Assembly of DNA with Histones and Nonhistone Chromosomal Proteins in Vitro†

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ABSTRACT: We have examined, by protein binding assays, thermal denaturation, and circular dichroism, the possible effects of histones on nonhistone chromosomal protein (NHCP) interactions with DNA. For these studies, we have fractionated mouse Krebs II chromosomal proteins into three discrete fractions: M_0, 5 M urea-soluble NHCP; M_1, 5 M urea-1 M NaCl-soluble NHCP from 5 M urea-extracted chromatin; and M_3, 5 M urea-3 M NaCl-soluble chromosomal proteins from 5 M urea-1 M NaCl-extracted chromatin. These fractions contain heterogeneous populations of NHCP, and were found to differentially affect histone binding to DNA by methods of reconstitution, or by direct binding of M_0, M_1, or M_3 to urea-salt reconstituted DNA with histones. M_0 was found to exert a unique effect on the thermal denaturation and circular dichroic spectra of DNA-histone complexes. M_0 from Krebs II chromatin was also found to compete for DNA sites in the presence of M_0 from mouse liver chromatin. In addition, in 5 M urea, pH 8.0, histone binding to DNA reached saturation at 1.85 mg/mg of DNA, higher than the in vivo ratio of 1.00 mg/mg of DNA. Saturation of histone binding to DNA occurred only in the presence of 5 M urea, resulting in a reduction of nonspecific histone-histone interactions on DNA.

It has been suggested by various investigators (Kornberg, 1974; Van Holde et al., 1974; Baldwin et al., 1975, Pardon et al., 1975) that histones assemble in a chromatin subunit by forming an octamer wound about a DNA of 200 base pairs, where histone F1 is suggested to be on the outside of the globular subunit and it is involved in superpacking of DNA in chromatin (Oudet et al., 1975), or cross-linking between subunits in the same chromatin chain. Both the active and the inactive portion of the genome seem to be arranged in a subunit fashion (Arnold and Young, 1976). We have, therefore, sought in our studies to determine whether we could affect the structure of histone-bound DNA by various classes of salt-urea fractionated NHCP,[1] thereby supporting a model for gene regulation in which certain classes of NHCP function to influence the physical structure of chromatin for transcription of various DNA sequences. The nonhistones, a very heterogenous group of proteins, have been implicated in certain positive control of RNA synthesis (Teng et al., 1971; Kostraba and Wang, 1972; Stein et al., 1972; Shea and Kleinsmith, 1973; Barrett et al., 1974); hence, it is of significance to examine how histones and nonhistones interact with DNA to influence chromatin structure and presumably result in gene activation or repression. More recently (Lapeyre and Bekhor, 1976), we have observed that, in 0.24 N NaCl, histones increased the quantity of certain classes of NHCP that could bind to DNA. In some of our experiments, we have undertaken binding assay methods to explore more closely how classes of chromosomal proteins interact with each other and with DNA, either by reconstitution (Bekhor et al., 1969) or by direct mixing (Lapeyre and Bekhor, 1976). We have fractionated NHCP into three groups depending on their dissociation properties from DNA in 5 M urea in the presence of increasing amounts of NaCl. We have also investigated how histones, in urea, influence the binding of various classes of NHCP to DNA.

Materials and Methods

Cells, Preparation, and Labeling. Murine Krebs II (KII) ascites tumor cells were propagated according to the method of Matthews and Korner (1970) in the peritoneal cavity of adult Swiss-Webster mice. KII cells, utilized for labeling, were harvested from mice on day 7, diluted, and placed in a spinner culture at about 10^6 cells/ml and incubated at 37 °C for 48 h. The medium used for all labeling experiments is calcium-free Ham's F-10, buffered with 20 mM Hepes (Sigma), pH 7.35, and supplemented with 100 units of penicillin-100 μg of Streptomycin/ml (Grand Island Biologicals) and 10 μM each of thymidine and leucine. Cells were labeled with either L-[4,5-^{3}H]leucine (33-44 Ci/mmol, New England Nuclear) at 10 μCi/ml, or L-[U-^{14}C]leucine (280 mCi/mmol New England Nuclear) at 0.5 μCi/ml.

Chromatin Preparation and Fractionation of Chromosomal Proteins. Chromatin was prepared essentially according to the methods of Bhorjee and Pederson (1973) with modifications. Either KII cells or mouse livers were isolated and immediately placed in a 4 °C solution of cell wash buffer (NET: 0.1 M NaCl, 1 mM EDTA, 0.01 M Tris, pH 8.0, at 4 °C). Cells were harvested by low-speed centrifugation in a refrigerated Sorvall at 1500 rpm for 5 min and washed two times in NET. Cells were then suspended in RSB buffer (NaCl, 0.01 M NaCl; 1.5 mM $MgCl_2$, 0.01 M Tris, pH 8.0, at 4 °C, containing 0.2% NP40 (Shell Chemical Co.)) and homogenized for a total of 20 strokes in a Teflon-glass homogenizer. Nuclei were recovered by centrifugation at 1000*g* for 5 min and washed three times in 10 volumes of RSB without NP40. Nuclei were suspended in TPD (0.01 M Tris, pH 8.0, 0.2 mM DTT (Sigma),

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[1] Abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; NHCP, nonhistone chromosomal proteins; BSA, bovine serum albumin; TPD, 0.01 M Tris, 1 mM phenylmethanesulfonyl fluoride, 0.2 mM dithiothreitol, pH 8.0; NET, 0.1 M NaCl, 1 mM EDTA, 0.01 M Tris, pH 8.0; RSB, 0.01 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris, pH 8.0-0.2% NP40; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CD, circular dichroism; OD, optical density.

TABLE I: Fractionation and Classification of Chromosomal Proteins Extracted from Krebs II Chromatin.

Fraction No.	Conditions of Extraction	Designation	μg Extracted/mg of DNA (= 1.0 equivalent)	Average Mol Wt ($\times 10^{-4}$)
1	34-h 60 × 10^3 rpm supernatant from medium 0 suspended chromatin	M_0	260	8.6
2	42-h 60 × 10^3 rpm supernatant from medium 1 suspended medium 0 pellet	M_1	640	6.5
3	42-h 60 × 10^3 rpm supernatant from medium 3 suspended medium 1 pellet	M_3	75	9.4
4	42-h 60 × 10^3 rpm supernatant from medium 1 suspended medium 0 pellet, and separated from NHCP on Bio-Rex 70.	Histones	1000	1.5

1 mM PMSF) at a concentration of 7 × 10^7/ml, allowed to swell, and were homogenized a total of 40 strokes in a tight-fitting Kontes Teflon–glass homogenizer. Crude chromatin was sedimented through 1.7 M sucrose in TPD for 2 h at 25 × 10^3 rpm in a Beckman SW27 rotor. Purified chromatin was twice suspended in 8 volumes of TPD with a Teflon–glass homogenizer and pelleted by centrifugation for 10 min at 20 000*g*. Chromatin was sheared in 5-ml portions at 20 OD_{260}/ml in the omnimixer microcup with three 1-min bursts at full speed followed by removal of aggregates by centrifugation at 20 000*g* for 10 min. Chromosomal proteins were fractionated from unsheared chromatin essentially as described by Bekhor et al. (1974), with certain modifications into various urea–salt soluble fractions. Freshly prepared, purified, unsheared chromatin was first suspended at 25 OD_{260}/ml in medium 0 (5 M urea in TPD) and left to swell for 2 h at 0 °C. The urea-soluble proteins were separated from the chromatin by centrifugation for 34 h at 60 × 10^3 rpm in a Beckman 60 Ti rotor at 0 °C. These urea-soluble proteins will be termed here M_0 proteins. Medium 1 proteins were prepared by suspending the medium 0 extracted pellet in medium 1 (medium 0 containing 1 M NaCl) and centrifuging for 42 h at 60 × 10^3 rpm in the Beckman 60 Ti rotor. Medium 3 proteins were prepared by suspending the medium 1 extracted pellet in medium 3 (medium 0 + 3 M NaCl) and again spinning for 42 h at 60 × 10^3 rpm. All protein fractions prepared were either used directly in subsequent experiments or frozen in aliquots in liquid N_2. DNA for reconstitution was prepared as previously described (Lapeyre and Bekhor, 1976).

Separation of Histones and Nonhistone Proteins. Several methods of separation were attempted and the most successful one, in our hands, was that of Levy et al. (1972). The medium 1 protein fraction containing at least 99% of the histones solubilized from chromatin was dialyzed extensively against medium G (5 M urea, 0.3 M guinidine-HCl (Mann, ultrapure), 0.1 M sodium phosphate, pH 7.0, 1 mM PMSF, 6 mM 2-mercaptoethanol) for Bio-Rex 70 separation (Na^+ form, 200–400 mesh, Bio-Rad). The medium 1 protein fraction was passed through a Bio-Rex 70 column equilibrated with starting buffer. NHCP were eluted in medium G, while histones remained bound. Histones were eluted with medium 3. Recovery in proteins was about 90%. All proteins used for reconstitution were dialyzed up to medium 3. Proteins were concentrated, if required, in an Amicon high-pressure cell.

Preparation of DNA. DNA was isolated from purified Krebs II chromatin by the sodium dodecyl sulfate–Pronase–chloroform–isoamyl alcohol technique, using autodigested Pronase (Calbiochem). Chromatin was incubated with 50 μg/ml of Pronase in 1% sodium dodecyl sulfate, 10 mM Tris, 0.1 M NaCl, 1.0 mM EDTA, pH 7.4, at 37 °C for 30 min, followed by one extraction with equal proportions of redistilled phenol and chloroform–isoamyl alcohol (24:1, v/v), and two extractions with chloroform–isoamyl alcohol. After overnight dialysis against deionized water, chromatin was incubated with 20 μg of RNase (boiled)/ml and 10 units of RNase T_1 (Worthington)/ml at 37 °C for 10 min in 10 mM Tris, pH 7.4. Subsequently, the DNA solution was made 1% in sodium dodecyl sulfate and later digested with 50 μg of pronase/ml, as described above. The DNA was deproteinized as above, ethanol precipitated, dissolved in 0.01 N NaCl, and dialyzed against deionized water for further use.

Chromosomal Protein–DNA Complexes and Binding Assays. Labeled proteins were bound to DNA either by reconstitution as previously described (Bekhor et al., 1969) or by direct binding as described below. Input equivalents of the protein (either histones or nonhistones) varied from 0.1 to 10 equivalents/unit of DNA, depending on the experiment. An equivalent is defined as the amount in mass units of a protein in a class that would be solubilized from a given amount of DNA (Table I). In reconstitution, various concentrations of proteins and DNA (75–100 μg/ml) in medium 3 were gradient dialyzed to medium 0 or 0.24 TPD (0.24 N NaCl in TPD). Complexes in urea (in experiments where we reconstitute DNA with M_1 or M_3 proteins and histones) were recovered by centrifugation in a Beckman SW65 rotor at 65 × 10^3 rpm for 20 h. Complexes in 0.24 TPD (in experiments where we reconstitute M_0 proteins with DNA and histones) were separated from free protein by centrifugation for 30 min at 30 × 10^3 rpm in a Beckman 40.3 rotor, followed by two washings in 0.24 TPD. For radioactivity measurements, the pellets were dissolved in 1% sodium dodecyl sulfate, and counted in Triton X114–xylene cocktail (Anderson and McClure, 1973).

Direct binding of NHCP to DNA–histone complexes was done as follows. DNA was reconstituted with known concentrations of histones by gradient dialysis from medium 3 to medium 0. The DNA–histone complexes were recovered from medium 0 by centrifugation in the Beckman SW65 rotor at 65 × 10^3 rpm for 20 h. The DNA–histone pellet was later suspended into 0.24 TPD, and dialyzed against 0.24 TPD for direct binding with various classes of NHCP in 0.24 TPD.

Thermal Denaturation. Thermal denaturation was carried out in a Gilford Model 240 spectrophotometer utilizing chart print-out and a Haake thermoregulator. Samples were heated at 0.6 °C/min and monitored at 260 nm for hyperchromicity. An automatic reference compensator was utilized to correct for solvent absorbance changes. All samples were melted in 0.25 mM EDTA, pH 8.0. Data were plotted at 1.0 °C intervals at normalized hyperchromicity, according to Li and Bonner (1971). T_m designates the temperature at midpoint of helix-coil transition of DNA or chromatin as determined from the thermal denaturation data.

Circular Dichroism. Circular dichroic spectra were measured with a Cary 61 circular dichroic spectropolarimeter calibrated with 0.1% *d*-10-camphorsulfonic acid. Spectra were

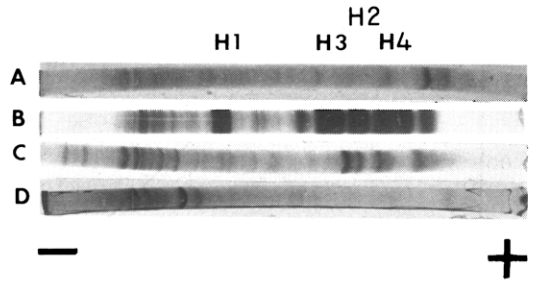

FIGURE 1: Electrophoretic mobility of various chromosomal protein fractions on sodium dodecyl sulfate-urea-polyacrylamide gels. (A) M_0 NHCP; (B) M_1 chromosomal proteins prior to chromatography on Bio-Rex 70; (C) M_1 NHCP following chromatography on Bio-Rex 70; (D) M_3 NHCP. H designates the position of the lysine- and arginine-rich histones, as seen in gel B.

recorded from 210 to 320 nm at ambient temperatures in either 0.24 TPD or TPD. The programmable baseline was set for zero, utilizing the buffers as zero reference points. Slit program was set for a bandwidth of 4 mm, and a time constant of 3 s, yielding a scan rate of 0.2 mm/s. Mean residue ellipticity [θ] is shown as deg cm^2/dmol on the basis of the DNA residue concentration. The mean DNA residue molecular weight used in our calculation was 330. For these measurements, we used DNA-chromosomal protein complexes of 0.5–1.0 OD_{260}/ml with OD_{320} not greater than 0.05 unit, indicating negligible or no light scattering.

Other Procedures. Electrophoresis of both histones and nonhistones was carried out in the sodium dodecyl sulfate discontinuous buffer system of Laemmli (1970). Protein was determined by the method of Hartree (1972), and DNA by the method of Burton (1956).

Results

Binding Studies. As described under Methods, defined classes of labeled NHCP were prepared by sequential dissociation of M_0, M_1, and M_3 from DNA (Table I). Essentially all of the histones were found in medium 1 proteins. Medium 1 proteins were then separated into M_1 NHCP and histones by the method of Levy et al. (1972). Figure 1 shows the polyacrylamide disc gel electrophoretic pattern of the various classes of NHCP which we have obtained from Krebs II chromatin. These proteins were used in subsequent binding studies of histones and NHCP to DNA under various conditions.

Figure 2 shows that histone binding to DNA in medium 0 reaches saturation at 1.85 mg/mg of DNA at 2 equivalents input of histones or greater. In other experiments not reported here, we observed a saturation level of 1.5 mg of histones/mg of DNA. That the amount of bound histones to DNA at saturation is higher than the in vivo ratio of about 1.0 (Table I) is not surprising, as we have reported in 1973 (Bekhor, 1973) that native chromatin can be further titrated with histone I, resulting in an increase in chromatin thermostability. These results suggest that native chromatin is not necessarily saturated with histones. When M_1 or M_3 NHCP are present at 1-equivalent inputs (Table I), higher input concentrations of histones are required to reach saturation (Figure 2). We also find that for M_1 proteins higher inputs of histones are required for saturation than observed for M_3 proteins. This cannot be due to aggregations of histones and nonhistones, since in 5 M urea these proteins can be separated from each other on Bio-Gel A-0.5m (unpublished data). The effect of M_1 on histone binding to DNA may be due either to its greater stoichiometry

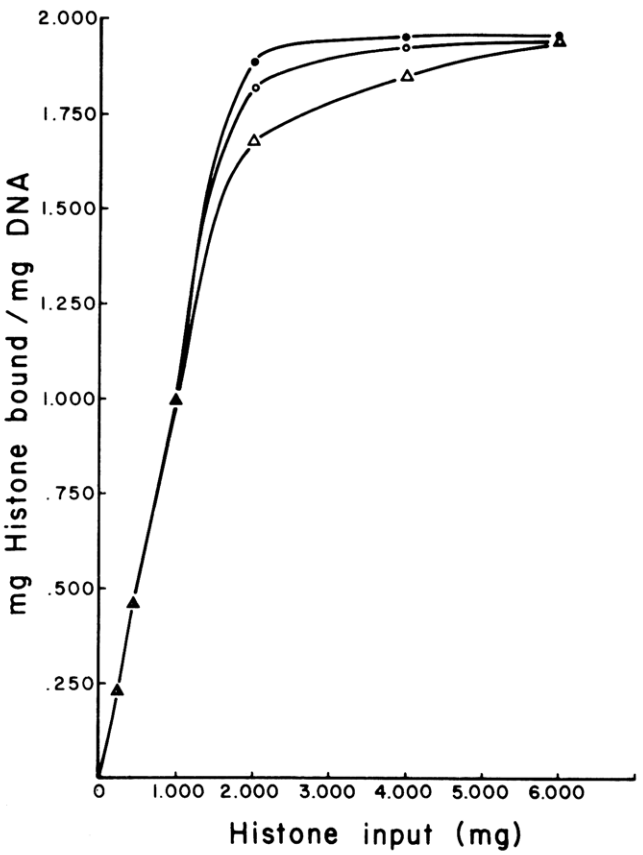

FIGURE 2: Effects of varying amounts of input histones on the amounts of KII histones bound to unsheared (high-molecular-weight) DNA in medium 0 in the absence and presence of 1 equivalent of various classes of KII NHCP. (●) No NHCP; (○) M_3 NHCP; (△) M_1 NHCP. Complexes were isolated as described under Methods. All NHCP were labeled with [^{14}C]leucine, while histones were labeled with [^{3}H]leucine. Data are normalized to mg of protein/mg of DNA.

in chromatin (Table I), or to the probability that the histone binding sites may be both chemically and/or conformationally related to M_1 protein binding sites, as M_1 and histones dissociate from DNA under the same conditions. As shown by Lapeyre and Bekhor (1976), when the reconstitution is carried out to 0.24 TPD, histones do not demonstrate saturation binding to DNA, probably indicating that the additional binding in 0.24 TPD may be due to nonspecific histone aggregation on DNA. Thus, 5 M urea seems to eliminate nonspecific protein-protein interactions in DNA-histone complex formation (Figure 2).

Histones clearly influence the number of available DNA binding sites for NHCP. In Figure 3, we show the influence of histones on M_1 protein binding to DNA, where DNA, histones, and M_1 were reconstituted from medium 3 to medium 0, as described under Methods. We find that M_1 binding to DNA is decreased in the presence of 1.0 and 2.0 mg of histones. The data in Figure 3 also indicate that, with 2 mg of histones/mg of DNA, M_1 binding to DNA-histone complexes is decreased by about 40%. However, for M_3 (Figure 4), 6 mg of histones/mg of DNA are required to decrease M_3 binding to DNA by about 35%. Therefore, higher inputs of histones are needed to affect M_3 dissociation from DNA. These results demonstrate that M_3 is very tightly bound to DNA and cannot be manipulated by histones, while M_1 binding is more histone dependent. This finding is in agreement with our previous kinetic analysis on NHCP binding to DNA in the presence of histones (Lapeyre and Bekhor, 1976).

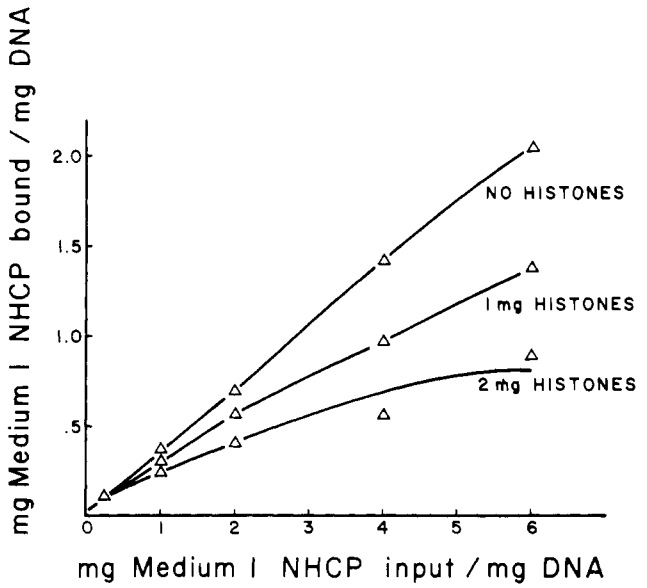

FIGURE 3: KII medium 1 NHCP binding curves to unsheared DNA in medium 0 in the presence of 0, 1, and 2 mg of histones/mg of DNA. Data is normalized to mg of protein/mg of DNA.

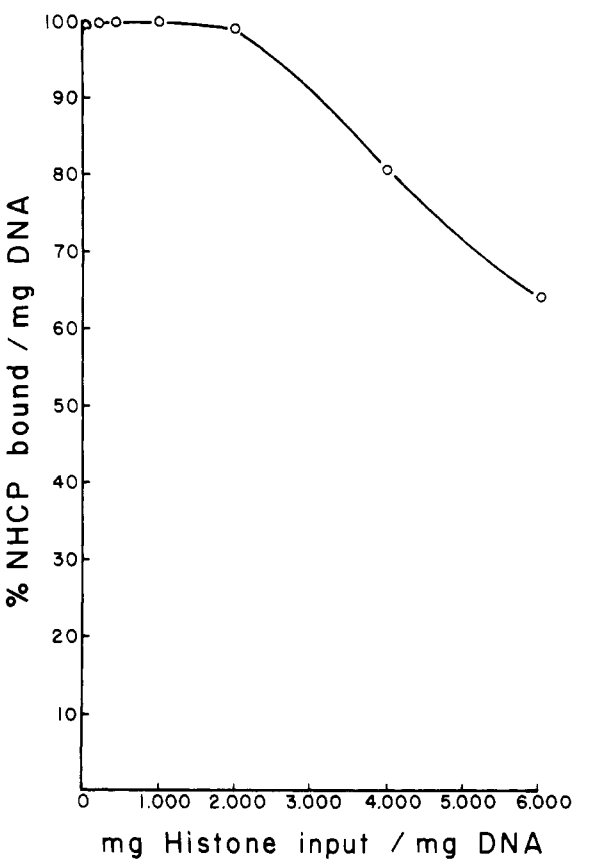

FIGURE 4: A plot of the percent of Krebs II M_3 [^{14}C]leucine NHCP bound to DNA in medium 0 with increasing inputs of [^{3}H]leucine histones. One equivalent of M_3 NHCP (O) was present during reconstitution.

It became clear that we should also design experiments to demonstrate whether tissue-specific (and thereby gene-specific) M_0, M_1, and M_3 can be detected by competition binding to DNA–histone complexes. Since Krebs II NHCP can be labeled to a high specific activity (500–900 cpm/μg of protein) with [^{3}H]leucine, we compared the binding of labeled Krebs II NHCP to DNA as affected by competition either with unlabeled Krebs II NHCP fractions (to establish baseline curves) or unlabeled mouse liver NHCP fractions. Figure 5 shows that, at inputs of less than 1 equivalent of unlabeled Krebs II M_0, the competition for binding to DNA with labeled Krebs II M_0 follows the expected theoretical curve; however, at 1 equivalent input and higher, competition for binding to DNA–histone complexes of unlabeled Krebs II M_0 does not follow the expected theoretical value. This may be accounted for by a high nonspecific binding of M_0 to DNA–histone complexes, as previously indicated (Lapeyre and Bekhor, 1976). Therefore, comparison of this type of competition with M_0 from other tissues may be valid for values which are under 1 equivalent. Based on this analysis, we find that there is little competition between labeled Krebs II M_0 and unlabeled liver M_0, at levels of less than 1 equivalent input of liver M_0. This may suggest that M_0 contains, in part, tissue-specific proteins which are capable of interacting specifically with DNA–histone complexes. On the other hand, we could not obtain a similar type of competition between M_1 or M_3 proteins from heterologous tissues.

Circular Dichroism and Thermal Denaturation. Circular dichroism and thermal denaturation provide, respectively, information concerning the conformation of the DNA–protein complex and its thermostability. All CD and thermal denaturation experiments described here were carried out in TPD and in 0.25 mM EDTA, pH 8.0, respectively. We employ these methods to probe for physical changes in the DNA helix caused by chromosomal proteins that may be related to functional control of transcription. Figure 6 and Table II summarize the CD data for various combinations of and its thermostability. All CD and thermal denaturation experiments, describe DNA and chromosomal proteins. Protein-free DNA has a positive molar ellipticity at 277 nm of 10:600 deg cm^2/dmol. This corresponds to DNA in 100% B conformation (Hanlon et al., 1972). Native chromatin, on the other hand, is 49% B, indicating the presence of DNA in a mixed B and C conformation. Circular dichroism of chromatin (Shih and Fasman, 1970; Hjelm and Huang, 1974; Wilhelm et al., 1974) shows that the presence of histones in chromatin causes a reduction in the positive CD spectra of DNA near 275 nm and a big negative CD spectra near 220 nm. We have also reported (Lapeyre and Bekhor, 1974) that, when chromatin is fractionated into condensed (heterochromatin) and diffuse (euchromatin) fractions, their CD spectra show, respectively, a decrease and an increase in DNA-B character relative to that of unfractionated chromatin. This is in agreement with the results reported by Slayter et al. (1972) and Polacow and Simpson (1973). Therefore, it may be inferred that by CD spectra analysis we can obtain an estimate of the percent of DNA in B conformation found in chromatin or DNA–chromosomal protein complexes in solutions which show negligible light scattering with absorption at 320 nm of less than 0.05 absorbancy unit/ml.

To determine which class of chromosomal proteins can alter the degree of packing of the DNA–helix and to what extent, we either reconstituted DNA with equivalent quantities (Table I) of the various classes of NHCP and histones in 0.24 TPD, or carried out direct mixing (binding) of NHCP to reconstituted DNA–histone complexes as described under Methods, then dialyzed the complexes overnight either against TPD or 0.25 mM EDTA, pH 8.0, for CD and T_m measurements, respectively. When 0.5 equivalent of histone is reconstituted with DNA, the ellipticity of DNA at 277 nm decreases from 10 600 to 7900 deg cm^2/dmol; 1 equivalent of histones decreases the ellipticity to 5200 deg cm^2/dmol, indicative of a further change in DNA structure towards more C form. When a mixture of

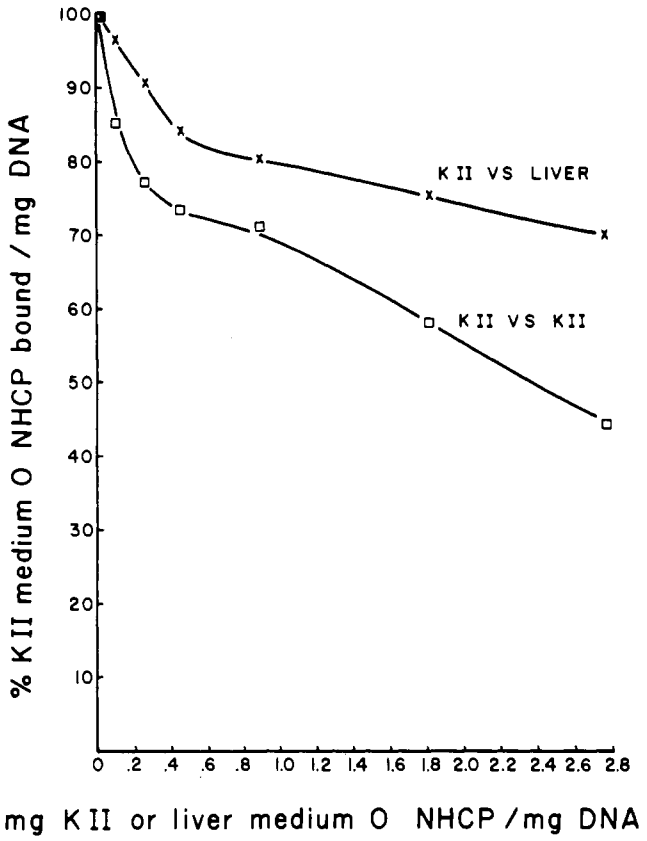

FIGURE 5: Competition binding curves between labeled KII medium 0 NHCP and unlabeled KII medium 0 NHCP (□), and between labeled KII medium 0 NHCP and unlabeled liver medium 0 NHCP (X) in 0.24 TPD. In this experiment, 100 μg of unsheared KII DNA, 1 equivalent of KII histones, and 1 equivalent of [^{14}C]labeled KII medium 0 NHCP were reconstituted to 0.24 TPD with varying amounts of either unlabeled KII medium 0 NHCP or unlabeled liver medium 0 NHCP. Results by reconstitution or direct binding were essentially the same. Complexes were isolated as described under Methods.

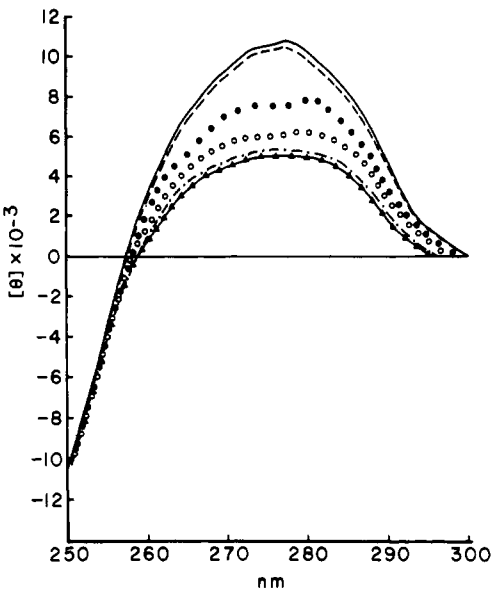

FIGURE 6: Circular dichroic spectra of KII-sheared DNA (—); sheared DNA with 1 equivalent of either M_0, M_1, or M_3 NHCP, or a combination of all three, or with BSA (- - -); DNA reconstituted with 0.5 equivalent of histones (●); DNA reconstituted with 1 equivalent of histones plus either M_0 NHCP, or + M_0 + M_1 + M_3 NHCP (○); all NHCP were bound to DNA at 1 equivalent input; DNA + 1 equivalent of histones ±1 mg of BSA/mg of DNA (- · -), and control sheared chromatin (▲). All samples were either reconstituted from medium 3, or the binding was done by direct addition of NHCP to reconstituted histone-DNA complexes, as described under Methods. CD measurements were taken in TPD, where there was no turbidity. We were able to get structural collapse of the DNA helix by slowly adding purified histones (at 50 μg/ml) to DNA (100 μg/ml) in 0.24 TPD and allowing them to interact for 1-2 h at 0-4 °C. If histones were added too quickly or at a high concentration, or the DNA was not sheared, structural collapse of the DNA was not observed, and the complexes precipitated.

1 equivalent of each fraction of NHCP (M_0, M_1, and M_3) is added to DNA already bound to 1 equivalent of histones, the ellipticity increases to 6000 deg cm^2/dmol relative to that obtained for DNA reconstituted with 1 equivalent of histones. Thus, the percent of B conformation increases from 51 in the absence of NHCP to 59 with NHCP. A similar change is produced when 1 equivalent of M_0 is bound to DNA-histone complexes but not with a nonspecific protein, such as bovine serum albumin. The change in CD at 277 nm from 5200 to 6000 is theoretically significant and cannot be assumed to represent artifactual effects without additional experimentation. On the contrary, we find that this increase in CD, as affected by M_0, correlates with an increase in the transcriptional activity of DNA-histone complexed with M_0 (Bekhor and Samal, 1976). Nicolini et al. (1975) demonstrated that, in synchronized HeLa cells, increase in the transcriptional activity could be correlated with the increased positive ellipticity during different stages of the cell cycle. This observed increase in the positive ellipticity does not imply loss of histones from that chromatin, but it may be due to modulation in nucleoprotein structure affected by NHCP (Nicolini et al., 1975). It is thus concluded that a particular class of NHCP (M_0) may, by its binding to DNA-histone complexes, modify the interactions between histones and DNA.

Thermal denaturation parallels the CD data presented above (Figure 7, Table II). DNA exhibits a T_m of 44.5 °C in 0.25 mM EDTA, pH 8.0. When 1 equivalent of histones is reconstituted with DNA, the T_m shifts to 81.5 °C (Table III). When all three classes of NHCP are added with the histones to DNA by reconstitution, the T_m decreases to 79.4 °C, very close to that of native sheared chromatin (79.6 °C), and 2.1 °C below that of the histone-DNA complex. Medium O NHCP decreases the T_m to 79.2 °C. On the other hand, M_1 and M_3 have very little effect on the T_m of DNA-histone complexes (Table III).

TABLE II: Effects of Histones and Nonhistones on the Molar Ellipticity of DNA.

Sample	$[\theta]_{277}$ ± 100	%B Conformation[a]
DNA	10 600	100
DNA + 1 equiv of[b] NHCP or 1 mg of BSA[c]/mg of DNA	10 400	99
DNA + 0.5 equiv of histones	7 900	76
DNA + 1 equiv of histones with and without 1 mg of BSA/mg of DNA	5 200	51
DNA + 1 equiv of histones and 1 equiv of total NHCP or 1 equiv of M_0 NHCP	6 000	59
KII Chromatin	4 900	49

[a] %B = $(([\theta]_{277} - [\theta]_{277}^{C})/([\theta]_{277}^{B} - [\theta]_{277}^{C}))100$ (Hanlon et al., 1972). [b] Equivalent (Table I). [c] BSA, bovine serum albumin.

Discussion

In these studies, we have used two methods for binding

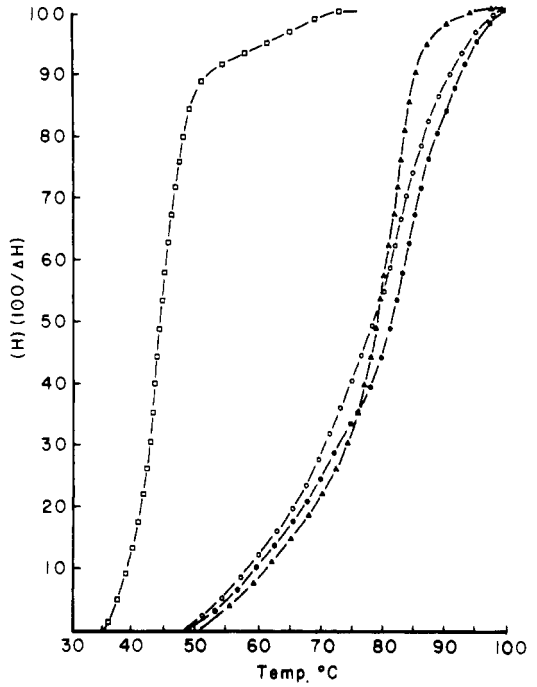

FIGURE 7: The normalized melting profile curves of DNA (□–□); control chromatin (▲); DNA + 1 equivalent of histones ± 1 equivalent of M_3 NHCP, or ± 1 mg of BSA/mg of DNA (●); DNA + 1 equivalent of histones + 1 equivalent of M_0 NHCP, or + 1 equivalent of M_0 + M_1 + M_3 NHCP (○).

histones and nonhistones to DNA: (1) by reconstitution, as previously described (Bekhor et al., 1969), and (2) by a method of direct binding, introduced earlier (Lapeyre and Bekhor, 1976). In the early studies on chromatin reconstitution (Bekhor et al., 1969; Huang and Huang, 1969) very little was known as to how a denaturing reagent, such as urea, can indeed affect restoration of chromatin which has been totally dissociated to its molecular building units. Previously, Lapeyre and Bekhor (1976) established that: histone binding to DNA is essential for sequence-specific nonhistone chromosomal protein binding to DNA; that the histones can augment specific binding of NHCP and limit their nonspecific binding; that in reassociation of chromosomal proteins to DNA about 90% of NHCP require histones to bind either simultaneously along with NHCP to DNA or that they must first bind to DNA for NHCP to bind to their recognizable DNA sites. We also find that histones can bind in excessive amounts (5–6 times the native histone–DNA levels) to DNA when the association of histones to DNA is allowed to take place by dialysis from 5 M urea–1 M NaCl to TPD or 0.24 TPD. On the contrary, when histones are allowed to interact with DNA in 5 M urea (as shown in Figure 2), the histones bind to DNA to saturation levels from 1.5 to 2 times the native histone–DNA levels found in chromatin; therefore, aggregation of histones on DNA does not occur in 5 M urea. Our data suggest that the most important factor in reconstitution is the formation of correct ionic- and hydrophobic-type binding, either between DNA and histones or histone octamers and DNA without precipitating the histones on DNA and, thus, blocking NHCP from binding to their specific sites. We know that, when reconstitution is done in NaCl alone, the resulting complex is rather dissimilar to native chromatin (Bekhor et al., 1969). Since urea seems to be essential for the correct binding of histones to DNA, we have been able to carry out "reconstitution" by mixing various fractions of NHCP with DNA–histone complexes (Figures 6 and 7) in 0.24 TPD.

TABLE III: Effects of Chromosomal Proteins on the Thermal Denaturation of DNA.

Sample[a]	T_m (°C)
DNA	44.5
Urea reconstituted chromatin	79
Native chromatin	79.6
DNA + 1 equiv of H	81.5
DNA + 1 equiv of H + 1 equiv of M_0	79.2
DNA + 1 equiv of H + 1 equiv of M_1	81.2
DNA + 1 equiv of H + 1 equiv of M_3	81.5
DNA + 1 equiv of H + 1 equiv of M_0 + M_3	79.2
DNA + 1 equiv of H + 1 equiv of M_0 + M_1 + M_3	79.4
DNA + 1 equiv of H + 1 mg of BSA/mg of DNA	81.5

[a] H, histones; or M_0, M_1, and M_3, see Table I.

Therefore, our method of fractionation of NHCP now allows us to examine possible structural and functional effects induced by these NHCP classes and their subfractions on reconstituted DNA–histone complexes, thus providing a model system for assembly of chromatin.

Presently, in vitro chromosomal protein–DNA complex formation has led to hypotheses on the possible functions of various classes of NHCP, both in repression and derepression of genetic activity. These fractions of NHCP include phosphorylated NHCP (Shea and Kleinsmith, 1973), and "loosely-bound" NHCP (Kostraba et al., 1975). In addition, Chiu et al. (1975) reported that "tightly-bound" NHCP, isolated from reticulocyte chromatin, may enhance the synthesis of globin mRNA. M_0, M_1, and M_3 are fractionated on the basis of their hydrophobic properties and increased binding affinities to DNA. It is therefore expected that studies on such fractions would be universally applied to chromatin structure and function. Further, M_0, M_1, and M_3 were found to contain phosphate groups at approximately the same levels, as measured by $^{32}PO_4$ incorporation into M_0, M_1, and M_3 (unpublished data; examined by polyacrylamide disc gel electrophoresis). Therefore, phosphorylated NHCP would contain proteins of different binding affinities to DNA, and, henceforth, would exhibit functions which may vary from tissue to tissue. The "loosely-bound" NHCP, 0.35 N NaCl-soluble NHCP, cannot be equated to our M_0, M_1, and M_3, and may contain many NHCP's found in M_0, M_1, and M_3, as we have shown by polyacrylamide disc gel electrophoresis for the 0.24 TPD-soluble proteins (Lapeyre and Bekhor, 1976). The tightly-bound NHCP's of Chiu et al. (1975) are probably equivalent, in part, to our M_1 and M_3 fractions.

For both types of binding studies, our data examine stable ligand interactions between histones, salt-fractionated classes of labeled NHCP, and DNA. We find that histone binding to DNA is influenced by the presence of NHCP (Figures 2, 3, 4, and 6). One equivalent of M_1 decreases the histone bound to DNA in the range of 1–4 mg of histone input/mg of DNA, more so than M_3, and may be attributed to their greater number or similarities in the nature of their binding to DNA. M_3, however, seems to be more strongly bound to DNA than M_1. Thus, the various urea–salt fractionated classes of NHCP might have different general functional and structural roles. Figure 5 indicated that M_0 from two tissues can compete to a certain extent with each other for stable DNA binding sites. This type of a competition allowed us to compare Krebs II and mouse liver M_0, and there appears to be either qualitative or quantitative differences between them. In addition, CD data (Figure 6 and Table II) indicate that M_0 can increase the

percentage of DNA in B conformation from 51 to 59% in the presence of 1 equivalent of histones. Therefore, particular NHCP classes can interact specifically with DNA-histone complexes to regionally modify histone-DNA interactions, resulting in less condensation with a presumed concomittant increase in the transcriptional activity.

In all of these studies, we utilized methods of fractionation for NHCP according to their binding affinities to DNA to allow us to develop a uniform, systematic, and universal method for the fractionation of NHCP whereby losses in NHCP are limited. We believe that our approach to examining classes of NHCP's, which include almost all of NHCP's that one can isolate from chromatin based on their binding properties to DNA and *in vitro* reconstituted DNA-histone complexes, and by testing for their effects on the physical and biological structure of these complexes, will aid in classifying the nonhistones in groups. The nonhistones are rather numerous and the methods which we have introduced seem to provide us with such an approach. We have, so far, succeeded in an initial and a general method of fractionation by which we obtained three fractions: M_0, M_1, and M_3. These groups of proteins are being collectively tested for their binding properties (Lapeyre and Bekhor, 1976), and for their effects on the physical and biological function of chromatin isolated from different tissues, as done here and elsewhere (Samal and Bekhor, 1976).

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