Evidence for *N*-Acetoxy-*N*-2-acetylaminofluorene Induced Covalent-like Binding of Some Nonhistone Proteins to DNA in Chromatin†

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ABSTRACT: The in vitro reaction of the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene with calf thymus chromatin has been examined. After dissociation of modified chromatin in 8 M urea–2% sodium dodecyl sulfate, we could separate by centrifugation a DNA–protein complex fraction (as a pellet) and an unbound protein fraction in the supernatant. The number of milligrams of protein bound per milligram of DNA rose with the number of fluorenyl residues bound to DNA. The buoyant density of DNA containing fractions of modified chromatin declined with the extent of modification, but these values rose to that of the control upon proteinase K treatment, indicating that these fractions were actually DNA–protein complexes. Examination of carcinogen bound to protein fractions showed that the unbound proteins and bound proteins had about three and five times more fluorenyl residues per milligram of protein, respectively, than the acid-soluble proteins. Electrophoretic examination of proteins liberated from the protein–DNA complex by DNase I digestion showed that seven proteins designated a–g were bound to DNA but at differing rates. Confirmation of binding was revealed by a decrease or loss of these proteins in the unbound protein fraction removed from modified chromatin by the urea–$NaDodSO_4$ treatment. All these findings indicate that the carcinogen acts as a divalent reagent cross-linking proteins which are noncovalently complexed to DNA in chromatin. A mechanism for such a reaction is proposed.

Carcinogenic aromatic amines are activated in vivo to electrophiles which covalently bind to nucleic acids, proteins, and carbohydrates (Miller & Miller, 1967; Farber et al., 1967; Miller, 1970). In the case of AAF[1] the active metabolite appears to be an ester of N-OH-AAF (Cramer et al., 1960; Miller & Miller, 1967, 1969) and the synthetic ester, N-AcO-AAF, which reacts directly with DNA and proteins, is currently used for in vitro studies. The two electrophilic species of N-AcO-AAF that have been proposed are the amidonium ion and, ortho to it, the carbonium ion at position 3 (Miller, 1970).

Two guanine derivatives have been isolated and characterized from the reaction of N-AcO-AAF with DNA. In the major product the nitrogen atom of AAF is covalently linked to carbon 8 of guanine (Kriek et al., 1967; Miller et al., 1966), while in the minor one the linkage is between carbon 3 of AAF and nitrogen 2 of guanine (Kriek, 1974).

N-AcO-AAF also binds to proteins and the studies of DeBaun et al. (1970) and of Barry & Gutmann (1973) implicate methionyl and lysyl residues, respectively. While other residues are undoubtedly involved, these have not been identified with certainty.

Metzger & Daune (1975; Metzger, 1976) found in studying the reaction of N-AcO-AAF with calf thymus chromatin in vitro that, as more carcinogen was bound, there was a progressively reduced recovery of both histones and DNA indicating that the two had been cross-linked. Electrophoretic analysis did not reveal a preferential binding of any individual histone. We directed our attention, therefore, to the possible cross-linking of specific nonhistone proteins to the DNA. Evidence will be presented that this occurs and that the linkage is very likely covalent.

Materials and Methods

Reagents. All chemicals were reagent grade. [9-^{14}C]-N-AcO-AAF (38 mCi/mol) was synthesized as previously described (Lotlikar et al., 1966). Proteinase K was purchased from Boehringer Mannheim and DNase I (2650 units/mg) was purchased from Worthington Biochemical Corp.

Molecular weight markers for $NaDodSO_4$–polyacrylamide gel electrophoresis (range 14 300–71 500) were obtained from BDH Chemicals, Ltd.

Chromatin. It was isolated essentially by the method described by Axel et al. (1973). Calf thymus glands were disrupted with a Dounce homogenizer in 10 mM Tris-HCl buffer, pH 8.0, 0.25 M sucrose, 0.1 mM $MgCl_2$. The homogenate was filtered through five layers of gauze, and the nuclei were collected by centrifugation. They were washed twice in the same buffer containing 0.5% Triton X-100 (v/v) and then three times in buffer lacking the detergent. Chromatin was obtained from the nuclei by a stepwise reduction in ionic strength from 50 to 0.5 mM Tris-HCl, pH 7.9. After the chromatin solution was dialyzed against 0.1 mM sodium citrate buffer, pH 7.0, containing 0.1 mM $PhCH_2SO_2F$, its protein to DNA ratio was 1.5 ± 0.1 and the histone to DNA ratio was 1.1 ± 0.1 (w/w).

Reaction of Chromatin with N-AcO-AAF. The procedure was similar to that described by Miller et al. (1966). [9-^{14}C]-N-AcO-AAF, dissolved in a minimum volume of absolute ethanol, was added to a chromatin solution that had an absorbance at 260 nm of about 20, and the mixture was incubated at 37 °C for 3 h under argon in the dark. Unreacted carcinogen was removed by three ethyl ether extractions and by dialysis. Different degrees of chromatin modification were obtained by increasing the carcinogen to DNA ratio in the incubation mixture. A control sample was always treated at the same time without carcinogen.

Isolation of Chromatin Fractions. Acid-Soluble Proteins. The chromatin solution was acidified to 0.25 N with 1 N HCl. After the solution was stirred at 4 °C for 15 min, the precipitate was pelleted by centrifugation for 2 min at 12000*g* in an Eppendorf microcentrifuge. The supernatant was

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[1] Abbreviations used: AAF, *N*-2-acetylaminofluorene; N-OH-AAF, *N*-hydroxy-*N*-2-acetylaminofluorene; N-AcO-AAF, *N*-acetoxy-*N*-2-acetylaminofluorene; $NaDodSO_4$, sodium dodecyl sulfate; DNase I, deoxyribonuclease I, EC 3.1.4.5; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

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dialyzed against distilled water and lyophilized. This fraction contained the acid-soluble proteins, of which the histones constituted the major component.

Unbound Protein Fraction. To dissociate proteins not linked to DNA by carcinogen, total chromatin was dialyzed against 100 volumes of 2% $NaDodSO_4$–8 M urea–0.1 M sodium phosphate (pH 7.0) for 48 h with five to six changes of buffer. Because micelle formation prevents $NaDodSO_4$ from diffusing freely across dialysis membranes, 0.1 volume of a 20% $NaDodSO_4$ solution was added to the sample inside the bag before dialysis. Then the DNA–protein complex was pelleted for 24 h at 48 000 rpm in a SW 50.1 rotor at 20 °C leaving the unbound proteins in the supernatant.

Bound Proteins. These were liberated by DNase I digestion (1 unit mL^{-1} (mg of DNA)$^{-1}$ at 37 °C for 18 h) of the protein–DNA complex that had been solubilized in 0.01 M $MgCl_2$, 0.01 M sodium phosphate buffer, pH 7.0. Perchloric acid was added to an aliquot to a final concentration of 0.8 M, and the mixture was centrifuged. From the absorbance of the supernatant at 260 nm multiplied by 0.6 to correct for hyperchromicity, the percentage of hydrolysis was determined.

Total DNA. To extract the DNA quantitatively, it was necessary to first digest the chromatin with proteinase K (25 μg/OD unit at 37 °C for 2 h) in 2% $NaDodSO_4$–0.01 M sodium phosphate buffer, pH 7.0. Then the digest was extracted with a phenol–chloroform–isoamyl alcohol mixture (24:24:1, v/v/v) and the DNA precipitated by addition of 2 volumes of cold ethanol. It contained less than 0.5% protein by weight.

Cesium Chloride Isopycnic Centrifugation. Solid CsCl was added to the modified chromatin solution until its density was about 1.65 g/cm^3. The mixture was centrifuged for 72 h at 20 °C in a Beckman Model L2 65B centrifuge in a 50 Ti rotor. When we used the relation $d\rho = 6.47 \times 10^{-10} \times \omega^2 \times r dr$ (Fasman, 1976), a speed was calculated to provide a density of 1.72 g/cm^3 at the bottom of the tube and was usually about 25 000 rpm depending upon the initial density. After centrifugation, 0.5-mL fractions were collected, and their refractive indices and absorbances at 260 nm were measured.

Electrophoresis. The $NaDodSO_4$-discontinuous system described by Maizel (1971) was used. Proteins were run on a slab gel (10% acrylamide–0.3% bisacrylamide) for 3 h at 100 V. Phenol red was used as the tracking dye, and molecular weight markers between 14 300 and 71 500 were run simultaneously. The gel was stained overnight with a 0.5% solution of Coomassie Blue R250 in a 50:50 (v/v) methanol–water mixture containing 7% acetic acid. After destaining in 5% methanol–7% acetic acid, the gel was scanned with a Joyce-Loebl microdensitometer. Proteins were quantitated on the scans by dividing the area under each band by the total area of all the bands. By this method, the variations of the relative amount of a particular protein in the different samples can be detected independently of the total quantity of protein loaded on the gel.

Determination of the Extent of Chromatin Modification. The amount of labeled carcinogen bound to various chromatin fractions was measured by liquid scintillation counting in 3a40 cocktail (Koch-light Laboratories, Ltd.). The counts were corrected for quenching by the H number methods (Horrocks, 1977).

The DNA was quantitated from its absorbance at 260 nm and by making use of a molar extinction coefficient/nucleotide residue of 6700 L M^{-1} cm^{-1}.

Proteins were measured by the Lowry method (Lowry et al., 1951).

Table I: Fluorenyl Residues Bound to Chromatin and the Amount of Protein Remaining Associated with the DNA

expt no.	chromatin (fluorene residues bound per 100 nucleotides[a])	DNA–protein complex[b] (mg of protein/mg of DNA)
1	0	0.16 ± 0.1
	2.2 ± 0.2	0.19 ± 0.01
	4.7 ± 0.4	0.20 ± 0.01
	8.4 ± 0.6	0.21 ± 0.01
2	0	0.13 ± 0.1
	3.8 ± 0.1	0.19 ± 0.1
	7.8 ± 0.1	0.24 ± 0.02
	14.6 ± 0.1	0.31 ± 0.03

[a] The fluorene residues are bound to both DNA and proteins. [b] DNA–protein complex is the pellet obtained by centrifugation of the chromatin dissociated in 8 M urea–2% $NaDodSO_4$.

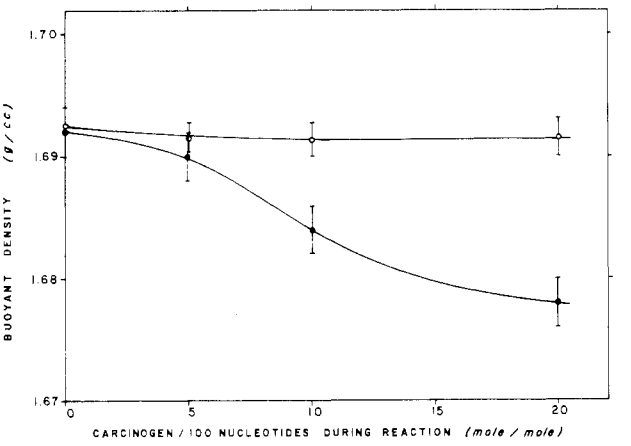

FIGURE 1: Variations of the buoyant densities of DNA containing fractions from increasingly modified chromatin samples. (●) DNA containing fractions from chromatin dissociated in 5 M CsCl. (○) Same fractions after proteinase K treatment. Isopycnic centrifugation was performed as described in Materials and Methods.

Modification was expressed in nmol of carcinogen per mg of protein for the fractions composed only of proteins or in carcinogen per 100 nucleotides (mol/mol) for the fractions containing DNA (chromatin, DNA–protein complex, and DNA).

Results

Increase of Protein Content and Decrease of Buoyant Density of DNA Fractions after Modification. Table I shows the amount of protein remaining associated with DNA after urea–$NaDodSO_4$ dissociation of chromatin samples modified to different extents. Comparison of the data shows that this amount increases with the rise in the number of fluorenyl residues bound to chromatin.

Following dissociation of chromatin in 5 M CsCl, the buoyant densities of the DNA-containing fractions were determined (Figure 1). These values decreased with increasing modification. After incubation of each sample with proteinase K, the buoyant densities returned to that of the control whose value remained unaffected by the enzyme treatment.

Relative Binding of Carcinogen to Chromatin Fractions. The modifications of total chromatin, the protein–DNA complex, and the DNA are presented in Figure 2. Because N-AcO-AAF binds to both proteins and DNA, the amount of carcinogen bound per 100 nucleotides was highest for total chromatin. Removal of proteins by 8 M urea–2% $NaDodSO_4$ did not reduce these values to those for DNA (lowest curve)

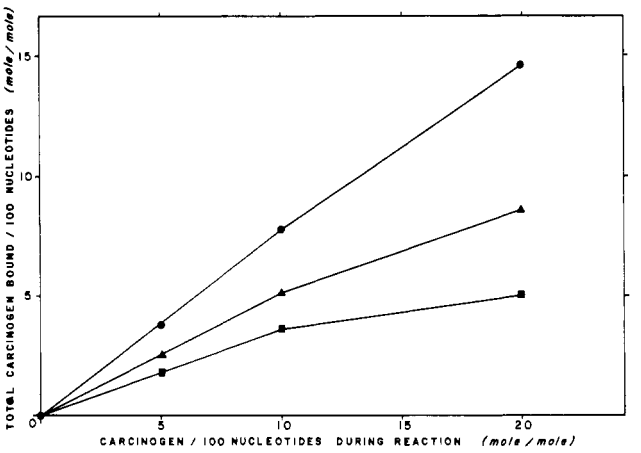

FIGURE 2: Binding of carcinogen to total chromatin (●), DNA–protein complex (▲), and DNA extracted from chromatin (■) as a function of the amount of N-AcO-AAF per 100 nucleotides during the reaction. In the DNA fraction the carcinogen was bound exclusively to the bases, while, in the chromatin and DNA–protein complex, it was bound to both DNA and proteins. The DNA–protein complex was pelleted by centrifugation after dissociation of the chromatin in 8 M urea–2% $NaDodSO_4$. The DNA was prepared from chromatin by proteinase K treatment, phenol extraction, and ethanol precipitation.

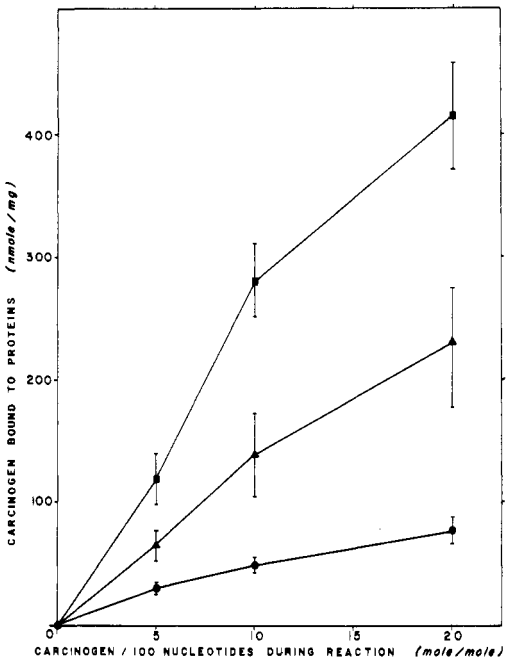

FIGURE 3: Fluorenyl residues bound to the different chromatin protein fractions as a fraction of the amount of carcinogen per 100 nucleotides during the reaction. The binding is expressed as nmol of fluorenyl residues per mg of protein. (●) Acid-soluble proteins. Prepared by 0.25 N HCl extraction of chromatin. (▲) Unbound proteins. Dissociated by 8 M urea–2% $NaDodSO_4$ found in supernatant after centrifugation. (■) Bound proteins. Liberated from the DNA–protein complex by DNase I digestion.

as would have been expected had there been no complex formation.

N-AcO-AAF binding to three protein fractions, acid-soluble proteins, unbound proteins, and bound proteins, is presented in Figure 3. The bound proteins had five times more, and the unbound proteins had three times more, fluorenyl residues per milligram than did the acid-soluble proteins.

Specific Binding of Some Nonhistone Proteins to DNA. To ascertain whether specific nuclear proteins were bound to DNA by the carcinogen, the unbound and complexed proteins were analyzed by polyacrylamide gel electrophoresis (Figure 4). In the control sample from the bound proteins liberated by DNase I digestion (Figure 4B), four very weak bands were discerned: a, b, c, and d having molecular weights, respectively, of 67 000, 60 000, 39 000, and 37 000. From the electropherograms it can be seen that the intensities of these bands increase as more AAF is bound to chromatin, and, in the most modified sample, three new proteins, e, f, and g, having molecular weights of 35 000, 28 000, and 23 000, were detectable. The presence of proteins in the control sample is not unexpected since the pellet following centrifugation is apt to have some protein contaminating the DNA. Most of this material was histones, reflecting the composition of chromatin and was found at the bottom of the polyacrylamide gel (mol wt ≤ 17 400). The minute amounts of proteins a–d observed in the control may reflect their close associations with DNA and hence their enhanced capacity for cross-linking by carcinogen. The increased binding of a–d seen in the scans above the control is not specious since each sample loaded on the gel originated from an equal amount of DNA that was measured by absorption at 260 nm.

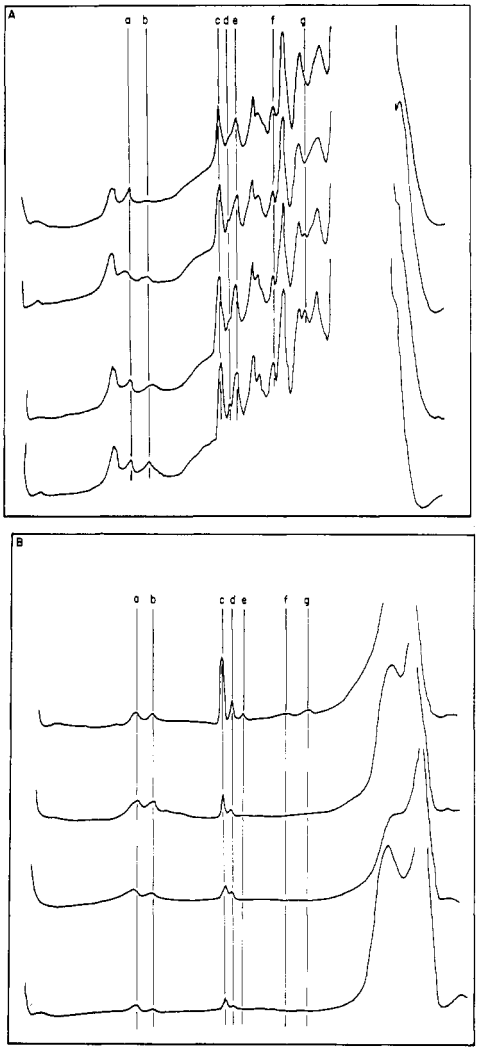

FIGURE 4: Densitometer scans of unbound proteins (A) and bound proteins (B) run on $NaDodSO_4$–polyacrylamide gels. The lowest scans represent unmodified chromatin (control), and the ones above it are for modified chromatin having 2.2, 4.7, and 8.4 fluorenyl residues bound/100 nucleotides, respectively. The molecular weights of bands a to g were (a) 67 000, (b) 60 000, (c) 39 000, (d) 37 000, (e) 35 000, (f) 28 000, and (g) 23 000. Unbound and bound protein fractions were obtained as described in the legend to Figure 3.

We expected the corresponding bands, a–g, in the unbound protein fractions to decrease or disappear (Figure 4A). Indeed

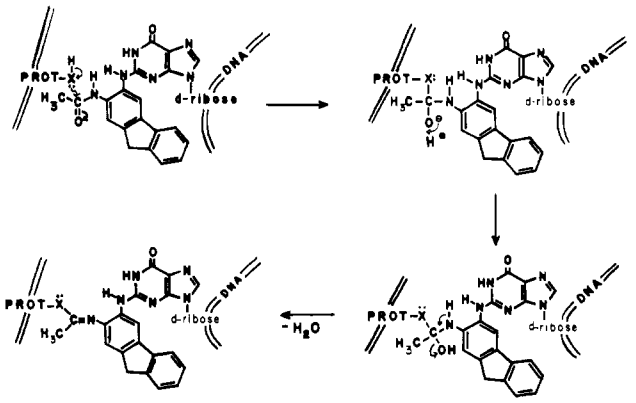

FIGURE 5: Proposed mechanism for covalent linkage of protein to DNA by N-AcO-AAF. X refers to a nucleophilic atom, for example, an ϵ nitrogen of lysine or the oxygen atom of serine. The reaction with a sulfur atom from methionine or cysteine probably yields an unstable derivative. As the fluorene moiety of the 3-(guanin-N^2-yl)-2-AAF lies in the minor groove of DNA, the acetyl group is accessible to nucleophilic amino acid residues. This is not the case for the *N*-(deoxyguanosin-8-yl)-2-AAF where the fluorene ring lies "protected" inside the DNA.

on the scans one observes that bands b and g disappear, and a and d decrease. Measurement of the relative areas under e and f shows that they decrease with increasing modification of the chromatin. The change in intensity of band c was not detectable. Of interest were the differences observed in the rate of disappearance of b, e, f, and g. That for f and g was rapid compared with that for e, which decreased only in the most modified sample. In contrast the decrease of b was proportional to the chromatin modification.

Because histone H1 migrates anomalously in the $NaDodSO_4$ system (Rodriguez & Becker, 1976) and has the same apparent molecular weight as protein d, the latter may be H1, but the other bound proteins are certainly nonhistones.

Discussion

N-AcO-AAF induced complex formation between DNA and histones in chromatin has been observed before (Metzger & Daune, 1975; Metzger, 1976). This complex was neither dissociated by 0.25 N HCl nor by phenol–$NaDodSO_4$. In the latter case the DNA was found associated with the proteins in the interphase. If, before extraction, the modified chromatin was first digested with proteinase K, all the DNA was recovered in the aqueous phase, leaving no doubt that the DNA in the interphase was complexed with proteins.

In the present study we have shown that the protein–DNA complex in modified chromatin is resistant to dissociation by both high cesium chloride concentration and by 8 M urea–2% $NaDodSO_4$. Taken together, these findings support the contention that N-AcO-AAF acts as a divalent reagent cross-linking protein to DNA in chromatin.

Additional evidence for the divalency of the carcinogen can be culled from the literature. Maher et al. (1968) reported that N-AcO-AAF cross-links complementary strands of DNA. These findings were confirmed by the light-scattering measurements of Fuchs & Daune (1972), who found no drop in the molecular weight of modified DNA after denaturation in contrast to the decline observed for unmodified DNA. Morin et al. (1977) reported that, while the carcinogen complexed lysozyme to DNA, it did not cross-link two native DNA molecules.

The binding of complementary DNA strands but not of two different DNA molecules stresses the need for close association of two molecules for linkage to occur. This notion can be accommodated by the mechanism shown in Figure 5 for the covalent coupling of protein to DNA where a nucleophilic atom of the protein molecule (for example, the ϵ nitrogen of a lysine residue or the oxygen of a serine residue) attacks the carbonyl groups of the N^2 derivative of AAF with guanine to form a stable tetrahedral compound (Rogers & Bruice, 1974), which may lose water to yield a Schiff base.

The necessity of a given spatial arrangement for the cross-linking reaction can account for the specific binding of some nonhistone proteins to DNA. The differences observed in the amount of modification needed to bind these proteins can reflect different geometrical arrangements in their association with DNA. Another possibility is that the binding of the first proteins (f and g) induces some conformational change in the chromatin which facilitates the binding of protein e.

Other ultimate carcinogens are also known to produce such protein–DNA complexes (Nietert et al., 1974; Morin et al., 1977). Ultraviolet light induces protein–DNA cross-links (Todd & Han, 1976), and in this case also the complex formation is dependent upon the geometry of the association between the two molecules.

While carcinogen induced DNA–protein complex formation has been reported several times, in the case of N-AcO-AAF its specificity with respect to nonhistone proteins has hitherto not been observed. As structure and function in chromatin are closely related, this specific binding may impair some nuclear function and may be one of the steps leading to a defect in cellular control.

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Analysis of Chromatin Reconstitution†

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ABSTRACT: The ability of high molecular weight chicken erythrocyte chromatin to spontaneously self-assemble into native-like material, after dissociation by high ionic strength and reassociation by salt gradient dialysis, was critically examined. The native conformational state of the reassembled nucleoprotein complex was regenerated to the extent reflected by circular dichroism spectra and the thermally induced helix–coil transition of the nucleoprotein DNA. However, the internucleosomal packing of ~205 base pairs of DNA per repeating unit, as probed by digestion with micrococcal nuclease, was not regenerated upon reassembly and was replaced by a packing of ~160 base pairs per repeating unit. Thus, high molecular weight chromatin containing only lysine-rich histones (H1 and H5) and core histones (H2A, H2B, H3, and H4) is *not* a true self-assembling system in vitro using the salt gradient dialysis system used herein. Circular dichroism and thermal denaturation studies on core chromatin (lysine-rich histones removed) showed that core histones *alone* are not capable of reassembling high molecular weight DNA into native-like core particles at low temperature (4 °C). Reassembly at 21 °C restored the circular dichroism but not the thermal denaturation properties to those characteristic of undissociated core chromatin. Nonetheless, micrococcal nuclease digestions of both reassembled core chromatin products were identical with undissociated native core chromatin. Reassembly in the presence of the complete complement of histones, followed by removal of the lysine-rich histones, did regenerate the thermal denaturation properties of undissociated native core particles. These results indicated multiple functions of the lysine-rich histones in the in vitro assembly of high molecular weight chromatin.

In the past few years the chromatin field has witnessed a significant increase of structural information. Eukaryotic interphase chromatin consists of a basic repeating unit known as the nucleosome which is composed of about 200 base pairs of DNA, a protein octamer consisting of two each of the four core histones H2A, H2B, H3, and H4, and probably one or two molecules of lysine-rich histone H1 (or H5 from avian erythrocytes) (Kornberg, 1974; Noll, 1974; Olins et al., 1976, 1977). The nucleosome may be described in terms of two major morphological domains differing in susceptibility to digestion with micrococcal nuclease (Hewish & Burgoyne, 1973; Ramsay-Shaw et al., 1976) and appearance in electron micrographs (Olins & Olins, 1974). One domain consists of the core particle made up of 140 base pairs of DNA which are wrapped around an octamer of core histones. The second domain is a somewhat variable length of DNA, connecting core particles, termed the linker. Although H1 is not an integral part of the core particle, binding sites to it have not been eliminated. Excellent reviews by Kornberg (1977) and Felsenfeld (1978) have been published.

Our knowledge of chromatin to date is predominately morphological. The recent low resolution crystallographic analysis of Finch et al. (1977) shows that a proteolytically degraded nucleosome core is a flat particle of dimensions about 110 × 110 × 57 Å. The DNA is proposed to wrap around the disk of core histone octamer in a uniform superhelix of ~80 base pairs per superhelical turn with a pitch of ~28 Å. This model is supported by neutron-scattering studies on intact core particles (Baldwin et al., 1975; Pardon et al., 1977). Our knowledge of the location and structural role of H1 is less complete. Nuclease digestion studies have implicated H1 in binding to linker DNA (Noll & Kornberg, 1977, and references cited therein) where it may play a role in determining internucleosomal spacing (Morris, 1976a,b; Wilhelm et al., 1977). Several studies (Renz et al., 1977; Campbell & Cotter, 1977) have provided evidence for H1 mediated condensation of the linear array of nucleosome cores into higher orders of structure.

Very little is known about the details of histone–histone and histone–DNA contacts within the core particles. Intranucleosomal architecture may be inferred from knowledge of the types of histone–histone complexes involved in the assembly pathway. Kornberg (1974) originally proposed that the arginine-rich H3:H4 tetramer forms the basis of the nucleosome

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