Distribution of H1 histone in chromatin digested by micrococcal nuclease

Jim W. Gaubatz and Roger Chalkley

Department of Biochemistry, School of Medicine, University of Iowa, Iowa City, IA 52242, USA

Received 8 June 1977

ABSTRACT

The relative amount of H1 histone associated with isolated nucleosomes from calf thymus was determined as a function of the extent of DNA digestion by micrococcal nuclease. Generally the amount of H1 histone associated with mononucleosomes decreases with increasing digestion until 60% of the original H1 remains associated with DNA 150 base pairs or less in size. Coincidentally, H1 histone increases relative to the other histones in aggregated material that sediments through sucrose gradients to form a pellet. However, the level of H1 histone remains at control values for oligonucleosomes (dimer to hexamer) over the 30% digestion range studied. An increase in ionic strength to 0.3 M NaCl in the density gradient reveals a different pattern of H1 binding, whereby the amount of H1 reflects the average size of the DNA fragments with which it is associated. Although there is significant binding to nucleosomes *per se*, it appears that the major ionic involvement of H1 is with internucleosomal spacer DNA.

INTRODUCTION

During the past several years, physical and biochemical data have accumulated (1,2,3) substantiating earlier work which indicated chromatin is comprised of a repeating substructure (4,5). Nuclease digestion of chromatin from higher eukaryotes yields a series of nucleoprotein particles in which DNA fragments correspond to multiples of 180-200 base pairs (1,6,7). DNA fragments of 140-150 base pairs represent the most nuclease-resistant portion of the repeating unit (7,8). It is generally thought that 140 base pairs of DNA envelop non-H1 histones to form the nucleosome (3,7,9). The nucleosomes are connected by regions of spacer DNA, 40-60 base pairs in length, that are relatively more nuclease-sensitive (10). Several models of chromatin structure propose that the nuclease-sensitive, spacer DNA is the site for H1 histone binding (11,12,13,14).

Nucleosomes can be isolated by density gradient sedimentation (1,15) or gel filtration (7,16). There is considerable disagreement over whether or not H1 histone is present in isolated mononucleosomes. Several investigators report little or no H1 histone is recovered with mononucleosomes (7,16,17,18). Other studies indicate that H1 histone is found with isolated mononucleosomes

although its relative content is lower, and it is associated with a 180-200 base pair DNA fragment and not the 140 base pair core (10,11,19). Thus it is considered that digestion of spacer-DNA releases H1 histone (7).

In the course of our work on chromatin structure, we have routinely observed H1 histone co-isolated with what appears to be 140-150 base pair nucleosomes. Since the distribution of H1 histone and the nature of its attachment to the chromosomal material are important factors in chromosomal structure, we have examined the extent and mode of H1 binding to isolated nucleosomes as a function of the amount of digestion with micrococcal nuclease and as a function of ionic strength.

MATERIALS AND METHODS

(a) Preparation of Nuclei and Chromatin

Nuclei were obtained from frozen calf thymus. The tissue was disrupted in 100 ml of 0.25 M sucrose, 0.01 M Tris HCl, 0.01 M $MgCl_2$ and 0.05 M $NaHSO_3$, pH 6.5 by homogenizing with a Waring Blender. The homogenate was filtered through cheesecloth, followed by filtration through Miracloth to remove connective tissue and undisturbed thymus tissue. Nuclei were collected by centrifugation at $1,100 \times g$ for 3 min. The nuclear pellet was suspended in the above buffer containing 1% Triton X-100 and washed by homogenizing in a Potter-Elvehjem homogenizer. This washing procedure was repeated 3 times. The final nuclear pellet was resuspended in buffer without $NaHSO_3$ and detergent and then resedimented. Chromatin was prepared from purified nuclei as described previously (20) except the centrifugation through sucrose was omitted. Nucleohistone was obtained from the chromatin gel by shearing with a Virtis Model "45" homogenizer for 1 min at the maximum setting.

(b) Micrococcal Nuclease Digestion of Nuclei

Nuclei were suspended in 5 mM Tris-HCl (pH 7.9), 0.5 mM $CaCl_2$ and 5% sucrose to a concentration of $0.5-1.0 \times 10^9$ nuclei/ml or approximately 3 mg DNA/ml. Micrococcal nuclease (Worthington Biochem. Corp.) was added to 150 units/ml or 50 units/mg chromatin DNA. Incubation was at $37^\circ C$ for the desired time. The reaction was terminated and nuclei were lysed by the addition of EDTA to a final concentration of 5 mM and by placing the lysate in ice. The kinetics of chromatin digestion were followed by removing duplicate samples and diluting the sample 20-fold with cold 1 M NaCl-1 M $HClO_4$; the undigested material was allowed to precipitate in ice for 10 min before being pelleted by centrifugation at $12,000 \times g$ for 10 min. The supernatant constituted the acid-soluble material, and was corrected for hyperchromicity. The corrected A_{260} was divided by the starting A_{260} units $\times 100$ to obtain the percent digestion.

(c) Sucrose Gradient Sedimentation

Samples of the digested chromosomal material were layered directly on sucrose gradients within one hour after their preparation. Fresh nuclear suspensions were always used and potential endogenous proteolysis of histones was monitored by taking aliquots for histone extraction at various steps during the preparation of nucleosomes. Endogenous nuclease activity was not detected under our digestion conditions. For analytical analyses of the digestion products, 5-20% linear sucrose gradients (5 ml) containing 1 mM EDTA - 1 mM cacodylate, pH 6.5 were used. Approximately 100 μ g of DNA were loaded per gradient, and the components were separated by centrifugation at 200,000 $\times$ g for 6 hr (SW 50.1 rotor, 40,000 rpm, 4°C). Sedimentation A_{260} profiles were determined by gradient displacement from the bottom with 60% sucrose; the effluent was pumped through an ISCO density fractionator equipped with a flow cell and log recorder. For preparative analyses, samples of digested nuclei (30 to 40 A_{260} units) were layered on 7-30% linear sucrose gradients (30 ml) containing 1 mM EDTA - 5 mM cacodylate, pH 6.5. In some cases, NaCl was incorporated into the gradient at either 0.3 M or 0.6 M. The preparative gradients were centrifuged in a Beckman SW 27 rotor at 25,000 rpm for 18-20 hr. Gradients were emptied by piercing the bottom and collecting drops to obtain 1-ml fractions. The maintenance of a pH at 6.5 and a low temperature are sufficient to retard endogenous protease activity during the preparation of nucleosomes from calf thymus nuclei, since no degradation was apparent in acrylamide gel electrophoresis of the histones.

(d) DNA Isolation and Analysis

DNA was purified from digested nuclei by adjusting the solution to 0.5% sodium dodecyl sulfate. Pronase B (Cal Biochem), previously heat-treated for 10 min at 80°C, was added to a final concentration of 100 μ g/ml, and the solution was incubated at room temperature for 1 hr. NaClO_4 was added to obtain a 1 M solution, and an equal volume of chloroform-phenol (1:1) was used to extract the DNA. DNA was isolated from sucrose gradients by the method given above or by placing each fraction (1 ml) in dialysis tubing, adding 100 μ g of Pronase, and dialyzing the fractions against an excess of 1 mM EDTA - 5 mM Tris, pH 7.9, overnight at room temperature. The dialysis bags were next placed in a greater than 5-fold volume of ethanol and left at 4°C overnight. The precipitated DNA was flushed from the dialysis tubing with ethanol and concentrated as a pellet by centrifugation. This method allows nearly quantitative recovery of the DNA. Isolated DNA fragment sizes were determined according to Maniatis *et al.* (21) on 3.5% and 5% acrylamide slab gels, employing the buffer system

of Peacock and Dingman (22). The gels were standardized using Hind II (Bethesda Research Laboratory) restriction endonuclease digestion of Φ X-174 RFI and wild type λ DNAs. The double stranded DNA chain lengths for Φ X-174 Hind II fragments that are given in the text are the average of two methods of calibration listed in Table 1, column A and B of Maniatis *et al.* (21). DNA was stained with 0.5 μ g/ml ethidium bromide for 15 min at room temperature. The DNA was visualized as florescent bands by illuminating with an ultraviolet lamp (UV Products), and the gels were photographed using an orange filter. DNA band migration distances were measured directly from large (3 x 4 in) negatives or from greatly enlarged (6 x 8 in) prints. In some cases, negatives of slab gels were scanned by a Beckman Model R-112 microdensitometer. The sizes of DNA isolated from sucrose gradients agreed precisely with total DNA purified by standard extraction techniques from the same digest.

(e) Histone Extraction and Analysis

Histones were extracted from pooled sucrose gradient fractions by the addition of 4 N H_2SO_4 to a final concentration of 0.4 N. The solution was sonicated to facilitate histone removal and dialyzed versus 0.4 N H_2SO_4 at 4°C to remove the sucrose. The precipitate was removed by centrifugation at 12,000 x g for 10 min. The supernatant was dialyzed against ethanol overnight at 4°C, and the histones recovered by centrifugation.

Electrophoresis was by standard methods using 9 cm acid-urea, polyacrylamide gels (23). The gels were stained with amido black, scanned at 600 nm, and the relative amount of H1 histone (paper weight of H1/total weight of all histones) was determined as described previously (24).

(f) Salt Extraction of H1 Histone

The major binding forces of H1 histone to DNA are ionic in nature, and H1 can be selectively dissociated by salts in relatively low concentrations (26). The efficiency of H1 histone extraction by sodium chloride was compared for nuclease-chromatin and nucleohistone. Chromatin in nuclei was solubilized by nuclease to 3-4% digestion; this aided in obtaining homogeneous preparations of chromatin and is defined as nuclease-chromatin (25). Equivalent amounts of nuclease-chromatin or nucleohistone were layered on 30 ml 15% sucrose solutions containing 1 mM EDTA - 10 mM cacodylate, pH 6.5 in the presence of varying amounts of NaCl. The chromatin was sedimented by centrifugation at 130,000 x g for 20 hr (SW 27 rotor, 25,000 rpm, 4°C). Less than 5% of the total A_{260} material loaded was recovered in the supernatant in all cases. Histones were extracted from the pellet, and the relative quantity of H1 histone at a given salt concentration was determined as outlined above.

RESULTS

H1 Histone Dissociation from Chromatin

Recent evidence indicates that chromatin prepared by conventional methods is not completely native, in as much as nuclease digestion products are not analogous to those obtained with nuclei (25). This calls into question any properties established using chromatin gels formed at low ionic strength and solubilized by mechanical shearing. Accordingly, we have compared the relative electrostatic interactions between H1 histone and chromosomal material prepared by either the nuclease method or by conventional shear methods (see Materials and Methods). The results, showing the amount of H1 histone still associated with the chromatin in the presence of increasing NaCl concentrations, are presented in Figure 1. H1 is less tightly bound to nuclease chromatin. The mid-point of the dissociation curve is approximately 0.35 M for total nuclease-chromatin, whereas it is 0.45 M for nucleohistone. The observed dissimilarities cannot be ascribed to differences in loading, pH or final ionic strength because these parameters were essentially the same in all experiments. Our results obtained with nucleohistone are in close agreement with previous work which has examined chromatin from other sources and employed other methods of salt extraction (27,28). We are not aware of any similar data for nuclease-chromatin. Of course, we would prefer to assay for H1 binding in nuclei, but unfortunately if the chromosomal material is not sheared, H1 histone becomes trapped in a viscous aggregate and cannot be removed completely even at salt concentrations in excess of 1 M (data not shown). One might assume that the H1-binding properties of nuclease-chromatin more closely resemble those of native chromatin due to the more gentle shearing and the fewer perturbations involved. However, this assumption is weakened when considering the binding of H1 since the 3-4% digestion of DNA (i.e., 7 base pairs per 200) may occur in the very region where H1 binds. Thus in terms of an analysis of H1 binding to chromatin we are faced with a dilemma in as much as (a) the nuclease-chromatin had a small fraction of its DNA digested to low molecular weight products but (b) in the preparation of nucleohistone, the chromatin had undergone extension during the gelling process and the formation of new sites for H1 binding during the extension cannot be excluded.

Micrococcal Nuclease Digestion Products

The digestion products of chromatin treated with micrococcal nuclease have been well characterized and documented (6-11,16,19). Our results are presented here because the time-dependent variation in the size of spacer DNA has relevance to H1 binding as a function of nuclease digestion. The

Figure 1. Amount of H1 histone remaining with either nucleohistone (●—●) or nuclease-chromatin (O—O) after extraction with different concentrations of NaCl. Each point represents the average of six determinations from two experiments.

variation in size of the different DNA fragments as a function of percent-digestion is shown in Figure 2. It can be seen that there is a gradual shortening of the DNA in each nucleosomal population until a stable plateau is reached or the species disappears from the digest.

The monomer DNA shows a loss (extrapolated back to zero percent digestion) of approximately 50 base pairs. A similar decrease is noted for the dimer material. Curiously, higher oligomers exhibit a much larger loss of

Figure 2. DNA size was determined as a function of percent acid-soluble DNA from micrococcal nuclease digests of nuclei. The DNA was purified and the fragments were sized on 3.5% and 5% polyacrylamide slab-gels using Hind II reaction products of Φ X-174 and λ DNAs. The DNA populations were assigned an order based upon the smallest fragment, i.e., monomer.

DNA amounting to approximately 100 base pairs. One interpretation is that the higher chromatin oligomers undergo a more extensive digestion of the free ends, so that the terminal nucleosomes become degraded. This may be due to H1 migration. H1 histone may be released early on in the digestion and may migrate and bind to larger chromatin fragments to protect them from further nuclease action (see below). However, if we assume that nucleosomes larger than a dimer suffer comparable digestion from the ends then the difference between their plateau levels should give a measure of the repeat size. In this instance, it is 200 base pairs. Another explanation for the low plateau value is that there might be heterogeneity in spacer DNA length, and those sections of chromatin containing shorter spacer segments are more resistant to cleav-

age and therefore selectively accumulate as digestion proceeds.

We find that the DNA repeat length for calf thymus chromatin is 200 base pairs. This result is derived from an analysis of subtracting the length of one repeat from the next highest repeat or from extrapolating to zero percent digestion; this is true for higher nucleosomal orders, e.g., trimer and tetramer. In agreement with Campton et al. (29) we find the repeat length revealed only in higher structure analysis. Thus, parity in the rate of digestion from the ends does not exist for all species. Indeed, digestion of spacer DNA from mononucleosomes proceeds with the greatest rapidity as other investigators have observed (7,30). For example, at 4% digestion monomer, dimer, trimer and tetramer have 175, 355, 570 and 770 base pair DNA chain lengths; this would correspond to 180, 215, and 200 base pair repeat distances. At 8% digestion the same four bands have average molecular weights of 160, 345, 545 and 740 base pairs which give repeating lengths of 185, 200 and 195 base pairs. Thus, an analysis of monomers and dimers would indicate a repeat DNA length of 180-185 base pairs, but the same analysis of trimers and tetramers would yield a structural repeat of approximately 200 base pairs.

Complications in interpretation, as mentioned above, may have led to some of the past confusion concerning the repeat length of DNA in chromatin (31). For the purpose of this paper, the item of interest is the rate of decrease in terminal spacer DNA. However, it is clear that several different approaches to the determination of the repeat length may give varying answers depending upon which size nucleosomes are used for analysis. For the reasons discussed above, we conclude that the repeat size in calf thymus chromatin is 200 base pairs.

Sucrose Density Gradient Fractionation of Nucleosomes

One can see the distribution of nucleosomes generated as a function of digestion by separating the reaction mixture on sucrose gradients. Figure 3 displays sedimentation analyses as a function of percent chromatin digested. The sedimentation coefficients of the major components in the gradients were computed by a method similar to Noll (32). It was found that $S_{20,w}$ values for monomer (11 S) dimer (16 S), etc. were in accordance with those found previously (15,19). By about 3% digestion, the amount of monomer, dimer, and trimer are roughly equal. At 30% digestion, almost all of the acid-insoluble material sediments at 11 S. Thereafter, the amount of monomer in the gradient decreases and subsequently accumulates as an aggregate that pellets under these conditions.

The products of a micrococcal nuclease digestion of calf thymus nuclei

Figure 3. Sucrose gradient analyses of chromatin particles from nuclease-chromatin digested to different extents with micrococcal nuclease.

were separated in sucrose gradients and pooled as shown in Figure 4. Histones were acid-extracted from the four fractions, and the relative amount of each histone subfraction was determined. From identical gradients run in parallel, DNA was purified and analyzed for the size of DNA fragments which coisolated with the histones. In Fig. 4-A, the nuclease-chromatin was sedimented through a gradient at low ionic strength (5 mM cacodylate, 1 mM EDTA); while in Fig. 4-B and Fig. 4-C, the ionic strength was increased by the incorporation into the gradient of 0.3 M NaCl and 0.6 M NaCl respectively. Equal aliquots of chromatin were loaded on all gradients and recovery was $\pm 10\%$ of the starting A_{260} material in all cases.

Figure 4. Sucrose gradient fractionation of micrococcal nuclease digested calf thymus nuclei (19% acid-soluble DNA). The digest (35 A₂₆₀ units) was sedimented through 30-ml, 7-30% linear sucrose gradients containing 1 mM Na₂ EDTA, 5 mM cacodylate-HCl, pH 6.5 and no salt (Fig. 4-A), 0.3 M NaCl (Fig. 4-B) or 0.6 M NaCl (Fig. 4-C). The histones and DNA fragments isolated from the various fractions are shown above and below the absorbance profile respectively. Fraction A refers to pelleted material, and the total A₂₆₀ recovered in each pellet is 6.0 for no salt, 11.8 for 0.3 M NaCl, and 4.3 for 0.6 M NaCl.

At low ionic strength (Fig. 4-A) all 5 histones were found in every fraction except the acid-soluble region of the gradient, from which no histones were recovered. The amount of H1 histone is reduced in the mononucleosome peak, but the other four histones are present in normal proportions. The DNA size in the gradient corresponds to a normal distribution about the nucleosomal peaks with which it would be expected to associate (6); so the separated particles appear to reflect accurately the size of the DNA. The mononucleosome region (fraction C) from which the histones were extracted contained only DNA that had an average length of 150 base pairs. Thus, we find some H1 histone cosediments even with core mononucleosomes containing little or no spacer DNA. In addition, there are discrete smaller DNA size classes present in intact mononucleosomes isolated from such gradients. This DNA is analogous in size to DNA fragments derived from limit digests described by others (8). In the low ionic strength medium, 17% of the A_{260} material sedimented as a pellet which would be expected to contain DNA greater than 1200 base pairs. Although not evident in the picture of Fig. 4-A, a small amount of DNA of a size normally found in monomeric and oligomeric subunits was recovered in the pellet. The smallest DNA molecules from the pellet migrated in acrylamide gels as a duplex band in the monomer region which correlated with 145 and 180 base pair DNAs. We assume this DNA is similar to the doublet DNA which Noll (1) and other investigators have reported (11).

Increasing the ionic strength to 0.3 M NaCl (Fig. 4-B) produced a release of monomeric (145-180 base pair) DNA from the pellet, but it also produced an attendant two-fold increase in the amount of oligonucleosomes which sediment as aggregate particles. This is at the expense of only the oligonucleosome species in the gradient. Several other important points emerge from Fig. 4-B. First, except for the monomeric DNA, all other DNA size classes appear to be distributed in a non-Gaussian manner so that they are skewed toward the bottom of the gradient. Secondly, the oligomeric DNA which sediments into the pellets is on the average 20 base pairs greater than the corresponding oligomeric DNA remaining in the gradient. Thirdly, all four non-H1 histones are found in normal amounts, but in 0.3 M NaCl, the amount of H1 histone is reduced to about one-half the normal chromatin level in the case of all fractions other than the pellet which now has a normal amount of H1 per unit DNA. The appearance of small DNA in the pellet fraction indicates that aggregation of oligomer nucleosomal material is occurring at this ionic strength. Thus it appears that nucleosomes which have been liberated by endonucleolytic cutting of spacer DNA can to some degree be held together (non-covalently crosslinked) by H1 histone. The

reasons H1 is implicated in crosslinking nucleosomes as opposed to non-histone proteins or non-H1 histones are (a) calf thymus chromatin is relatively deficient in non-histone proteins (33); (b) at 0.3 M NaCl, a substantial amount of the non-histone proteins are removed, but only 20-30% of H1 is removed from total nuclease chromatin (see Fig. 1 and ref. 26) and no detectable amounts of non-H1 histone are dissociated; and (c) the use of limited trypsin treatment to selectively cleave H1 molecules can reverse the sedimentation pattern from that of Fig. 4-B to approximately that of Fig. 4-C in which 0.6 M NaCl has removed all H1 histone from the nucleosomal particles (34). The fact that insignificant numbers of nucleosomes are cross-linked in a low ionic strength environment may be due to repulsive forces between nucleosomes which can be neutralized by adding salt. Furthermore, the ability of H1 to crosslink nucleosomes is optimal at approximately 0.15 M NaCl (data not shown) which is in good agreement with the precipitation properties of nucleosomal populations observed by others (35).

A further increase in ionic strength to 0.6 M NaCl (Fig. 4-C) yields significant shifts in sedimentation velocities whereby all oligonucleosomes have lower sedimentation coefficients. We did not detect any significant changes in the sedimentation rate of mononucleosomes under these conditions. This is in agreement with the observations of Noll and Kornberg (19). The lysine-rich histone is now located exclusively in the acid-soluble fraction D. The complete removal of H1 produces two additional effects: only chromosomal material containing DNA greater than 1200 base pairs sediments as in the pellet and the oligomeric subunits are sharply represented in a narrow range of the gradient. Approximately 12% of the total digest now sediments, indicating that at low ionic strength only 5% of the digest sediments as if it contained DNA much larger than that observed.

The Relative Amount of H1 Histone Found with Isolated Nucleosomal Species

The amount of H1 histone from mononucleosomes, oligonucleosomes and pelleted material compared to the amount of H1 from total nuclease-chromatin was determined as a function of the fraction of chromatin digested. This was investigated initially following fractionation in sucrose gradients at low ionic strength. Figure 5 shows the relative amount of H1 varies significantly between the three classes of chromosomal material. While all classes start at approximately 100% of control values at very early stage of digestion, and the oligonucleosomes remain at this level throughout the digestion range studied (4-30%), the amount of mononucleosomal H1 decreases to an

Figure 5. The relative amount of H1 histone found with isolated nucleosomes at low ionic strength. The amount of H1 histone from mononucleosomes (O), oligonucleosomes-dimer to hexamer (□); and pelleted chromosomal material (Δ) compared to the amount of H1 from total nuclease chromatin was determined as a function of percent chromatin digested. The nucleosomes were separated as described in the legend to Fig. 4. Brackets indicate the standard error found for 3 or more determinations.

apparent plateau of 60% at later stages. However, the pelleted material shows the presence of ever more H1 as a function of digestion, almost in a reciprocal manner to that observed for mononucleosomes. This would indicate either that H1 molecules are preferentially associated with larger, less extensively digested chromatin fragments or that migration of H1 is occurring during the digestion process.

It should be stressed that the pelleted material includes increasingly greater amounts of monomer and submonomer DNA fragments as digestion to greater than 20% acid-solubility ensues. In fact, greater than 30% digestion yields

less nucleosomal material in all regions of the gradient; yet more and more material pellets or precipitates until at the limit (50% acid-soluble DNA) only 20% of the digest remains in the gradient as mononucleosomes with an altered sedimentation velocity (approximately 10 S, see Fig. 3) and containing little or no H1 histones (data not shown). The remaining portion of the limit digest (30-35%) is isolated as a pellet in which the DNA sizes are distinctively smaller than the subunit repeat size (7,8) and contains the same spectrum of DNA fragments remaining in the monomer part of the gradient (data not shown). It remains to be determined whether H1 histone is migrating and crosslinking these particles at late stages of digestion, thereby selecting for nucleosomes lacking H1 which as a result remain in the gradient.

The data in Figure 5 are not representative of the total distribution of H1 molecules since they are not normalized for the amount of chromosomal material in each fraction. Such an analysis indicates that H1 histones are being released as a function of digestion. For example, at 9% digestion, the normalized amounts of total H1 histone are 31%-pellet, 55%-oligonucleosomes, and 7%-mononucleosomes. On the other hand, at 30% digestion, H1 histone is calculated to be present at 20%-pellet, 27%-oligonucleosomes, and 23%-mononucleosomes of the total amount. Therefore, it can be inferred that digestion to 9% acid-soluble DNA releases little or no H1, whereas digestion to 30% acid-soluble DNA releases 30% of H1 to the supernatant.

The observation that the amount of H1 coisolated with nucleosomal material in low ionic strength increases with the size of the particle and presumably with the molecular weight of the DNA suggests several possibilities: (a) there might be regions on the chromosome that are enriched in H1 and are afforded greater protection from nuclease action, (b) alternatively, H1 histones may migrate to chromatin fragments containing relatively more spacer DNA, or (c) H1 may serve to interconnect cleaved nucleosomal particles which would then cosediment as if they were a larger species. With regard to the later possibility of the crosslinking enrichment of H1, it seems improbable, for instance, from the data of Fig. 4, that less than 5% crosslinked smaller fragments (Fig. 4-A) could enrich the 12% larger, less digested chromatin (Fig. 4-C) by 32% in its relative content of H1 histone.

The Ionic Strength Dependence of H1 Binding to Nucleosomal Species

A measure of the strength of binding of H1 histone associated with nucleosomes at different degrees of digestion was determined in gradients containing 0.3 M NaCl which is close to the mid-point of H1 dissociation for

total nuclease-chromatin (Fig. 1). The results are shown in Figure 6. There are drastic changes in the amount of H1 binding to different fractions in the presence of 0.3 M NaCl. This is particularly telling when compared to the quantities of H1 bound at low ionic strength to the same components as seen in Fig. 5. The relative frequency of H1 histone in the pellet is now found to equal approximately its representation in total nuclease-chromatin. In oligonucleosomes, H1 histone decreased from levels similar to that of the pellet (at early digestion times) to values close to that of mononucleosomes (at late digestion times).

In Figure 7 the DNA fragments of mononucleosomes isolated at different ionic strengths were sized on 5% acrylamide slab gels. At 9% digestion, the average DNA length is 180 base pairs, proceeding to 150 base pairs at 19% digestion. Isolated in the presence of 0.3 M NaCl the 180 base pair monomers

Figure 6. The relative amount of H1 histone found with nucleosomes isolated in 0.3 M NaCl. The amount of H1 from mononucleosomes (○), oligonucleosomes-dimer to hexamer (□), and pelleted chromosomal material (△), expressed as a percentage of the amount of H1 from total nuclease chromatin was determined as a function of percent chromatin digested. The nucleosomes were separated as described in the legend to Fig. 4. Brackets indicate the standard error found for 3 or more determinations.

Figure 7. Microdensitometer scans of DNA fragments isolated by electrophoresis on 5% polyacrylamide slab-gels. Isolated populations of mononucleosomes from nuclei digested to 9%, 19%, or 30% acid-solubility were analyzed. The DNA obtained from gradients containing no salt or 0.3 M NaCl were essentially the same length and only one scan from each digest is shown. A Hind II- Φ X-174 DNA digest is included as a standard reference. The arrow represents the gel origin. Presumably the difference between the monomer DNA length found at 8% digestion (160 base pairs) in Fig. 2 and the 180 base pair length at 9% digestion is due to experimental variation.

contain 65% of the relative amount of H1 compared to total chromatin, whereas the monosomes containing 150 base pairs of DNA bind only 20% of its original H1 histone. Digestion to 30% does not result in any further loss of H1. The monomer populations differ only in their relative content of H1 and in their DNA size; so the loss of 30 base pairs of DNA results in a 45% reduction in the amount of bound H1. It is therefore concluded that the major electrostatic interaction of H1 histone along the chromatin fiber occurs with the spacer DNA.

We have also analyzed binding of H1 as a function of ionic strength to monosomes and oligomers lacking terminal spacer DNA. This was performed using a 13% digestion, at which stage external spacer DNA is largely gone

Figure 8. H1 dissociation curves from mononucleosomes (●—●) or oligomers dimers to hexamer (—) by NaCl. Nuclei were digested to 13% acid-soluble DNA with micrococcal nuclease, and the components were isolated as described in the legend to Fig. 4, except the salt concentration was varied. The relative amount of H1 histone is expressed as a percentage of the same value determined for total nuclease chromatin.

(Fig. 2). The results of such an analysis are shown in Figure 8 in which we see that the monosomes bind H1 less tightly than the oligomers. This presumably reflects the loss of the spacer DNA which is, in a proportional sense, almost as severe for di and trinucleosomes which make up the bulk of the oligomeric nucleosomes, as it is for the monosomes (see 30% digestion, Fig. 6). As expected if this analysis is repeated upon chromatin fragments with the terminal spacer DNA intact (after very brief digestion) then the H1 dissociation pattern more nearly resembles that of whole nuclease chromatin.

DISCUSSION

The results presented in the paper indicate that the strength of H1 binding to chromosomal material depends upon the integrity of the internucleosomal spacer DNA. As the spacer DNA is degraded by micrococcal nuclease the affinity of H1 for the chromatin is decreased. However, at low ionic strength, the nucleosome can still bind H1 even when all the spacer DNA is digested,

though the affinity of binding is significantly reduced compared to that of undigested material.

As digestion proceeds we see a redistribution of H1 so that the monosome becomes partially depleted and the higher molecular weight chromatin accrues extra H1. The extra histone associated with the higher molecular weight chromatin can be removed by treatment with 0.3 M NaCl, at the same time much of the binding of H1 to monosomes is removed. Oligomer nucleosomes respond in an interesting manner to treatment with 0.3 M NaCl. They appear to redistribute themselves in the gradient as a function of size of residual intact spacer DNA. Thus oligomer material lacking spacer DNA stays in the sucrose gradient in 0.3 M NaCl and shows a weak affinity for H1 comparable to that of monosomes lacking spacer DNA. On the other hand oligomer material with intact spacer DNA tends to aggregate and to cosediment in the pellet with chromatin containing larger DNA and to bind H1 in a more normal fashion.

Varshavsky *et al.* (11) and Whitlock and Simpson (10) have presented data that indicate H1 is found only with DNA species 180-200 base pairs long and not with DNA of 140-150 base pairs in size. The most likely explanation for this difference follows directly from the observations described above and is probably due to the labile nature of H1 attachment to nucleosomes as judged by its decreased binding in salt. Varshavsky *et al.* (11) washed their chromatin with 0.3 M NaCl, thereby potentially stripping H1 from areas of weaker interaction. Furthermore, and more importantly, it seems that the initial preparation of chromatin prior to nuclease digestion is an important factor in the extent of binding, as well as where it is bound.

When chromatin gels are formed at low ionic strength, not only is H1 bound more strongly than in briefly digested material, but the resulting nuclease digestion products are different from those obtained by digesting chromatin *in situ*. One sees an additional DNA fragment (which is relatively more stable) whose size has been variously estimated at 160-200 base pairs (7,8,11). This band-fragment can also be obtained by reconstitution experiments when histone H1 and/or H5 is present (36), and upon redigestion of isolated oligomers (7). We too find this dual population, e.g., a band at 140 base pairs progressing down from 200 base pairs, and a relatively stable band at 180 base pairs, if we digest chromatin gels or chromatin otherwise maintained in low ionic strength buffers for moderate to long times. Moreover, this process appears to be facilitated by mechanical shear. This distinct 180 base pair DNA band was found, albeit in very small amounts, in

the material that pelleted at low ionic strength, which in this instance, we assume represents a background of chromatin from nuclei that lyzed during the reaction. We speculate that during the process of gel formation and chromatin extension, it is possible for H1 histone, which is external to the nucleosome, to slide or migrate to reassociate along a greater stretch of the predominately naked spacer DNA. Thus owing to a molecular rearrangement of H1 in extended chromatin, H1 could protect with greater efficiency regions of DNA not normally accessible in vivo, and this could also increase the overall affinities between H1 and DNA.

Assuming that there are eight non-H1 histones per nucleosome (7,9), we may calculate the average number of H1 molecules found with nucleosomes at different stages of digestion and different ionic strengths. This is accomplished by using the relative amount of H1 found in our experiments and the specific extinction coefficient known for H1 (24). At low ionic strength this value ranges from 1.3 H1 molecules per 200 base pairs of DNA for oligomers early in the reaction to 0.8 H1 molecules per 150 base pairs of DNA for monomers late in the digestion reaction. This suggests that even for limited fragments of nucleoprotein fibers, there are areas of H1 enrichment.

Due to the faster rate of nuclease digestion (10,19) and to the decreased sedimentation velocity of oligonucleosomes lacking H1 histone (19 and Fig. 4-C) one indicated role for H1 is in packaging spacer DNA or at least in maintaining its condensed conformation. Models on chromatin structure have rarely tried to position H1 with any accuracy in relation to nucleosomes or spacers. We may now ask whether H1 molecules span the nucleosome to interact with spacer DNA on adjacent sides or do they lie on the spacer DNA and interact with adjacent nucleosomes. Although the data presented here would not distinguish between these models, other studies have led us to suggest that H1 histone spans the nucleosome interacting with a part of spacer DNA on each side (34). This model predicts that H1 spans the nucleosome to attach to spacer DNA on adjacent sides at one site proximal to the nucleosome and at one site distal to the nucleosome. In this model, the highly conservative apolar region of H1 (37) would be the portion of the molecule expected to interact with the nucleosome. This would be consistent with the preferential binding of H1 to superhelical DNA (38). Conversely, the short lysine-rich, N-terminal end of H1 (39) would be predicted to bind to the proximal DNA-spacer site while the long, lysine-rich, C-terminus (39) would be predicted to bind to the distal DNA-spacer site. The attachment of the first H1 molecule could thus determine the attachment sites for the next H1 his-

tone. This arrangement of H1 molecules along the chromatin fiber could potentially maintain spacer DNA in a compact form, as well as link together cleaved nucleosomes.

ACKNOWLEDGEMENTS

We thank Brian Nichols for the gift of Φ X-174 DNA. We wish to acknowledge and thank Kathy Huber for her skillful assistance. We thank the members of our lab for their helpful comments on the manuscript. Jim Gaubatz is a postdoctoral fellow supported on a Tumor Biology Training Grant from NIH CA 09119-03 to the University of Iowa.

REFERENCES

1. Noll, M. (1974) *Nature* 251, 249-251.
2. Olins, A. L. and Olins, D. E. (1974)
3. Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) *Cell* 4, 281-300.
4. Hewish, D. R. and Burgoyne, L. A. (1973) *Biochem. Biophys. Res. Commun.* 52, 504-510.
5. Pardon, J. F., Wilkins, M. H. F. and Richards, B. N. (1967) *Nature* 215, 508-509.
6. Finch, J. T., Noll, M. and Kornberg, R. D. (1975) *Proc. Nat. Acad. Sci. USA* 72, 3320-3322.
7. Shaw, B. R., Herman, T. M., Kovacic, R. T., Beaudreau, G. S. and Van Holde, K. E. (1976) *Proc. Nat. Acad. Sci. USA* 73, 505-509.
8. Axel, R. (1975) *Biochemistry* 14, 2921-2925.
9. Kornberg, R.D. (1974) *Science* 184, 868-871.
10. Whitlock, J. P. and Simpson, R. T. (1976) *Biochemistry* 15, 3307-3314.
11. Varshavsky, A. J., Bakayev, V. V. and Georgiev, G. P. (1976) *Nucleic Acids Res.* 3, 477-492.
12. Jackson, V., Hoffmann, P., Hardison, R., Murphy, J., Eichner, M. E., and Chalkley, R. (1977) in Molecular Biology of the Mammalian Genetic Apparatus-Its Application to Gene Expression, Carcinogenesis and Aging, ed. T'so, P. (Elsevier North-Holland Biochemical Press, Amsterdam) pp. 281-299.
13. Noll, M. (1976) *Cell* 8, 349-355.
14. Li, J. H. (1975) *Nucleic Acids Res.* 2, 1275-1289.
15. Rill, R. L., Oosterhof, D. K., Hozier, J. C. and Nelson, D. A. (1975) *Nucleic Acids Res.* 2, 1525-1538.
16. Honda, B. M., Baillie, D. L., and Candido, E. P. M. (1975) *J. Biol. Chem.*, 250, 4643-4647.
17. Olins, A. L., Carlson, D., Wright, E. and Olins, D. E. (1976) *Nucleic Acids Res.* 3, 3271-3291.
18. Goodwin, G. H., Woodhead, L. and Johns, E. W. (1977) *FEBS Letters* 73, 85-88.
19. Noll, M. and Kornberg, R. D. (1977) *J. Mol. Biol.* 109, 393-404.
20. Panyim, S., Bilek, D., and Chalkley, R. (1972) *J. Biol. Chem.* 246, 4206-4215.
21. Maniatis, T., Jeffrey, A. and de Sande, Van, H. (1975) *Biochemistry* 14, 3783-3794.
22. Peacock, A. and Dingman, C. (1967) *Biochemistry* 6, 1818-1827.
23. Panyim, S. and Chalkley, R. (1969) *Arch. Biochem. Biophys.* 130, 337-346.
24. Hoffmann, P., Eichner, M. E., Chalkley, R. (1977) *Nucleic Acids Res.*, in press.

25. Noll, M., Thomas, J. O. and Kornberg, R. D. (1975) *Science* 187, 1203-1206.
26. Hnilica, L. S. (1972) *The Structure and Biological Function of Histones*, Ed., CRC Press, Cleveland, Ohio.
27. Bartley, J. A. and Chalkley R. (1972) *J. Biol. Chem.* 247, 3647-3655.
28. Ohlenbusch, H. H., Olivera, B. M., Tuan D. and Davidson, N. (1967) *J. Mol. Biol.* 25, 299-315.
29. Campton, J. K., Bellard, M. and Chambon, P. (1976) *Proc. Nat. Acad. Sci. USA* 73, 4382-4386.
30. Lohr, R., Corden, J., Tatchell, K., Kovacic, R. T. and Van Holde, K. E. (1977) *Proc. Nat. Acad. Sci. USA* 74, 79-83.
31. Cotter, R. (1977) *Nature* 266, 119.
32. Noll, H. (1967) *Nature* 215, 360-363.
33. Chalkley, R. and Jensen, R. H. (1968) *Biochemistry* 7, 4380-4387.
34. Gaubatz, J. W., Hardison, R., Eichner, M. E. and Chalkley, R. *Molecular Human Cytogenetics, ICN-UCLA Symposia*, in press.
35. Whittaker, R. G. and Blanchard, B. J. (1977) *Fed. Proc.* 36, 784.
36. Camerini-Otero, R. D., Soliner-Webb, B., and Felsenfeld, G. (1976) *Cell* 8, 333-347.
37. Bustin, M., Rall, S. C., Stellwagen, R. H. and Cole, R. D. (1969) *Science* 163, 391-393.
38. Vogel, T. and Singer, M. F. (1976) *J. Biol. Chem.* 251, 2334-2338.
39. Jones, G. M. Y. and Cole, R. D. (1972) In *Structure and Biological Function of Histones*, L. S. Hnilica, ed. p. 19, CRC Press, Cleveland.