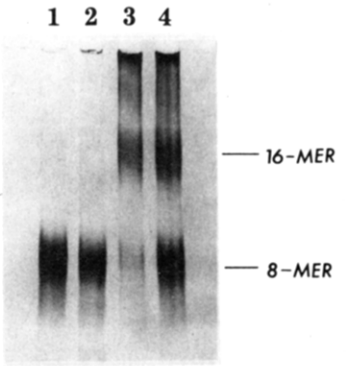

FIGURE 4: Cross-linking of histones in core histone–core particle complexes. Electrophoresis on a 5% polyacrylamide–$NaDodSO_4$ gel of cross-linked histones extracted from complexes formed from core particles and 1.0 (lane 3) or 0.5 (lane 4) equiv of added core histones. Cross-linked histones from core particles alone (lane 1) or core histones alone (lane 2) are also shown. Polyglutamic acid was present in all samples. The positions of octamers and 16-mers are indicated.

ionic strength solution. Lane 2 shows nucleosome core particles that were loaded in the 0.1 M NaCl and polyglutamic acid containing buffer. Under these conditions, the electrophoretic mobility of the core particle (labeled N_0 for the nucleoprotein particle containing zero equivalents of added core histones) is not altered, and only a small amount of DNA is dissociated from the particle by the electric field. With increasing amounts of core histones added, an increasing fraction of the DNA appears in discrete nucleoprotein complexes with lower mobilities than core particles, suggesting that several new types of particles formed. Two of these bands are here labeled N_1 and N_2, respectively, in order of decreasing electrophoretic mobility for the sake of identification. The identities of these complexes will be inferred from additional experiments, presented below. When polyglutamic acid is omitted from samples containing added histones, discrete bands are not observed at all (not shown).

The same gel, stained for protein with Coomassie blue, is shown in Figure 3B. It is apparent that the low-mobility complexes stain more intensely for protein than do nucleosome core particles for equal DNA-staining intensities. For example, compare bands N_0 and N_1 of lane 4 for the two stains. This result suggests that the discrete complexes formed have higher histone to DNA ratios than core particles.

To further study these apparent histone–nucleosome core particle complexes, we subjected soluble (in the presence of polyglutamic acid) mixtures to protein cross-linkng with dimethyl suberimidate. Figure 4 shows the mobilities of the resultant cross-linked proteins on a $NaDodSO_4$-containing gel. Lane 1 shows that for nucleosomes alone, the histones cross-linked into octamers. Lane 2 shows that core histones alone, under these conditions, also cross-linked into octamers, as expected (Stein et al., 1979). Lane 3 shows that a mixture of core particles plus 1 equiv of core histones cross-links predominantly into 16-mers and higher aggregates, whereas lane 4 shows that a mixture of core particles plus 0.5 equiv of core histones cross-links predominantly into octamers and 16-mers. The large amount of 16-mers formed in lane 4 and the absence of complexes intermediate between 8- and 16-mers suggest that histones interact strongly with nucleosomes as excess octamers.

To demonstrate that the 16-mers arose from a complex of a nucleosome with an excess octamer, rather than from a two-nucleosome aggregate, we prepared a cross-linked sample as in Figure 4, lane 4, and fractionated the sample by sucrose gradient sedimentation. The gradient profile exhibited a peak

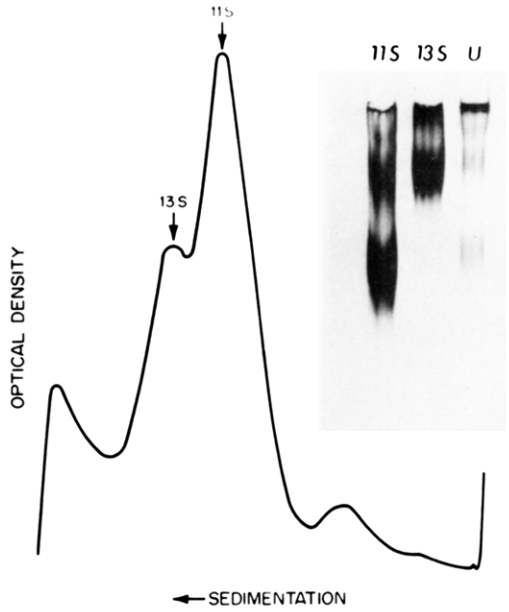

FIGURE 5: Fractionation of cross-linked complexes. Sucrose gradient profile of cross-linked nucleoprotein particles resulting from treating a mixture of core particles and 0.5 equiv of added core histones with dimethyl suberimidate in the presence of polyglutamic acid. The inset shows the cross-linked histones extracted from an unfractionated portion of the sample (lane U) and from the 11S peak and the 13S peak on a 5% polyacrylamide–$NaDodSO_4$ gel.

at approximately 11 S and one at 13 S (Figure 5). The molecular weights of the (cross-linked) histones contained in fractions from each peak are shown in the inset. The 11S fraction contained predominantly octamers, whereas the 13S fraction contained predominantly 16-mers with a small amount of higher molecular weight material. We measured the protein to DNA ratios for fractions taken from the center of each peak. The 11S fraction had a protein to DNA ratio of 1.2 ± 0.1, the value expected for nucleosome core particles. The 13S fraction had a ratio of 3.5 ± 0.1, consistent with nucleosomes containing excess octamers. Also, it can be seen that whereas the octamer and 16-mer bands of the unfractionated sample (lane U) are of nearly equal intensity, the area under the 13S peak in the sucrose gradient profile, which monitors DNA, is very nearly half that in the 11S peak. Therefore, these experiments indicate that the 13S particle is a nucleosome with an excess (cross-linked) histone octamer. Moreover, the results obtained by cross-linking are entirely consistent with the electrophoretic properties of un-cross-linked nucleoprotein particles and suggest that bands N_1 and N_2 of Figure 3 correspond to core particles complexed with one and two excess octamers, respectively.

Transfer of Excess Octamers from Nucleosomes to DNA. Excess octamers of nucleosome–octamer complexes appear to remain associated with nucleosomes when the concentration of DNA added to the solution is low (Figure 2). Here, we examine whether excess octamers can be transferred to DNA, when complexes are incubated with higher relative concentrations of DNA. Mixtures of nucleosome core particles and 2 equiv of core histones were preincubated, as in Figure 3, and then incubated with increasing amounts of chicken erythrocyte DNA. The resultant nucleoprotein complexes were analyzed on a polyacrylamide gel, shown in Figure 6. In the control sample, lane 1, incubated without DNA, the octamer–nucleosome complex (N_1) is the predominant particle observed. With increasing amounts of high molecular weight DNA added, the intensity of N_1 decreases, and N_0 (nucleosome core particles) concomitantly increases. Thus, the results are

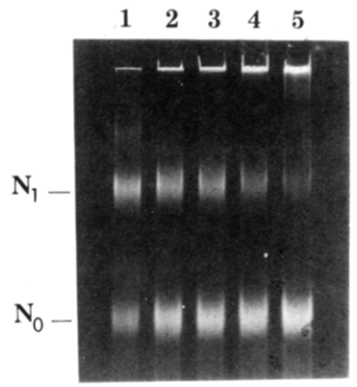

FIGURE 6: Transfer of excess octamers from core particles to DNA. Portions of a sample containing core histone–core particle complexes (containing 2 μg of DNA each) were incubated with 0, 2, 4, 10, or 20 μg of higher (than core particle size) molecular weight DNA, and the nucleoprotein samples were run on a 4% polyacrylamide gel. N_0 denotes core particles, and N_1 denotes complexes of core particles with excess histone octamer. Reaction conditions are described under Materials and Methods.

consistent with the transfer of excess octamers from octamer–nucleosome complexes to high molecular weight DNA. Upon removal of the excess octamer, ordinary nucleosomes are formed which have the mobility N_0. The high molecular weight DNA and also complexes of histones with this DNA do not enter the gel appreciably.

This experiment suggests that excess octamers are more weakly bound than the core octamer in nucleosomes. Nucleosomes incubated with a 10–fold weight excess of DNA, under the conditions used in Figure 6, do not release their octamers (data not shown). Therefore, the two octamers in the nucleoprotein complex N_1 are not equivalent. This point is examined more thoroughly below.

Exchange of Histones in Excess Octamers with Nucleosomal Histones. At ionic strengths in the physiological range, the core histones in chromatin do not appear to exchange appreciably between one nucleosome and another (Cremisi & Yaniv, 1980; Louters & Chalkley, 1984). However, histones in excess octamers might be able to exchange more readily with nucleosomal histones in the same nucleoprotein particle. Such intraparticle exchange seems particularly plausible for H2A and H2B since these histones are less tightly bound in the nucleosome (Ohlenbusch et al., 1967; Burton et al., 1978).

To assay for possible intraparticle histone exchange, we performed an experiment similar to the one shown in Figure 6, except that ^3H-labeled core histones were mixed with unlabeled nucleosome core particles, forming complexes, and the complexes were incubated in a buffer containing 0.1 M NaCl for 1 h at room temperature. High molecular weight DNA (a 5-fold weight excess) was then added, and the mixture was incubated for an additional hour. If intraparticle exchange occurred, the nucleosomes released from the complex should now contain some labeled histones, detectable by fluorography.

This experiment is shown in Figure 7. The ethidium bromide stained gel (A) shows that almost all of the nucleosomal DNA was complexed with excess octamers upon incubation with ^3H-labeled core histones (lane 1). Subsequent removal of excess histones is evidenced by the substantial increase in the intensity of band N_0 (core particles). The fluorogram shown in panel B shows that the complexes N_1, N_2, and N_3 (lane 1) contained progressively more label per unit amount of DNA, consistent with the binding of one, two, and three excess octamers, respectively. Lane 2 shows that the nucleosomes released from the particles after removal of excess histones (band N_0) did not incorporate label. Thus,

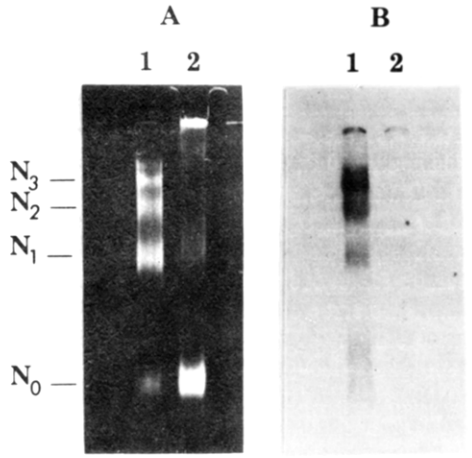

FIGURE 7: Assay for exchange of histones in excess octamers into nucleosome core particles. Complexes of core particles with about 3 equiv of added ^{3}H-labeled core histones (lane 1) were incubated with DNA (5 times the weight in core particles) to remove excess octamers from the core particles (lane 2). (A) Ethidium bromide stained gel; (B) fluorogram. N_1–N_3 denotes particles containing one to three excess histone octamers, respectively; N_0 denotes core particles.

intraparticle exchange did not occur under the conditions used in these experiments. Moreover, the result of this experiment clearly confirms the nonequivalence of excess and nucleosomal histones.

When we performed similar experiments at 0.3 M NaCl, we did observe some histone change. Additionally, under these conditions, labeled histones became incorporated into leftover uncomplexed nucleosomes (in the absence of added DNA), consistent with the transfer of excess octamers between nucleosomes, in addition to intraparticle histone exchange (data not shown).

Migration of Excess Octamers over the Surface of Chromatin. We have shown that in 0.1 M NaCl interactions between histones and chromatin fragments are substantial and can inhibit the deposition of these histones onto DNA that has been added to the solution (Figure 2). Of course, at a replication fork, nascent DNA is contiguous with chromatin. Therefore, it is of interest to know whether excess octamers on chromatin could migrate over the chromatin surface onto a contiguous length of DNA under conditions where octamers cannot dissociate from the chromatin molecule.

To test this idea, we have ligated ^{3}H-labeled DNA onto chromatin fragments that were preincubated with core histones in the presence of polyglutamic acid. The efficiency of nucleosome formation on the ^{3}H-labeled DNA, relative to appropriate controls, was then assessed by digesting the sample with micrococcal nuclease and measuring the amount of protected 146 base pair DNA produced by fluorography.

In a preliminary control experiment, we prepared samples containing increasing amounts of core histones (0, 2, or 5 μg) and 10 μg (DNA content) each of chromatin fragments of average length about 25 nucleosomes (but with sizes ranging from about 3 to 80 nucleosomes). We then added 1 μg of linearized ^{3}H-labeled ϕX174 RF DNA (about 5000 base pairs) to each sample and incubated the samples 2 h at room temperature under our ligation conditions, but in the absence of DNA ligase. The samples were then digested, fairly extensively, with micrococcal nuclease, and the DNA fragments were run on a 5% polyacrylamide gel. The results of ethidium bromide staining are shown in Figure 8A, lanes 1–3. In lane 4, only 0.5 μg of core histones was premixed with ^{3}H-labeled DNA *before* 10 μg of chromatin was added; the sample was then treated in the same way as the others.

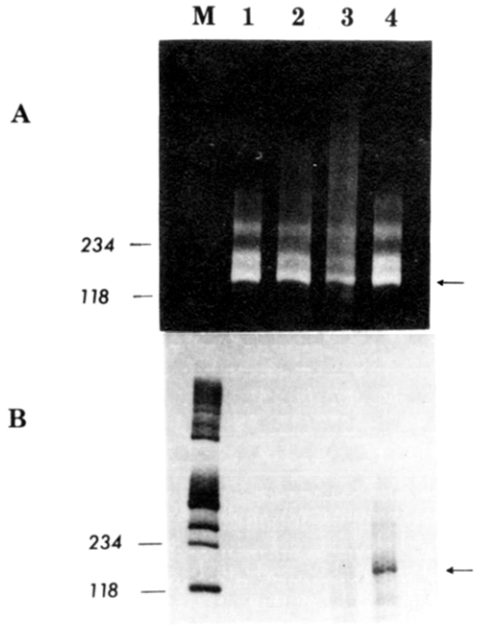

FIGURE 8: Micrococcal nuclease digestion of samples containing chromatin–core histone complexes and unligated ^{3}H-labeled DNA. Samples in lanes 1–3 contained chromatin first complexed with 0, 0.2, or 0.5 equiv of excess core histones per nucleosome and then incubated with ^{3}H-labeled DNA. Lane 4 contained 0.05 equiv of excess core histones per nucleosome, but the histones were premixed with the ^{3}H-labeled DNA before addition of the chromatin. All samples contained polyglutamic acid. (A) Ethidium bromide stained gel; (B) fluorogram. Lane M contained DNA fragments from a *Hae*III digestion of RF ϕX174 DNA; sizes of two fragments in base pairs (bp) are indicated. The arrow denotes the position of core particle length DNA (145 bp).

Figure 8A shows that the chromatin was digested predominantly to mononucleosomes in all cases; lanes 2 and 3 show that the chromatin samples containing excess histones were somewhat more resistant to digestion. The fate of ^{3}H-labeled DNA is shown in the fluorogram of the gel (Figure 8B). Whereas a strong 145 base pair band was produced for the sample where histones were added to the DNA (lane 4), the labeled DNA was digested to considerably smaller fragments in the other samples, under the same conditions, even when 10 times the amount of histones (as used in lane 4) was added to the chromatin. Milder nuclease digestions produced a series of bands on the fluorogram in all samples, arising apparently from preferential micrococcal nuclease cutting sites on the *ϕX174* RF DNA (Hörz & Altenburger, 1981; Dingwall et al., 1981). This control experiment shows that nucleosome formation on the ^{3}H-labeled DNA can easily be detected and that excess histones remain on the surface of the chromatin, under these conditions.

We next prepared a sample by adding 5.0 μg of core histones to 10 μg of chromatin, as in Figure 8 lane 3, but DNA ligase was included during the incubation. Additionally, we prepared a control sample to which DNA ligase was added but which did not contain excess histones. To assess the extent of DNA–chromatin ligation, the DNA from portions of each sample and an unligated control (lane 1) was run on a 1% agarose gel; the fluorogram is shown in Figure 9A. Since the same amount of ^{3}H-labeled DNA was applied to each lane, the decrease in the band intensity at 5000 base pairs and the corresponding appearance of higher molecular weight labeled DNA indicate that DNA–chromatin ligation occurred. DNA–DNA ligation (which did not occur) results only in the appearance of discrete bands that are multiples of 5000 base pairs (not shown). The small amount of material found in

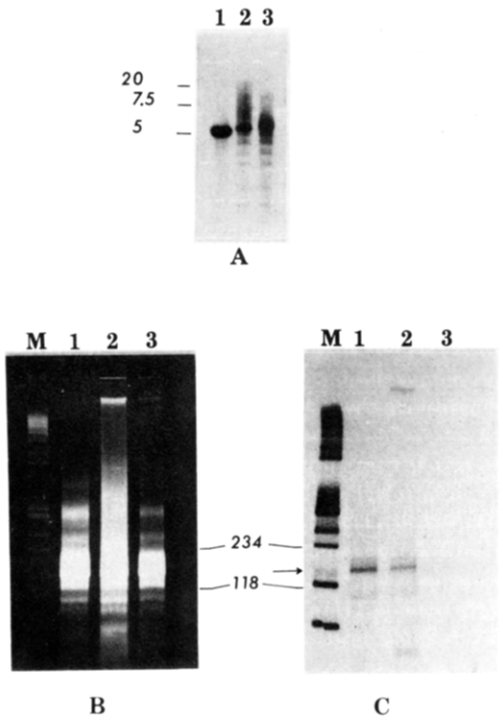

FIGURE 9: Analysis of ^{3}H-labeled DNA ligated onto chromatin or core histone–chromatin complexes. (A) Fluorogram of a 1% agarose gel used to measure the fraction of ^{3}H-labeled DNA ligated to chromatin samples. Lane 1 contained DNA extracted from a mixture of chromatin–core histone complexes and ^{3}H-labeled DNA not incubated with DNA ligase. Lane 2 contained DNA extracted from a sample prepared as in lane 1 but which was incubated with DNA ligase. Lane 3 contained DNA extracted from a mixture of a chromatin sample, without excess histones, and ^{3}H-labeled DNA that was incubated with DNA ligase. The DNA size calibration (kilobase pairs) was determined from unlabeled restriction fragments run on the same gel. (B) Micrococcal nuclease digestion of ligated samples; ethidium bromide stained gel. Lane 1 contained nuclease-resistant DNA from a reference sample prepared as in Figure 8, lane 4. Lane 2 contained nuclease-resistant DNA from the sample described in lane 2 of panel A, and lane 3 contained nuclease-resistant DNA from the sample described in lane 3 of panel A. (C) Fluorogram of the gel shown in (B). All samples contained polyglutamic acid. Lane M contained DNA fragments from a *Hae*III digestion of RF ϕX174 DNA; the sizes of two fragments in base pairs (bp) are indicated. The arrow indicates the position of (145 bp) core particle DNA.

bands of lower molecular weight than 5000 base pairs appears to arise from contaminating nuclease activity. By densitometry of a less exposed film, we estimate that about 70% of the DNA molecules ligated to chromatin fragments in the sample which contained excess histones (lane 2). Ligation is also apparent in the control sample (lane 3), but the extent is difficult to estimate due to the lower molecular weight of the resultant DNA produced.

The remaining portions of samples 2 and 3, in addition to a control sample prepared by directly adding 0.5 μg of core histones to 1.0 μg of ^{3}H-labeled DNA, as in Figure 8, lane 4, were digested with micrococcal nuclease. The ethidium bromide stained gel (Figure 9B) shows that all samples were digested primarily to mononucleosomes. The fluorogram (Figure 9C) shows that a 145 base pair band is present for the ligated sample that contained chromatin with excess histones (lane 2), but not in the control (lane 3). By densitometry, we estimate that the 145 base pair band in lane 2 is about 37% as intense as in the direct histone addition control sample (lane 1).

From the data in Figure 9, we can roughly estimate what fraction of the octamers bound to the surface of a chromatin fragment migrated onto contiguous DNA. Figure 9A (lane 2) indicates that the ^{3}H-labeled DNA was ligated onto chromatin fragments containing an average length of about 5000 base pairs. The distribution of chromatin lengths is broad, ranging from several hundred up to more than 10000 base pairs (not shown). Since the chromatin fragments contained, on the average, 0.5 μg of excess histones per microgram of DNA, complete transfer of these histones onto the 5000 base pair DNA should result in an average histone to DNA ratio (on the labeled DNA) of 0.5 (w/w). Taking into account that only about 70% of the labeled DNA molecules were ligated onto chromatin fragments (Figure 9A, lane 2), complete octamer migration should result in approximately 70% as much labeled 145 base pair protected labeled DNA as in the sample to which histones were directly added at a weight ratio of 0.5 (Figure 8B, lane 4, and Figure 9C, lane 1). The observed value, 37% (Figure 9C, lane 2), indicates that approximately half of the octamers migrated over the surface of the chromatin onto a contiguous piece of DNA. Using lower values for the fraction of DNA ligated to chromatin, or for the size of the ligated chromatin, would give a higher fraction of migrating octamers.

Discussion

The reasons for the observed solubility properties of chromatin are poorly understood. Moreover, the solubility behavior is complex. For example, it is well-known that mechanically sheared chromatin, solubilized at low ionic strength, is extremely insoluble at physiolgocial ionic strength (Zubay & Doty, 1959). Also, chicken erythrocyte chromatin prepared by nuclease digestion of nuclei and subsequent lysis at low ionic strength behaves similarly, at least for most of the material (Fulmer & Bloomfield, 1981; A. Stein, K. Holley, J. Zeliff, and T. Townsend, unpublished observations). In contrast, chromatin fragments prepared by nuclease digestion of nuclei and lysis at near-physiological ionic strengths are quite soluble at physiological ionic strength (in the absence of divalent ions) (Ruiz-Carrilo et al., 1980). The differences between soluble and insoluble chromatin fragments must be subtle because the protein compositions and gross structures of these macromolecules appear to be indistinugishable (Fulmer & Bloomfield, 1982; Ausio et al., 1984).

The precipitation of *soluble* high molecular weight chromatin or core particles (in the absence of polyglutamic acid) by added histones might result rom an increased charge neutralization of DNA phosphates. We do not know the mechanism by which polyglutamic acid solubilizes complexes of nucleosomes and excess histones. Possibly the polyanion forms ion atmospheres (in the Debye–Hückel sense) around areas of histone positive charge, thereby lessening the additional DNA charge neutralization. Alternatively, perturbation of the counterion or hydration layers of chromatin or histones by polyglutamic acid may be responsible for solubilization. As discussed above for chromatin fragments alone, the changes involved in solubilizing nucleohistone complexes may be subtle.

We have shown that the core histones interact with nucleosomes as excess octamers. This interaction is very similar to what has been observed using somewhat elevated salt concentrations (0.5–0.6 M) to solubilize those nucleohistone complexes in the absence of polyglutamic acid (Voordouw & Eisenberg, 1978; Stein, 1979). Because similar interactions occur at 0.1 and 0.6 M salt concentrations, such interactions should be expected to occur at the ionic strength that exists in the cell nucleus, a value which is not precisely known.

The prepackaging of nascent core histones into octamers in the cell nucleus, if it occurs, could serve to facilitate chromatin assembly if the octamers could be efficiently

transferred to newly synthesized DNA. On the other hand, the high affinity of excess octamers for nucleosomes might be expected to inhibit chromatin assembly, as we showed here for a simple in vitro system containing chromatin, histones, and closed circular DNA (and polyglutamic acid). These two experimental findings, however, do not lead to a conflict if octamers on the surface of chromatin are mobile and can migrate onto a contiguous stretch of DNA such as that which exists at a replication fork. Here, we have shown that such octamer migration can occur in vitro, and therefore, this model (Stein, 1979) is at least a plausible one.

Under the conditions used in most of our experiments (ionic strength close to 0.10 M), exchange between the histones in excess octamers and the histones in nucleosomes did not occur (Figure 7). Recently, Louters & Chalkley (1984) studied the exchange of histones added to chromatin over a range of ionic strengths using other methods. They found that approximately 10% of H2A and H2B exchanged into nucleosomes in chromatin at 0.1 M salt (in 25 min, 25 °C) and that the value increased to about 25% at 0.3 M salt concentrations.

We do not think that our findings are in conflict with this study because, for one thing, polyglutamic acid was present in our system. This acidic polypeptide stabilizes histone octamers (Stein et al., 1979). Despite this difference in conditions, at 0.3 M NaCl, we did detect exchange between added core histones and those of nucleosomes. Additionally, they found that exchange was about 3-fold less efficient in mononucleosomes (the system that we examined) than in chromatin. Moreover, the finding by Louters & Chalkley (1984) that exchange of added H2A and H2B into nucleosomes requires the presence of H3 and H4 is very consistent with our finding here that added histones interact with nucleosomes as excess octamers.

Because of the possible exchange of histones H2A and H2B in excess octamers with histones in nucleosomes under certain conditions, our migrating octamer model for chromatin assembly is not inconsistent with the observation that some of the newly synthesized H2A and H2B do not incorporate into nascent nucleosomes at the replication fork (Senshu et al., 1978; Worcel et al., 1978; Jackson & Chalkley, 1981; Annunziato et al., 1982). Thus, a fraction of the newly synthesized H2A and H2B could be lost by exchange as histone octamers migrate over the surface of chromatin. The fraction of excess octamer H2A and H2B that exchanges in vitro appears to be very sensitive to the ionic strength (Louters & Chalkley, 1984; A. Stein, K. Holley, J. Zeliff, and T. Townsend, Unpublished observations). This sensitivity might possibly be a contributing factor to the variability reported in different chromatin replication studies (Leffak et al., 1977; Senshu et al., 1978; Worcel et al., 1978; Jackson & Chalkley, 1981; Annunziato et al., 1982; Leffak, 1984).

Finally, our model does not require that newly made histones remain continually in contact with the surface of chromatin until deposition. It simply provides a mechanism, consistent with in vitro studies, whereby histone–chromatin interactions might facilitate, rather than inhibit, chromatin assembly.

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Registry No. Poly(glutamic acid), 25513-46-6; poly(glutamic acid), SRU, 24991-23-9.

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