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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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by condensing the DNA into a prenucleosome kernel which is transformed into the completed nucleosome upon binding the slightly lysine-rich histones H2A and H2B. A central role of H3 and H4 in nucleosome formation is supported by studies employing nuclease digestion (Camerini-Otero et al., 1976; Sollner-Webb et al., 1976), X-ray diffraction (Boseley et al., 1976), closed circular DNA supercoiling (Camerini-Otero & Felsenfeld, 1977; Bina-Stein & Simpson, 1977) and electron microscopy (Bina-Stein & Simpson, 1977). A recent report by Wilhelm et al. (1978) indicates that H3 and H4 organize DNA into spherical-like particles of ~110-Å diameter prior to the binding of H2A and H2B during in vitro reconstitution. An alternate view of the assembly mechanism involving a heterotypic tetramer consisting of one each of the core histones, based on the possible existence of such a kinetic unit free in solution, has been proposed by Weintraub (Weintraub et al., 1975).

Reconstitution represents one of the most powerful techniques in biochemistry for examining the roles of individual components in a complex macromolecular assembly. Full potential of the approach is realizable only when each individual component can be purified and reintegrated into the macromolecular complex in a native site. These studies are valid only when the reconstituted product is shown to be structurally and functionally identical with the native starting material. In the absence of a suitable biologically functional assay for efficient reconstitution, chromatin has been subjected to an array of physical and indirect biochemical tests. Van Holde and co-workers (Tatchell & Van Holde, 1977) have shown that nucleosome core particles can be self-assembled from salt-extracted histone complexes and 140 base pair DNA with ~80% yield. The purified reconstituted core particles appeared identical with native core particle by a variety of independent criteria. Reconstitution of chicken reticulocyte chromatin via a denaturing pathway (5 M urea) in the presence of excess histone and nonhistone protein has resulted in a transcribable globin gene by *Escherichia coli* RNA polymerase (Godski & Chae, 1978; see other examples cited therein). Both individually purified histones, obtained via denaturing conditions, and salt-extracted histone complexes, used in reconstitution of high molecular weight chromatin, have resulted in the appearance of nucleosomes in electron micrographs (Oudet et al., 1975), characteristic X-ray diffraction maxima (Bosely et al., 1976), and *limit* nuclease digestion products (Camerini-Otero et al., 1976). However, the uniform 200 base pair repeat of native chromatin resulting from digestion with micrococcal nuclease has been reported lost upon reconstitution (Yaneva et al., 1976; Steinmetz et al., 1978). It has been suggested (Laskey et al., 1977) that an as yet unidentified thermolabile factor is required to regenerate the proper internucleosomal spacing. Garel et al. (1976) have demonstrated that reconstitution with acid-extracted histones results in a mixture of well-organized nucleosomal subunits and nonspecific histone–DNA complexes. Both the specificity and ionic strength dependence of sequential histone complex binding to DNA were found to be different for acid-extracted histones and salt-dissociated histone complexes (Wilhelm et al., 1978). Reconstitution studies by Leffak & Li (1977) using acid-extracted histones and a variety of reconstitution pathways have shown that neither a mixture of all five histones nor a mixture of the four core histones is capable of reassembling high molecular weight DNA into native chromatin-like material at physiological histone/DNA ratios as judged by circular dichroism and thermal denaturation. Such data suggest that the structural integrity of acid-extracted histones is not completely regained upon "renaturation", under the conditions used.

The ability to reassemble high molecular weight chromatin into native-like material at high efficiency is a prerequisite to a multitude of sophisticated studies on the eukaryotic genetic apparatus. True self-assembly requires that the native material represents a minimum in free energy with respect to the components present in the finished product and that the environment allows this minimum to be spontaneously located. Two questions concerning chromatin reconstitution remain open at present. To what efficiency can the subunit structure of chromatin be regenerated upon reassembly? Is the native inter- and intranucleosomal structural integrity preserved upon reassembly? Such questions are nontrivial to answer. We believe that employment of a variety of independent criteria in parallel comparative studies between native and reassembled material is necessary to assess the success of reconstitution. Various physical and biochemical probes are sensitive to specific subsets of the physical properties of the system. True self-assembly can be inferred only if all examined biochemical and physical properties of the reassembled system are identical with the native starting material. A critical examination, employing a variety of criteria, of the simple nondenaturing method of reconstitution via dissociation/reassociation of high molecular weight chromatin by changes in ionic strength has not been reported.

We report studies herein on the self-assembly of nonsheared high molecular weight chromatin isolated by micrococcal nuclease digestion of chicken erythrocyte nuclei (Ramsay-Shaw et al., 1976). Chromatin prepared in such a manner contains no nonhistone proteins and is devoid of proteolytic degradation. The most fundamental level of chromatin organization is obtained by removal of the lysine-rich histones (H1 and H5) resulting in chains of core particles referred to herein as core chromatin. Both core chromatin and the next level of complexity, chromatin containing all five histones (whole chromatin), were subjected to nondenaturing dissociation/reassociation via ionic strength alteration. This was accomplished by dialysis to 2 M NaCl and then returning to low ionic strength by gradient dialysis. The quality of reassembly was assessed by comparisons with the appropriate native starting material by three independent criteria. (1) Conformational properties of the DNA and histones were compared by circular dichroism. (2) A measure of the total interaction energy involved in packaging the DNA by histones was compared by thermal denaturation monitored by hyperchromicity at 260 nm. (3) The presence of nucleosome subunits and the repeat length were analyzed by digestion with micrococcal nuclease.

These studies indicate that (a) reassembly of native core histone–DNA interactions requires the participation of lysine-rich histones and (b) whole chromatin, containing only core and lysine-rich histones, is *not* a true self-assembling system in vitro by salt gradient dialysis.

Materials and Methods

Isolation of Chromatin. Chicken erythrocyte nuclei were prepared by the method of Ramsay-Shaw et al. (1976). Chromatin was solubilized by brief digestion of nuclei with micrococcal nuclease (EC 3.1.4.7, Worthington) in 0.3 M sucrose, 0.01 M Tris-HCl, 0.75 mM $CaCl_2$, pH 7.2, at 37 °C with ~15 units of enzyme/mL for ~5 min or at 0 °C with ~100 units/mL of enzyme for 10–20 min. The latter digestion resulted in a higher yield of high molecular weight chromatin whose properties were indistinguishable from the former. Digestions were terminated by addition of 0.1 M

EDTA[1] to a final concentration of 10 mM EDTA. Digested nuclei were pelleted at 12000*g* for 15 min followed by resuspension in ≃5 volumes of 0.25 mM EDTA, pH 7.0, and the chromatin was extracted by standing overnight at 4 °C. Extracted nuclear debris was pelleted at 3000*g* for 10 min. Supernatants from both the 12000*g* and 3000*g* fractionations were pooled, dialyzed vs. 10 mM Tris-HCl, 0.25 mM EDTA, pH 7.8, and fractionated on a 3.5 × 47 cm Bio-Gel A-150m (Bio-Rad), 50–100 mesh gel filtration column equilibrated with 10 mM Tris-HCl, 0.25 mM EDTA, pH 7.8, at 4 °C. Pooled void peak fractions were used in this study and will be referred to as whole chromatin.

Removal of H1 and H5. It was first shown by Ohlenbusch et al. (1967) that lysine-rich histones may be selectively dissociated from chromatin by NaCl. Approximately 3–7 mg of whole chromatin in ~4 mL was dialyzed vs. 1 L of 0.6 M NaCl, 10 mM Tris-HCl, 0.25 mM EDTA, pH 8.0, at 4 °C for ~24 h. Subsequent fractionation on a 1.3 × 110 cm Bio-Gel A-150m, 50–100 mesh gel filtration column equilibrated with the above solution resulted in two well-resolved peaks. Pooled void peak fractions were returned to low ionic strength by continuous flow exponential gradient dialysis as described by Carroll (1971), with V_1 = 500 mL; C_1 = 0.6 M NaCl, 10 mM Tris-HCl, 0.25 mM EDTA, pH 8.0; V_2 = 2000 mL; C_2 = 10 mM sodium phosphate, 0.25 mM EDTA, pH 7.0; flow rate ~50 mL/h, followed by exhaustive dialysis vs. either 1.0 mM sodium phosphate, 0.25 mM EDTA, pH 7.0, or 0.25 mM EDTA, pH 7.0, at 4 °C. This material will be referred to as core chromatin.

Dissociation and Reconstitution. Complete dissociation of whole and core chromatin (~0.5 mg/mL) was achieved by dialysis vs. 1 L of 2 M NaCl, 10 mM sodium phosphate, 0.25 mM EDTA, pH 7.0, at 21 or 4 °C. Reassociation of chromatin components at the same temperature of dissociation occurred upon continuous flow with approximately the linear gradient dialysis described by Carroll (1971), with V_1 = 500 mL; C_1 = 2 M NaCl, 10 mM sodium phosphate, 0.25 mM EDTA, pH 7.0; V_2 = 1 L; C_2 = 1 M NaCl, 10 mM sodium phosphate, 0.25 mM EDTA, pH 7.0; V_3 = 1 L; and C_3 = 10 mM sodium phosphate, 0.25 mM EDTA, pH 7.0, followed by a 1-L exponential gradient with C_2 = 10 mM sodium phosphate, 0.25 mM EDTA, pH 7.0, and exhaustive dialysis vs. either 1.0 mM sodium phosphate, 0.25 mM EDTA, pH 7.0, or 0.25 mM EDTA, pH 7.0.

Spectroscopic Analysis. Absorption spectra were recorded on a Cary 14 spectrophotometer at 21 °C. Circular dichroism (CD) measurements were made at 5 °C with a Cary 60 with a 6001 circular dichroism accessory as previously described (Adler et al., 1971). Concentrations of DNA nucleotide residues in whole and core chromatin were determined by absorption at 258 nm using ϵ_{258} = 6800 cm^{-1} (mol of nucleotide)$^{-1}$. Samples used were (1.5–2.0) × 10^{-4} M nucleotide residue in 0.25 mM EDTA, pH 7.0. Reported spectra represent averages of three independent preparations.

Thermal Denaturation. Chromatin samples were thermally denatured in 1.0 mM sodium phosphate, 0.25 mM EDTA, pH 7.0, at OD_{260} (25 °C) ~ 0.9–1.0. A jacketted 1-cm pathlength quartz cuvette (Hellma 1-160B QS) was filled with the sample which had been degassed by bubbling in helium and tightly sealed with a Teflon stopper. Optical density at 260 nm and the cell temperature were continuously monitored by a Cary 14 spectrophotometer and a linear thermal probe (Mandel & Fasman, 1974) located immediately adjacent to the circulant exit. The temperature was increased at a rate of ~0.2 °C/min as described by Mandel & Fasman (1974). Approximately four sets of data points (optical density and temperature) per °C increment were processed on a PDP-11 computer to plot hyperchromicity (h) vs. temperature (T) and dh/dT vs. T on a Complot plotter. The derivative was obtained by successive 11–19 point quadratic least-squares fits. Thermal denaturation data from two to four independent preparations were averaged by the following algorithm. Separate data sets [$h(T)$, T] were combined by intercalation with respect to temperature. A block of n (number of data sets to be averaged) consecutive temperature and corresponding hyperchromicity points were replaced by their average. Derivatives were calculated from the averaged data as described above. The dh/dT denaturation envelope was resolved into component thermal transitions by Gaussian curve fitting on a Du Pont 310 curve resolver. A T_m is defined as the temperature of maximum dh/dT for each transition and was reproducible to ca. ±0.7 °C. The total hyperchromicity was 38 ± 1%. Area analyses of component transitions were reproducible to ca. ±2% of the total hyperchromicity. The ratio OD_{350}/OD_{260} was less than 0.01. No increase in OD_{350} was detected throughout the melting transition.

Thermal denaturation of core chromatin samples was monitored by circular dichroism at 280 nm as described by Mandel & Fasman (1974). Temperature was increased at a rate of 0.2 °C/min. Averages of three to four independent preparations are presented below.

Digestion of Whole and Core Chromatin with Micrococcal Nuclease. Native and reassembled whole chromatin (OD_{260} ≃ 1.0, ≃ 3 mL) were digested with 4 units of micrococcal nuclease/mL at 0 °C for 20 min and with 20 units of micrococcal nuclease/mL at 37 °C as a function of time in 5 mM Tris–acetate, 20 mM ammonium acetate, 0.2 mM EDTA, pH 7.8, made 0.4 mM in $CaCl_2$ immediately prior to addition of the enzyme. Native and reassembled core chromatin (OD_{260} ~ 1.0, ~ 3 mL) was digested with 3 units of micrococcal nuclease/mL at 37 °C for 20 min in 10 mM Tris-HCl, 0.75 mM $CaCl_2$, pH 7.3. Stock solutions of micrococcal nuclease were prepared in water at 5000 units/mL and stored at −70 °C. Enzyme activities were determined as described in the Worthington Enzyme Manual. Tenfold dilutions of enzyme were prepared in 0.1% BSA immediately prior to digestion. Digestions were quenched by addition of 0.1 M EDTA to a final concentration of 10 mM. The extent of digestion was measured by the acid solubility of oligonucleotides as described by Camerini-Otero et al. (1976).

DNA Extraction and Electrophoresis. DNA from nuclease digestions and starting material (OD_{260} ~ 1.0, 1 mL) was prepared for molecular weight analysis by removal of proteins via digestion with 0.1 mg of proteinase K/mL (E. Merck) in 1 M NaCl, 0.5% $NaDodSO_4$ at 37 °C for 1 h, followed by two extractions with an equal volume of phenol–chloroform–isoamyl alcohol (24:24:1) and two extractions with chloroform–isoamyl alcohol (24:1). The aqueous extract was dialyzed vs. 2 × 5 L glass-distilled H_2O at 4 °C for 24 h and then lyophilized. Dry samples were dissolved in one-tenth strength electrophoresis buffer at ~0.2 mg DNA/mL and allowed to stand at 4 °C overnight. The DNA from nuclease digests was analyzed on 1.4% agarose slab gels electrophoresed at 20 mA for 0.5 h, followed by 40 mA for ~2 h. The electrophoresis buffer was 0.04 M Tris–acetate, 5 mM sodium acetate, 10 mM EDTA, pH 7.9. The DNA from starting

[1] Abbreviations used: BSA, bovine serum albumin; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; NHP, nonhistone protein.

Table I

	protein/DNA (g/g)[a]	H4[b]	H3[b]	H2A[b]	H2B[b]	H1[b]	H5[b]
whole chromatin	1.05	1.00	1.42	0.91	1.04	0.36	1.05
core chromatin	0.79	1.00	1.40	0.92	1.05		

[a] Total protein to DNA ratios were reproducible to ±0.05 g/g. [b] Histone band area on polyacrylamide gels stained with Amido Black expressed relative to H4. Averages of four determinations were reproducible to ±0.1.

material was analyzed in the same manner except that the agarose concentration was 0.6%. Gels were stained with 0.4 μg of ethidium bromide/mL in water and photographed by transillumination with ultraviolet light.

For calibration purposes, *Hin*cII–*Hin*dIII (New England Biolabs) restriction enzyme fragments of λ DNA (Miles Laboratories) were coelectrophoresed with all products of nuclease digestions. The sizes of the λ/*Hin* fragments, under 1200 base pairs in length, were determined by electrophoretic comparison on 4% polyacrylamide slab gels with two DNA fragments of precisely known length (kindly provided by Dr. R. Schleif). The primary standard fragments were a 970 base pair fragment of λ DNA, isolated after *Hae*III digestion (Hirsch & Schleif, 1976), and a 203 base pair sequenced fragment, isolated from a *Hae*III digest of λ DNA containing the integrated *lac* regulatory region (Ogata & Gilbert, 1977). The sizes so determined for the λ/*Hin* fragments differ slightly from values published by Maniatis et al. (1975). However, the published values are inconsistent with the primary standard fragment lengths. The DNA from starting material was calibrated with *Eco*RI (kindly provided by Dr. P. Wensink) restriction enzyme fragments of λ DNA. The sizes of the λ/*Eco*RI fragments were taken from Thomas & Davis (1975).

Gel Electrophoresis of Histones. Electrophoresis of histones was performed on sodium dodecyl sulfate–polyacrylamide gels by the modified Laemmli (1970) method, as described by Maizel (1971). A 25-cm separating gel of 13% polyacrylamide was employed, with a 1-cm stacking gel of 3% polyacrylamide. Nucleoprotein samples were dissociated by addition of one-fifth volume of a mixture containing 10% sodium dodecyl sulfate, 25% 2-mercaptoethanol, 50% glycerol, 0.05% bromphenol blue, 0.313 M Tris-HCl, pH 6.8, and heated in a boiling water bath for 2 min (Olins et al., 1977). The cooled dissociated samples, containing 10–40 μg of histone, were applied directly to the gels, and electrophoresed at 50 V for 30 h. The gels were stained with 0.1% Amido Black in 20% ethanol, 7% acetic acid, and diffusion destained in the same ethanol–acetic acid solvent (Olins et al., 1977). Gels were scanned at 570 nm with a Zeiss spectrophotometer equipped with a linear transport device. Areas of the stained histone bands were determined with a Du Pont 310 curve resolver.

Protein/DNA Ratio. Total histone to DNA ratios were determined by Lowry protein analysis (Lowry et al., 1951) of chromatin samples, using purified chicken erythrocyte H4 as a standard and absorption at 258 nm using $\epsilon_{258} = 20$ cm^{-1} (g of DNA)$^{-1}$.

Miscellaneous. All dialyses were performed in Spectrapor 3 membrane tubing treated as previously described (Adler et al., 1971) (Spectrum Medical Industries, Inc.). All chemicals were reagent grade. Solutions were prepared at room temperature with glass-distilled water.

Results

Macromolecular Composition. We have focused our attention on the self-assembly of the basic structural components of chromatin: histones and DNA. Such material is obtainable free of proteolysis, from chicken erythrocytes. Histone composition of whole and core chromatin (H1 and H5 histones removed) is summarized in Table I. These staining ratios may be compared with histone staining ratios of chicken erythrocyte nuclei given in Table I of Cowman & Fasman (1978). The amounts of H1 and H5 relative to H4 are identical within experimental error for nuclei and whole chromatin. The protein/DNA (g/g) ratio of 1.05 obtained for whole chromatin is in excellent agreement with the value of 1.07 calculated for two lysine rich histones per 210 base pair repeating unit (Olins et al., 1976, 1977). Core chromatin contained 0.79 g of protein/g of DNA. The expected ratio for one core histone octamer per 210 base pairs is 0.77. Protein/DNA ratios of reassembled whole and core chromatin were identical with native material within experimental error. Only histone protein bands were visible on 13% polyacrylamide–NaDodSO$_4$ gels up to loads greater than 0.1 mg of total protein/gel. Relative staining ratios for core histones are identical for whole and core chromatin. No lysine-rich histone was detected in core chromatin by gels containing ~0.1 mg of total histone. The molecular weight of DNA in these preparations as determined by gel electrophoresis calibrated with λ/*Eco*RI DNA fragments ranged from 4×10^6 to 35×10^6 with a most probable value of 14×10^6. This corresponds to ~100 nucleosomes per chain.

Dissociation/Reassociation of Whole Chromatin. Dissociation of whole or core chromatin was accomplished by dialysis vs. 2 M NaCl (10 mM sodium phosphate, 0.25 mM EDTA, pH 7.0) at 21 or 4 °C. Reassociation occurred during continuous flow linear gradient dialysis to either 0.25 mM EDTA, 1.0 mM sodium phosphate, pH 7.0, or 0.25 mM EDTA, pH 7.0, at the identical temperature of the dissociation.

Circular Dichroism. A conformational evaluation of the reassembled materials was made by circular dichroism (CD). Optical activity at wavelengths above 250 nm corresponds entirely to DNA while the region below 250 nm represents a superposition of contributions from both histone peptide and DNA nucleotide. The peak value, $[\theta]_{283} = 4000 \pm 300$ deg cm^2/dmol, with shoulder, $[\theta]_{274} = 3700 \pm 300$ deg cm^2/dmol, and crossover at 261 nm for native whole chromatin (Figure 1) are in agreement with previous reports for whole chromatin (Shih & Fasman, 1970; Simpson & Sober, 1970; Permogorov et al., 1970; Henson & Walker, 1970). Free chicken erythrocyte DNA under these conditions displays a corresponding peak value of $[\theta]_{276} = 9200 \pm 300$ deg cm^2/dmol (data not shown). This reduction in ellipticity has been suggested to represent a contribution from a Ψ-type condensation of part of the B-form DNA around the histone octamer of the core particle (Cowman & Fasman, 1978). It is apparent in Figure 1 that the CD properties of the folded DNA are identical in native and dissociated/reassociated whole chromatin. The region below 250 nm is mainly due to the optical activity of the histone peptide chromophore. The two negative peaks at 208 and 222 nm with ellipticities of −45 000 ± 3000 and −36 000 ± 2000 deg cm^2/dmol of nucleotide, respectively, are characteristic of a substantial amount of

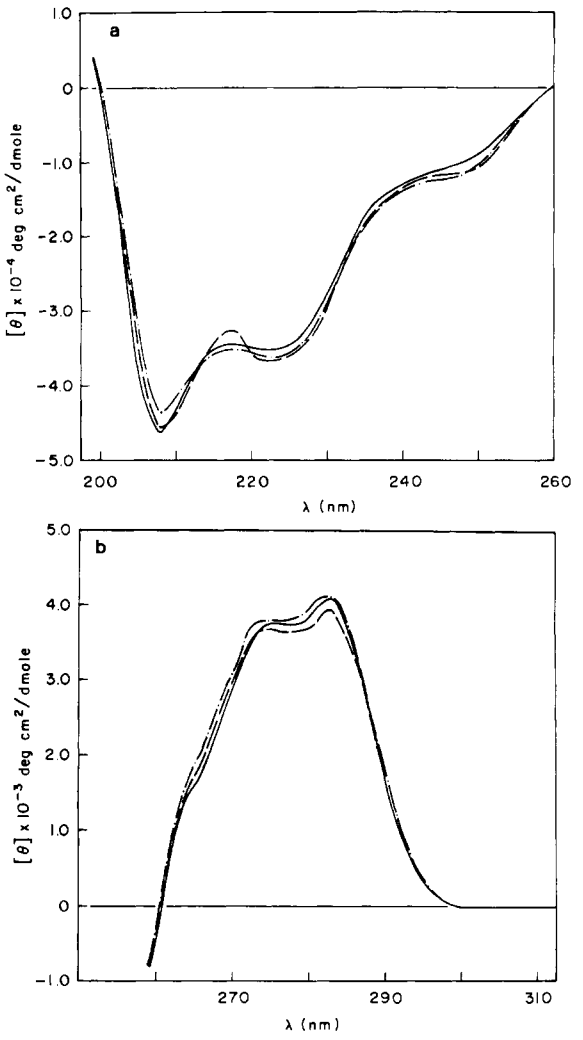

FIGURE 1: Circular dichroism spectra of native whole chromatin and products of dissociation/reassociation. Native whole chromatin (—); whole chromatin dissociated/reassociated at 4 °C (- - -) and 21 °C (-·-·) recorded in 0.25 mM EDTA, pH 7.0, at 5 °C. (a) Peptide region below 250 nm; (b) nucleotide region above 250 nm.

α-helical content of the histones. The CD spectrum for free chicken erythrocyte DNA at low ionic strength was subtracted from the spectra shown in Figure 1a (an operation of unknown validity) and the difference was converted to peptide concentration units using the measured histone/DNA ratios reported above. The resultant curve was fitted to a linear combination of model secondary structure basis spectra for the α helix and β sheet [poly(L-lysine); Greenfield & Fasman, 1969] and the random coil basis spectrum was taken as the average of the individual core histones in 10^{-3} N HCl (Fulmer & Fasman, unpublished data). This resulted in an estimated total histone conformation in whole chromatin of ~25% α helix, ~10% β sheet, and ~65% random coil. The CD properties in this region are identical within experimental error for native whole chromatin and the products of reassembly at both 21 and 4 °C. Thus, the histone secondary structure was not significantly different in reassembled and native whole chromatin.

Thermal Denaturation. Macromolecular conformational transitions reflect the forces which maintain the native structure. Perturbation of the DNA helix–coil transition by bound histones provides an energetic criterion for comparing native whole and core chromatin to the products of reassembly. Derivative plots of the thermal denaturation of native whole chromatin and dissociated/reassociated whole chromatin at

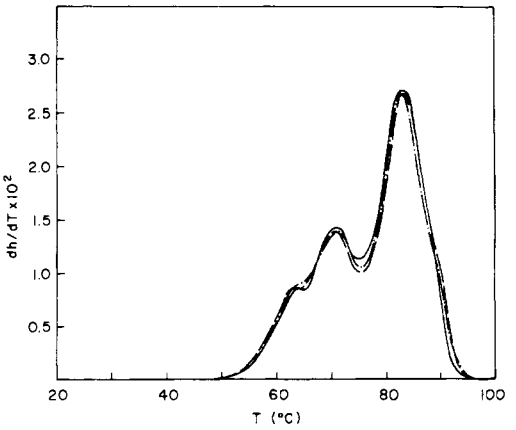

FIGURE 2: Derivative profiles of hyperchromicity melts, dh_{260}/dT vs. T, of native whole chromatin and products of dissociation/reassociation. Native whole chromatin (—); whole chromatin dissociated/reassociated at 4 °C (- - -) and 21 °C (-·-·) recorded in 1.0 mM sodium phosphate, 0.25 mM EDTA, pH 7.0.

4 and 21 °C are shown in Figure 2. The data are identical within experimental error. Three thermal transitions are observed: transition I with T_{m_I} = 62.5 °C represents ~14% of the total hyperchromicity; transition II with $T_{m_{II}}$ = 71.0 °C represents ~24% of the total hyperchromicity; and transition III with $T_{m_{III}}$ = 83.7 °C represents ~62% of the total hyperchromicity. A detailed analysis of these thermal transitions will be presented elsewhere (Fulmer & Fasman, manuscript in preparation). Recent reviews on thermal denaturation studies of chromatin by Ansevin (1978) and Li (1978) have been published.

Micrococcal Nuclease Digestion. Morphological characterization of native whole chromatin by digestion with micrococcal nuclease is shown in Figure 3a (track III). At least eight DNA fragments were observable which indicated a repeating unit of ~205 ± 10 base pairs of DNA when compared with the λ/Hin DNA fragments described in Materials and Methods. DNA fragment profiles of native whole chromatin were identical when nuclease digestions were carried out at either 0 or 37 °C. This is in agreement with previously published values for the repeating unit of chicken erythrocyte chromatin of ~210 base pairs (Morris, 1976b; Lohr et al., 1977; Rill et al., 1977; Wilhelm et al., 1977). Micrococcal nuclease digestion at 0 °C of whole chromatin dissociated and reassociated at either 4 or 21 °C did not result in discrete fragments of DNA, but rather a broad continuous distribution of DNA of decreasing mean molecular weight with increasing digestion time (data not shown). Micrococcal nuclease digestions at 37 °C of reassembled whole chromatins displayed identical kinetics for release of acid-soluble nucleotides as found for the native starting material (Figure 3b). A repeating nucleosomal subunit structure is barely discernible in the reassembled whole chromatins when digested at 37 °C. However, this subunit repeat has decreased to 160 ± 10 base pairs of DNA (Figure 3a, tracks I and II). Prolonged digestion of both native and reassembled whole chromatins resulted in ~140 base pair core particles (Figure 3a, tracks IV–VI). Thus, the amount of DNA in the reassembled core particle is identical with native starting material. Only the internucleosomal spacing has been altered upon reassembly. It is also noted that the background of DNA on gels of reassembled whole chromatin digests at 37 °C is significantly higher than on digests of native whole chromatin.

Dissociation/Reassociation of Core Chromatin. Micrococcal Nuclease Digestion. DNA fragments resulting from micrococcal nuclease digestion of core chromatin and products

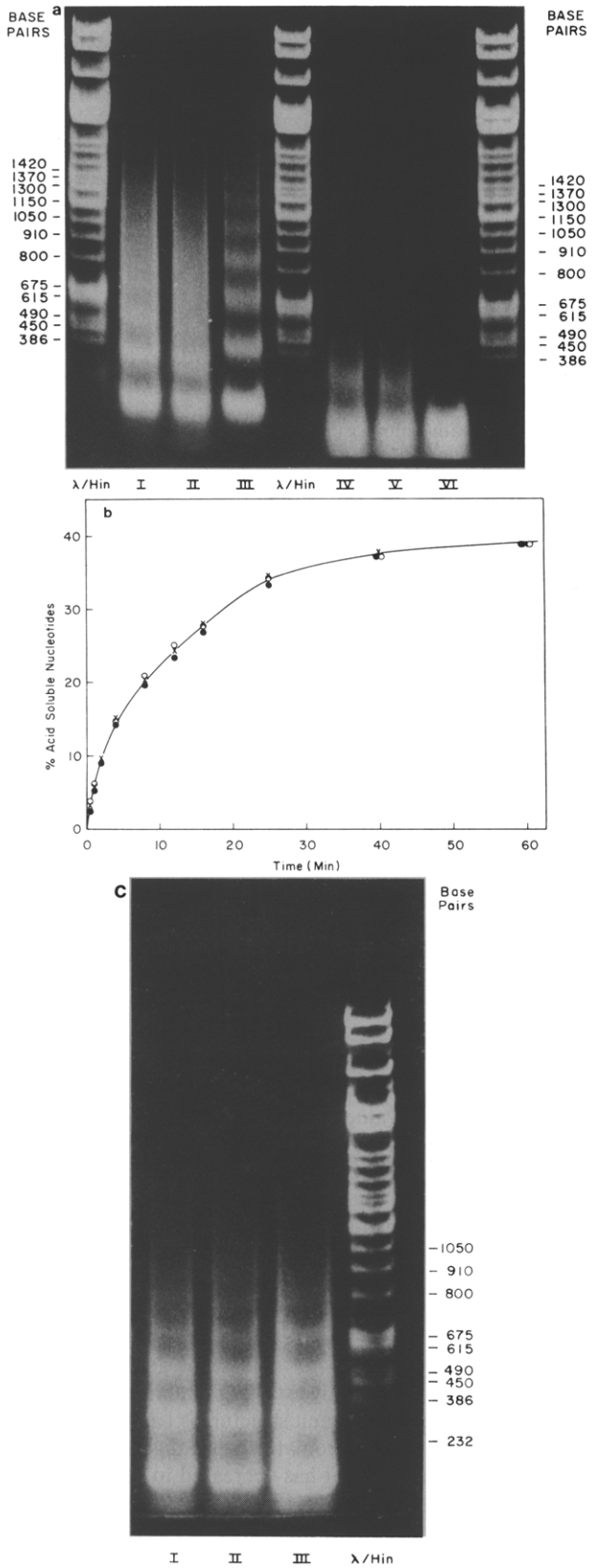

FIGURE 3: (a) Micrococcal nuclease digestion of whole chromatins. Whole chromatin reassembled at 21 °C digested to 6% (I) and 39% (IV) acid solubility; whole chromatin reassembled at 4 °C digested to 6% (II) and 39% (V) acid solubility; native whole chromatin digested to 6% (III) and 39% (VI) acid solubility. (b) Micrococcal nuclease digestion kinetics monitored by release of acid soluble nucleotides: (●) native whole chromatin; (×) whole chromatin reassembled at 4 °C; (○) whole chromatin reassembled at 21 °C. (c) Micrococcal nuclease digestion of core chromatins: I, core chromatin reassembled at 21 °C; II, core chromatin reassembled at 4 °C; III, native core chromatin.

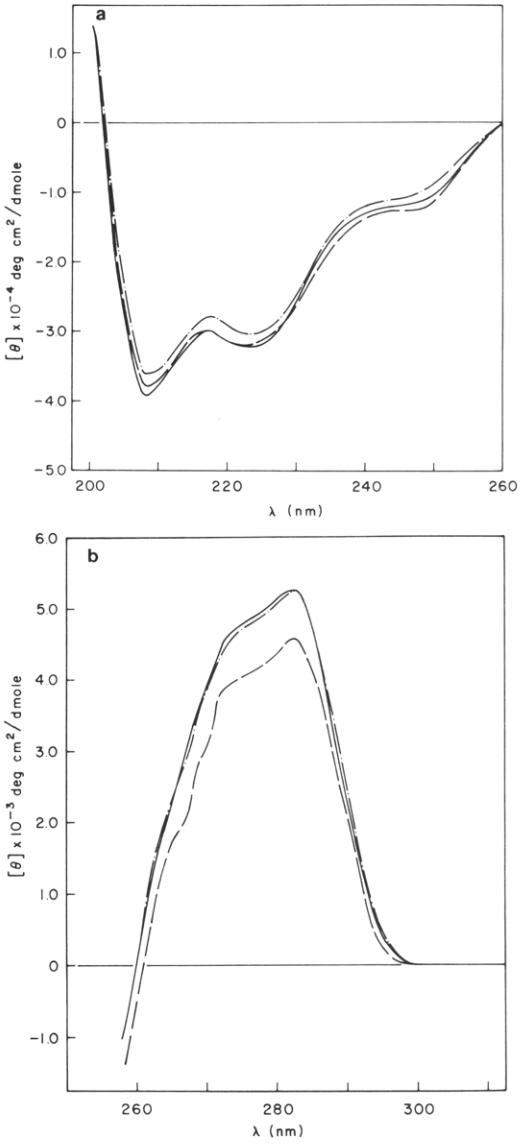

FIGURE 4: Circular dichroism spectra of native core chromatin and products of dissociation/reassociation. Native core chromatin (—); core chromatin dissociated/reassociated at 4 °C (- - -) and 21 °C (----), recorded in 0.25 mM EDTA, pH 7.0 at 5 °C. (a) Peptide region below 250 nm; (b) nucleotide region above 250 nm.

of reassembly at 4 and 21 °C are shown in Figure 3c. Both the kinetics of digestion measured by acid solubility (data not shown) and the distribution of digestion products (Figure 3c) were identical within experimental error for native and reassembled core chromatin. The length of the repeating unit of core nucleosome was found to be 150 ± 10 base pairs. This is compatible with a repeating unit of 137 ± 7 base pairs for core chromatin reported by Steinmetz et al. (1978).

Circular Dichroism. Conformational evaluation by CD of native core chromatin and reassembled products can be seen in Figure 4. Removal of lysine-rich histone from whole chromatin resulted in an increase of $[\theta]_{283}$ to 5200 ± 300 deg cm^2/dmol as previously reported (Simpson & Sober, 1970; Li et al., 1975). The shoulder at 274 nm has increased to $[\theta]_{274}$ = 4700 ± 300 deg cm^2/dmol while the crossover point has shifted to the blue by 1 nm, to 260 nm. The increase in ellipticity may represent the release of tertiary structural constraints placed upon the DNA by the lysine-rich histones (Li et al., 1975; Cowman & Fasman, 1978). Reassembly at 21 °C yielded a product with an identical CD spectrum in this spectral region. However, reassembly at 4 °C resulted in a

small but reproducible decrease in the peak ellipticity of the reconstitute to $[\theta]_{283}$ = 4500 ± 300 deg cm^2/dmol. The shoulder at 274 nm has dropped to 3800 ± 300 deg cm^2/dmol and the crossover point returned to 261 nm. Thus, the DNA was not condensed by the core histones at 4 °C in exactly the same manner as native core chromatin and core chromatin reassembled at the higher temperature. The peptide CD region, below 250 nm, is identical within experimental error for native and reassembled core chromatin at both temperatures of reassembly. Two negative peaks are observed at 208 and 222 nm with ellipticities of −38 000 ± 3000 and −31 000 ± 2000 deg cm^2/dmol of nucleotide, respectively, with a crossover at 202 nm. Analysis of histone secondary structure as described above for whole chromatin resulted in an average core histone conformation of ~36% α helix, ~2% β sheet, and ~62% random coil. This is in agreement with the pronounced α-helical and random coil character of core particles detected by infrared spectroscopy (Cotter & Lilley, 1977). Secondary structural estimates on core histones by analysis of CD and Raman spectra by Thomas et al. (1977) indicate ~50% α helix, 5–13% β sheet, and 36–51% random coil. Combination of the whole and core chromatin analyses in the present study allows one to calculate an approximate secondary structure for the lysine-rich histones on whole chromatin of ~28% β sheet and ~72% random coil with negligible α helix. This assumes that the core structure remains unaltered in the presence of the H1 and H5.

A sample reassembled at 4 °C was allowed to incubate at 21 °C for 2 days before remeasuring the CD spectrum. Instead of approaching the spectral characteristics of native core chromatin, the ellipticity decreased to a value of $[\theta]_{283}$ ~ 3800 deg cm^2/dmol. The low temperature reassembled core chromatin product appears to be locked into a different state at low ionic strength.

Thermal denaturation of native and reassembled core chromatin monitored at $[\theta]_{280}$ is shown in Figure 5. Denaturation profiles of native and reassembled core chromatin at 21 °C are essentially identical within experimental error. The ellipticity at 280 nm is independent of temperature from 5 to 45 °C. A subsequent decrease in ellipticity is observed with an apparent transition midpoint of ~55 °C. This has been attributed (Wilhelm et al., 1974a) to the melting of regions of DNA not directly stabilized by bound histones (linker regions). A second transition with a sharp increase in ellipticity with an apparent midpoint of ~72 °C is observed. This transition has been attributed (Ong & Fasman, 1976; Mandel & Fasman, 1976) to the release of the DNA tertiary structural constraints. A third transition is observed by a decrease in ellipticity upon complete denaturation of the DNA. The change in $[\theta]_{280}$ of core chromatin reassembled at 4 °C upon thermal denaturation is distinctly different than native material. The most obvious differences include the presence of a premelt below 50 °C (Wilhelm et al., 1974b) in core chromatin reassembled at 4 °C, different ellipticities at the temperature regions of overlap in component transitions, and the ellipticity of the final denatured state of $T > 95$ °C. Due to the complicated overlap of descending and ascending transitions in $[\theta]_{280}(T)$, it is not possible to quantitatively interpret the observed differences. However, it is clear that the low temperature reassembled core chromatin does follow a different denaturation path than native core chromatin. We must conclude that the details of histone–DNA packing are not the same as in native core chromatin.

Thermal unstacking of DNA base pairs in native and reassembled core chromatin monitored by hyperchromicity at 260 nm is shown in Figure 6. Native core chromatin displays three major thermal transitions: transition I with T_{m_I} = 55.5 °C represents ~47% of the total hyperchromicity; transition II with $T_{m_{II}}$ = 69.5 °C represents ~14% of the total hyperchromicity; and transition III with $T_{m_{III}}$ = 80.7 °C represents ~39% of the total hyperchromicity. A detailed analysis of these thermal transitions will be presented elsewhere (Fulmer & Fasman, manuscript in preparation). Although the relative areas of the corresponding thermal transitions in reassembled core chromatin at both 4 and 21 °C remain identical within experimental error to native core chromatin, the T_m's have shifted to lower temperatures. The reassembled product at 21 °C resulted in T_{m_I} = 55.0 °C, $T_{m_{II}}$ = 67.0 °C, and $T_{m_{III}}$ = 79.0 °C, while that reassembled at 4 °C displayed T_{m_I} = 54.0 °C, $T_{m_{II}}$ = 66.0 °C, and $T_{m_{III}}$ = 77.0 °C. The derivative profiles in Figure 6 represent averages of three to four independent preparations and the shifts in T_m's are outside of experimental error. It is apparent that the amount of DNA melting in each cooperative thermal transition is the same in native and reassembled core chromatin. We also note that the transition widths are unaltered upon reassembly at either temperature. Thus, the reassembled products appear to represent similar distributions about different mean energies of interactions between histone cores and DNA. The binding energy of association of these structural domains is reduced in the reassembled product at 21 °C compared with core chromatin and even less in the product at 4 °C. We must conclude that the details of histone–DNA packing in both reassembled products are not the same as in native core chromatin. Recent reviews on thermal denaturation studies of chromatin by Ansevin (1978) and Li (1978) have been published.

Whole chromatin reassociated at 4 °C was subsequently stripped of lysine-rich histone as described in Materials and Methods. The hyperchromicity melt of this material is also shown in Figure 6. It is essentially identical with that of native core chromatin. Thus, it appears that the lysine-rich histones play a special role in modulating the assembly of core particles along the chain of high molecular weight DNA.

Discussion

Chromatins obtained from a variety of sources all appear to contain the same basic histone structural components but differ in amounts of nonhistone protein (NHP). Whereas calf thymus and rat liver chromatin preparations generally contain respectively ~0.4 (Tuan et al., 1977; Hjelm & Huang, 1974) and ~0.7 (Stein et al., 1975; Defer et al., 1977) g of NHP/g of DNA, the chicken erythrocyte chromatin used in the present study shows no detectable proteins other than histones. Detailed reconstitution studies on this basic level of organization should precede subsequent explorations on the more complex and biologically relevant material containing all chromosomal components.

A recent study by de Murcia et al. (1978) has indicated that native whole chromatin should display $[\theta]_{283}$ ~2000 deg cm^2/dmol, while values of ~4000 deg cm^2/dmol represent shear damaged nucleosome core particles. This proposition was based on previously observed values of ~2000 deg cm^2/dmol (Mandel & Fasman, 1976; Lawrence et al., 1976) for mononucleosome core particles. However, Cowman & Fasman (1978) have shown that the peak ellipticity in the 280-nm spectral region is sensitive to the length of linker DNA and that the full repeating unit of ~200 base pairs displays optical properties essentially identical with the whole chromatin used in the present study. We suggest that the low ellipticity observed by de Murcia et al. (1978) is due to additional

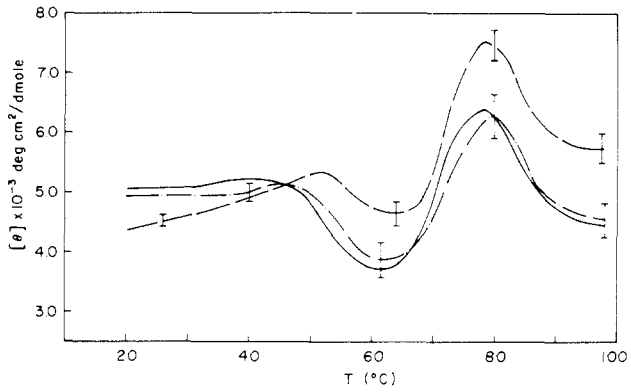

FIGURE 5: Thermal denaturation monitored by $[\theta]_{280}$ vs. T of native core chromatin and products of dissociation/reassociation. Native core chromatin (—); core chromatin dissociated/reassociated at 4 °C (- - -) and 21 °C (····) recorded in 0.25 mM EDTA, pH 7.0.

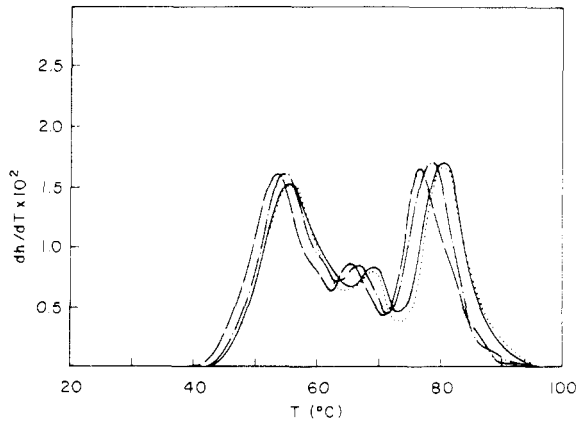

FIGURE 6: Derivative profiles of hyperchromicity melts, dh_{260}/dT vs. T, of native core chromatin and products of dissociation/reassociation. Native core chromatin (—); core chromatin dissociated and reassociated at 4 °C (- - -) and 21 °C (····) and reassembled whole chromatin at 4 °C subsequently stripped of lysine-rich histone (···) recorded in 1.0 mM sodium phosphate, 0.25 mM EDTA, pH 7.0.

tertiary folding of the linker region as evidenced by their electron micrographs.

Analysis of the CD spectra of the peptide region (below 250 nm) of whole and core chromatin by curve fitting to linear combinations of α-helix, β-sheet, and random coil conformations yielded the following results: ~36% α helix and ~62% random coil with negligible β sheet for the histones inside the core particle and ~28% β sheet and ~72% random coil with negligible α helix for the lysine-rich histones outside of the core particle. Such an analysis suffers from the questionable validity of the simple subtraction of the CD spectrum of DNA from the chromatin spectra and further assumes that no changes occur in either the core histones or DNA contributions to this spectral region upon removal of the lysine-rich histones. Thus, the above values are to be regarded as approximate. The results for the core histones are in qualitative agreement with previous studies on core particles employing infrared spectroscopy (Cotter & Lilley, 1977) and with CD and Raman spectroscopy studies on isolated core histones (Thomas et al., 1977).

It is apparent from several studies (Oudet et al., 1975; Boseley et al., 1976; Camerini-Otero et al., 1976) that histones separated from DNA even under denaturing conditions are capable of regenerating subunits morphologically similar to nucleosomes when recombined with DNA. However, the integrity of the reconstitute's inter- and intranucleosomal architecture has yet to be established. In the absence of high resolution diffraction data on intact material, we have employed biochemical, spectroscopic, and energetic comparisons to the simplest form of reconstitution: dissociation/reassociation in the absence of denaturants. Although such data are difficult to interpret on precise structural grounds, they provide rigorous guidelines in assessing the quality of reassembly.

The present study exemplifies the importance of comparative studies employing a variety of independent criteria. Conformational properties of histones and DNA in whole chromatin are regenerated upon in vitro reassembly by salt gradient dialysis to the extent reflected by circular dichroism. This is in agreement with the previous report of Stein et al. (1975). The energetics of histone–histone and histone–DNA binding as reflected in the thermally induced helix–coil transitions of the DNA in native and reassembled whole chromatin are indistinguishable. However, whole chromatin reassembled by salt gradient dialysis differs from native whole chromatin in internucleosomal spacing along the high molecular weight DNA as determined by digestion with micrococcal nuclease.

Removal of the lysine-rich histones from native whole chromatin, by dissociation with 0.6 M NaCl, resulted in the appearance of up to five discrete bands which were multiples of 150 ± 10 base pairs of DNA throughout all stages of digestion with micrococcal nuclease. Thus, a minimum in free energy is obtained upon removal of the lysine-rich histones via favorable interactions between core histone octamers which resulted in clusters of core particles separated by regions of free DNA of correspondingly increased length rather than random sliding of core particles along the DNA. Removal of lysine-rich histone at low ionic strength by tRNA displacement was reported (Noll & Kornberg, 1977) to preserve the ~200 base pair repeating unit. The higher ionic strength used in the present study may lower the activation energy for the spontaneous intercore particle association.

Studies on core chromatin show that core histones are incapable of reassembling native core particles on high molecular weight DNA in the absence of lysine-rich histones under the conditions investigated. Repackaging of the core particle DNA (as judged by CD) and reestablishment of native core histone–DNA interactions (as judged by thermal denaturation) were not achieved by reassembly of core chromatin at 4 °C. Reassembly at 21 °C fully accomplished the former, but only partially the latter. Thermal denaturation (Figure 6) of core chromatin which had been reassembled at 4 °C in the presence of the lysine-rich histones, which were consequently removed, indicates that native core histone–DNA interactions were restored only by reassembly in the presence of the lysine-rich histones, even though they are not an integral part of the core particle. These results support specific interactions between lysine-rich histones and the core particle. Recent cross-linking studies by Bonner (1978) and Glotov et al. (1978) have shown that H1 may be cross-linked to core histones in whole chromatin. Such interactions may participate in reassembling native *intra*nucleosomal architecture.

Given the results of the present study and observations by others, the authors view the in vitro assembly of whole chromatin as follows. Complete dissociation of histones from DNA is achieved in 2 M NaCl (Ohlenbusch et al., 1967). Lowering the ionic strength to ~1.2 M results in the binding of H3:H4 tetramers to DNA (Wilhelm et al., 1978). Further decrease in ionic strength to ~0.6 M results in the binding of two H2A:H2B dimers to each H3:H4-DNA prenucleosome kernal (Wilhelm et al., 1978; Camerini-Otero & Felsenfeld, 1977) to form core particles. The core particles are closely packed (150 ± 10 base pairs of DNA per repeating unit) along

the high molecular weight DNA via favorable interactions between adjacent core histone octamers. Lysine-rich histones bind to the closely packed core particles between 0.6 and 0.2 M NaCl. Binding of lysine-rich histones involve at least two structurally distinct macromolecular domains. The first domain involves a part of the lysine-rich histone and sites on or within the core particle. These interactions are supported by (a) the necessity of lysine-rich histone to regenerate the above examined physical properties of reassembled core chromatin; (b) the regeneration of the above examined physical properties of reassembled whole chromatin; and (c) the proximity of lysine-rich histone to core histone as defined by cross-linking studies (Bonner, 1978; Glotov et al., 1978). The interactions in this domain *are* reassembled into a native-like conformation. The second domain involves perhaps the remainder of lysine-rich histone and sites on the DNA of the linker region (Noll & Kornberg, 1977). Due to attractive forces between core particles, these interactions *are not* reassembled into a native-like conformation. The close packing of nucleosomes observed by nuclease digestion at 37 °C (160 ± 10 base pairs/repeating unit) in reassembled whole chromatins indicates that the binding energy of lysine-rich histone is insufficient to overcome these favorable intercore particle interactions. Thus, the lysine-rich histone may be forced to choose, at least in part, an aberrant binding site at low ionic strengths and temperatures which results in the loss of the differential susceptibilities of core and linker DNA to nuclease digestion at 0 °C.

A very thorough study by Tatchell & Van Holde (1977) has shown that 140 base pair core particles can be reassembled at 4 °C, pH 8.0, into products indistinguishable from native core particles by a variety of independent criteria. The assembly may be both temperature and ligand (H^+) linked and/or sensitive to the molecular weight of the DNA. One might expect the increased rotational and translational mobility of 140 base pair DNA over the high molecular weight DNA used in the present study to assist the assembly process. The apparent discrepancy may result from a structural relaxation of isolated core particles to a state which may be relocated upon reassembly or may simply reflect possible synergetic effects among core particles on high molecular weight core chromatin.

Our present conclusions on a temperature-dependent core chromatin reconstitution are supported by Steinmetz et al. (1978). Reconstitution at 4 °C resulted in higher backgrounds on DNA gels of micrococcal nuclease digestion products when compared with native core chromatin. The 4 °C reassembled product also displayed slight differences in DNase I digestion fragment distribution. Studies on reconstitution of H3 and H4 with closed circular DNA at 4 °C (Bina-Stein & Simpson, 1977) have shown that maximum superhelicity is induced only after prolonged dialysis against 0.6 M NaCl. The temperature dependence of core chromatin reassembly observed in the present study may represent a kinetic barrier (due to the nonequilibrium nature of continuous flow gradient dialysis) which may be overcome by the participation of lysine-rich histone in the assembly process. Equilibrium measurements of the assembly process as a function of temperature are currently in progress.

In summary, our results demonstrate that whole chromatin containing only lysine-rich and core histones is not a true self-assembling system in vitro by salt gradient dialysis under the conditions investigated herein. Reassembled whole chromatin bears several similarities to native whole chromatin as reported in previous studies (Oudet et al., 1975; Boseley et al., 1976; Camerini-Otero et al., 1976; Garel et al., 1976; Yaneva et al., 1976; Steinmetz et al., 1978; Godski & Chae, 1978; Wilhelm et al., 1978), but is not totally identical. The data support the structural integrity of core particles, on high molecular weight DNA, reassembled in the presence of lysine-rich histone and suggest that differences lie in intercore particle and/or lysine rich histone interactions. Laskey et al. (1977) have reported the requirement of a nonhistone thermolabile factor(s) present in a *Xenopus* egg extract to regenerate the native internucleosomal repeating pattern upon digestion with micrococcal nuclease. The failure of whole chromatin to reassemble properly in the present study supports the possible existence of such accessory factor(s) required for proper in vitro assembly.

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