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Circular Dichroism and Thermal Denaturation Studies of Subnucleosomes and Their Relationships to Nucleosome Structure†

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ABSTRACT: Chicken erythrocyte chromatin moderately digested with micrococcal nuclease yields several species of nucleosomes and subnucleosomes that are resolved by electrophoresis in the presence of 3 M urea. This report compares the circular dichroism spectra, thermal denaturation, and certain other properties of chromatosomes (trimmed nucleosomes containing 170-bp DNA and all five histones), nucleosome cores, and four subnucleosomes. One subnucleosome is a partial core lacking an H2a-H2b pair and 40-50 DNA base pairs (bp) from one end. The stoichiometries of the other subnucleosomes, which contain homotypic histones associated with short DNA fragments, are (H3)(H4)/70-80 bp, (H1)/60-70 bp, and (H2a)(H2b)/50-60 bp. The latter subnucleosomes originate from the ends of nucleosome cores. All properties of partial and whole nucleosome cores were nearly identical, indicating that the terminal H2a-H2b pairs do not make binding contacts with the residual core DNA or histones that are critical to the conformation of the remaining core structure. Analyses of histone contributions to the far-UV circular dichroism of subnucleosomes showed that H2a-H2b pairs and H3-H4 pairs in nucleosomes are both nearly 50% α helical and that their helix contents do not depend on the nucleosome integrity. These and other results suggest that homotypic histone pairs and the DNA they tightly bind define quasi-independent conformational subdomains within nucleosomes. H3-H4 pairs stabilized and reduced the 275-nm circular dichroism intensity of short DNA fragments much more effectively than H2a-H2b pairs. In addition, H3-H4 pairs stabilized considerably more DNA than predicted for simple electrostatic interactions. H1 also thermally stabilized short DNA more efficiently than H2a-H2b pairs, but slightly less efficiently than H3-H4 pairs, and modestly increased the 275-nm CD intensity relative to protein-free DNA. The properties of subnucleosomes generally support current concepts of the relative roles of different histones in the nucleosome structure and indicate that nonelectrostatic interactions between histones (particularly H3 and H4) and DNA are critical for nucleosome stability.

The primary structure of chromatin is determined by the organization of histones into discrete subunits, termed nucleosomes, that occur at regular intervals along DNA fibrils. Nucleosomes contain two conformationally distinct domains. The globular nucleosome core consists of 146 bp[1] of DNA wrapped about an octamer of two copies each of H2a, H2b, H3, and H4. Cores are linked by shorter, variable (ca. 40-60 bp) regions of DNA usually associated with histone H1 or H5 [reviewed by Kornberg (1977); Felsenfeld, 1978; Thomas, 1978; Rill, 1979; McGhee & Felsenfeld, 1980].

Studies of nucleohistones reconstituted from mixtures of histones and 146-bp or longer DNA have shown that H3-H4 tetramers are primarily responsible for organizing the nucleosome core structure, while H2a and H2b are required mainly to complete the core folding. H1 is not essential to the core structure but is involved in higher order folding of chromatin fibrils [reviewed by Rill (1979); McGhee & Felsenfeld, 1980]. Relatively little is known about the nature of interactions of specific histones with DNA, however, and there are no clear explanations of the relative structural roles of histone subtypes in terms of sequence differences.

We have addressed these questions, in part, by examining the thermal denaturation, circular dichroism, and certain other properties of four nucleohistone fragments, termed "sub-

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[1] Abbreviations: bp, base pairs; EDTA, ethylenediaminetetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; TEMED, tetramethylethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate.

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nucleosomes", that contain subsets of histones associated with DNA shorter than the core DNA length. The isolation and chemical characterization of these subnucleosomes, which originate from nucleosomes cleaved internally at specific sites by micrococcal nuclease, were described in the preceding paper (Nelson et al., 1982). Two subnucleosomes form a complementary pair, i.e., one is a partial core containing two copies each of H3 and H4, but only one copy each of H2a and H2b and 95–115 bp length DNA. The other contains an H2a–H2b pair associated with 50–60 bp length DNA. We have shown that the latter subnucleosome is derived from the ends of cores by analyzing nucleohistone fragments from DNase I digests of purified cores labeled on the 5′ ends with ^{32}P (Nelson et al., 1982). A third subnucleosome contains an H3–H4 pair associated with 70–80 bp length DNA, and the fourth contains a single H1 (a or b) associated with 60–70 bp length DNA.

Direct isolation from chromatin of "partial" cores lacking H2a and H2b plus associated DNA from one end provided a means to examine the influence of H2a and H2b on the structure of nucleosomes by determining the effects of their *absence* on properties of the remaining core fragment that are sensitive to DNA and histone conformations. Virtually no diffrences were noted between partial and intact cores, indicating that the terminal H2a–H2b pairs do not strongly influence the structure of the rest of the core.

Distinctive differences were noted in the circular dichroism and thermal denaturation of the smaller subnucleosomes. These differences reflect the disparate abilities of specific homotypic histones to locally affect DNA conformation and stability. Of particular interest is the finding that H3–H4 pairs were considerably more effective than H2a–H2b pairs in stabilizing DNA against thermal denaturation and altering the DNA circular dichroism spectrum. H3–H4 pairs stabilized much more DNA than could be accounted for by electrostatic interactions of basic residues in amino-terminal tails, or even the whole chains, indicating that nonelectrostatic interactions are involved in the tight DNA binding of H3 and H4.

Materials and Methods

Isolation and Digestion of Chicken Erythrocyte Nuclei. Chicken blood was removed from 3–4-week-old Babcock B-300 white leghorn male chicks by heart puncture using a syringe containing an equal volume of 0.05 M Na_2EDTA, 0.23 M NaCl, and 1 mM PMSF, pH 7.0.

Nuclei were isolated from the red blood cells by slight modification of the method of Blobel & Potter (1966) as described by Nelson et al. (1982). All steps were performed at 0–4 °C. After suspension in 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF, and 15 mM cacodylate (adjusted to pH 6.5 with Tris), nuclei (5 mg equiv of DNA/mL) were digested to 15–20% acid solubility with micrococcal nuclease (Worthington, 2 units/50 μg of DNA). The nuclear suspension was cooled in an ice bath and centrifuged, and then the digested nuclei were resuspended in electrophoresis buffer (10 mM Tris–Mops, 2 mM EDTA, 3 M urea, and 0.0025% bromothymol blue, pH 7.6), dialyzed overnight against the same buffer, and centrifuged at 10 000 rpm for 10 min to remove any residual nuclear debris and large chromatin fragments.

Particles larger than tetranucleosome size were removed from the clarified digestion mixture by step gradient centrifugation. The digest was divided into 12-mL polyallomer centrifuge tubes, underlayered with 15% sucrose in electrophoresis buffer containing 3 M urea, and centrifuged at 40 000 rpm at 6 °C in the IEC 283 rotor for 2 h. The upper layers containing mononucleosomes and small oligonucleosomes were pooled, made 10% sucrose, and dialyzed overnight against buffer containing 10% sucrose. (Note, all urea solutions were prepared with stock 10 M urea deionized by passage over a column of Amberlite MB-1 mixed-bed resin immediately prior to use.)

Preparative Electrophoresis of Nucleohistones. Mono- and oligonucleosomes were electrophoresed on 1.2 × 17 cm cylindrical gels of 8% polyacrylamide [acrylamide/bis(acrylamide), 80:1] containing 0.067% TEMED, 0.067% ammonium persulfate, 3 M urea, 10 mM Tris–Mops, 2 mM EDTA, and 0.0025% bromothymol blue, pH 7.6. Polymerized gels were overlayered with tray buffer (10 mM Tris–Mops, 2 mM EDTA, and 0.0025% bromothymol blue, pH 7.6) made 3 M in urea and preelectrophoresed at 200 V for 2 h. Sample volumes were 0.25–0.5 mL and contained 1 mg of DNA. Samples were electrophoresed at 6 °C for 9 h at 160 V (4 mA/gel).

Gels were stained briefly with ethidium bromide (1 mg/L tray buffer + 10% glycerol), and bands were excised with a razor under UV illumination. Particles were electrophoretically eluted from gel slices into dialysis tubing (150 V, 4 mA/tube, 6 °C) and then dialyzed exhaustively into 0.1 mM cacodylic acid and 0.02 mM EDTA (pH 7.0). Samples were concentrated to 20–40 μg of DNA/mL by using a Millipore molecular separator and then centrifuged at 16 000 rpm for 20 min to remove dust.

Chemical Characterization of Nucleohistones. Histone contents were determined by electrophoresis on 15-cm, 18% polyacrylamide gels containing $NaDodSO_4$ as described by Laemmli (1970) and modified by Bonner & Pollard (1975). Gels were stained with 0.25% Coomassie blue in 50% methanol and 10% acetic acid, diffusion destained in 50% methanol and 10% acetic acid, and then scanned at 580 nm with a Gelman densitometer.

Protein was measured by the Coomassie dye binding method (Bio-Rad) using nucleosome cores and purified histones as standards [see Nelson et al. (1982)].

DNA lengths were determined by electrophoresis on 12% polyacrylamide gels containing 7 M urea as described by Maniatis et al. (1975). DNA was isolated by using the method of Marmur as modified by Britten et al. (1974). After electrophoresis, gels were soaked overnight in formamide, stained with 0.25% Stains-all (Eastman) in 50% formamide, pH 7.4, diffusion destained in distilled water, and scanned at 610 nm with a Gelman densitometer. DNA fragments were sized by comparison to fragments from a DNase I digest of chromatin (Noll, 1974; Prunell et al., 1979).

Circular Dichroism Spectra. Absorption spectra were obtained on a Cary 15 spectrophotometer. Circular dichroic spectra of two preparations were recorded by using a JASCO ORD/UV-5 optical rotatory dispersion recorder with CD attachment and Sproul Scientific SS20 CD modification. Spectra of a third preparation were obtained on the Cary 60 recording spectropolarimeter in the laboratory of Dr. D. W. Urry at the University of Alabama in Birmingham. All spectra were recorded at room temperature in 0.1 mM cacodylic acid and 0.02 mM EDTA, adjusted to pH 7.0 with solid Tris.

T_m Measurements. Thermal denaturation profiles were obtained by using a Beckman Acta CII spectrophotometer. A Yellow Spring Instrument probe and thermivolt thermometer were used to give a continuous record of absorbance at 260 nm vs. temperature on a Houston Instrument Series 200 Omnigraph X–Y recorder. The temperature was programmed to increase at either 1.0 or 0.3 °C/min by using a calibrated

Table I: Composition of Nucleohistones

species	DNA[a] length (bp)	histones	μg of histone/ μg of DNA[b]
MN1u	170 ± 10	$(3,4,2a,2b)_2 5_1$	1.25
MNCu	145 ± 10	$(3,4,2a,2b)_2$	1.18
SN7u	105 ± 15	$(3,4)_2(2a,2b)_1$	1.19
SN6u	75 ± 9	$(3,4)_1$	0.54
SN5u	66 ± 9	$(1a \text{ or } 1b)_1$	0.50
SN4u	55 ± 5	$(2a,2b)_1$	0.79

[a] Averaged for three preparations. Variations indicate approximately the half bandwidth at half-height. Precision for the three preparations was ±5%. [b] Calculated from the histone stoichiometries and DNA lengths. Values determined based on the 258-nm absorbancies and the Bio-Rad protein dye binding assay agreed within ±10% [see also Nelson et al. (1982)].

Neslab Model TP-2 temperature programmer attached to a circulator bath. Sample buffer was 0.1 mM cacodylic acid and 0.02 mM EDTA, adjusted to pH 7.0 with solid Tris.

Derivative melting profiles were obtained by plotting the percent change absorption at 260 nm (corrected for thermal expansion) vs. temperature as described by Li & Bonner (1971). Each thermal denaturation curve shown is the average of three to four separate curves obtained from different preparations.

DNase I Digestion of 5′-End-Labeled Intact and Partial Cores. Samples were isolated by preparative electrophoresis of chromatin from nuclei digested to about 15% acid solubility. Labeling of the 5′ ends with [γ-^{32}P]ATP (New England Nuclear; 10–40 Ci/mmol) by polynucleotide kinase (Miles Lab.) was performed as described by Simpson & Whitlock (1976). Free ^{32}P was removed by sucrose density gradient sedimentation, and labeled particles were concentrated by using an immersible ultrafilter (Millipore). Samples were dialyzed against 10 mM Tris-HCl and 0.1 mM EDTA (pH 8.0) and warmed to 37 °C, and then $MgCl_2$ solution (3 mM final concentration) and DNase I (Worthington; 0.5 μg/50 μg of DNA) were added. Aliquots were removed at times indicated in the figure legends and pipetted into an EDTA solution containing unlabeled carrier DNA. Deproteinized samples were electrophoresed on denaturing gels as described above. Gels were autoradiographed on Kodak X-OMAT R film. Autoradiograms were scanned with a Joyce-Loebl Mark III densitometer.

Results

Compositions and Purities of Nucleohistones. Data are compared for six nucleohistones isolated from digests of erythrocyte nuclei. These are "trimmed" nucleosomes or "chromatosomes" (Simpson, 1978) containing all core histones, H5 and approximately 170-bp DNA, nucleosome cores containing 145–160-bp DNA, and the four subnucleosomes (SN4u–SN7u) described above (see summary in Table I).

Purities of samples were assessed by three methods. (1) Aliquots were electrophoresed on the gel system used for preparation. One set of gels was scanned at 260 nm; another set was stained with ethidium bromide. The particles migrated with mobilities identical with the corresponding particles in an unfractionated digest. Both gel scans and fluorescence patterns indicated that the samples were at least 90% pure. (2) Quantitative densitometry of histone gel electrophoretic patterns showed that each sample contained only the expected histones. (3) Electrophoresis under denaturing conditions showed that each nucleohistone contained a limited range of DNA lengths and that there was little overlap of the size ranges in different species [see preceding paper (Nelson et al., 1982)].

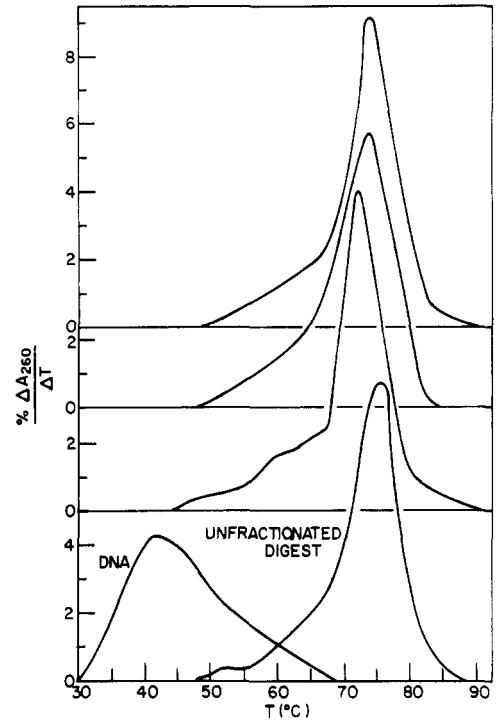

FIGURE 1: Derivative thermal denaturation profiles of MN1u, MNCu, SN7u, DNA, and a whole digest (top to bottom). Samples were denatured in 0.1 mM cacodylate, 0.02 mM EDTA, and 0.1 mM PMSF (pH 7.0) at 0.3 °C/min. Curves shown are averaged for three preparations (precision = ±5%).

Samples of all nucleohistones except SN5u remained clear after removal of urea and electrophoresed as single bands in the absence of urea. SN5u did not enter gels and yielded slightly turbid solutions indicative of aggregation in the absence of urea.

Thermal Denaturation. The thermal stability of DNA is greatly reduced at low ionic strengths (T_m = 44 °C under the conditions used here), but histone binding significantly increases DNA stability. This stabilization is not uniform; i.e., distinct denaturation phases are observed. These phases presumably reflect variations in binding affinities of different histone chain segments to double-stranded DNA [e.g., see Li & Bonner (1971)]. Thermal denaturation of nucleosome cores was biphasic, with a principal transition at 73 °C (Figure 1). Approximately 79% of the DNA denatured between 65 and 85 °C and the bandwidth (BW) at half-height of the derivative curve equals 10 °C. Almost identical data were reported by Weischet et al. (1978) for the denaturation of nucleosome cores under similar ionic conditions. Chromatosomes and partial cores (SN7u) denatured similarly to cores. The T_m (74 °C) and fraction of DNA denaturing between 65 and 85 °C (82%) were slightly greater for chromatosomes than cores, and the major transition was more cooperative (BW = 8 °C). The partial core was slightly less stable than cores (T_m = 72 °C), but the major transition was more cooperative (BW = 7 °C) and also accounted for 79% of the total DNA. The higher cooperativity may reflect the greater role of H3 and H4 in organizing the core DNA (see below).

All three of the smaller subnucleosomes (SN4u–SN6u) denatured in three discernible phases—a high-temperature phase with a T_m nearly identical with that of nucleosome cores (71–73 °C), an intermediate phase (T_m = 55–63 °C), and a low-temperature phase with a T_m similar to that of protein-free DNA (Figure 2). Two features of these denaturation curves are noteworthy. First, nearly the full length of DNA in each subnucleosome was stabilized more than protein-free DNA.

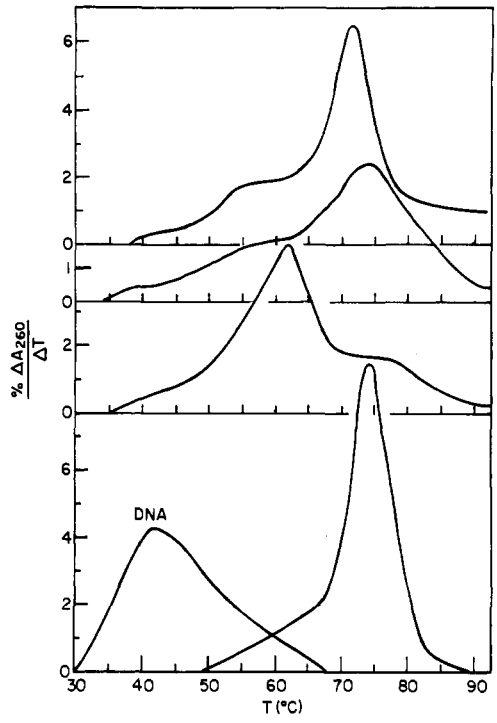

FIGURE 2: Derivative thermal denaturation profiles of SN6u, SN5u, SN4u, DNA, and MN1u (top to bottom); determined as in Figure 1.

Second, each of the three homotypic classes of histones clearly stabilized short DNA fragments in different ways.

Since the compositions of subnucleosomes are established, their denaturation can be interpreted semiquantitatively. For example, the average DNA length in SN4u is 55 bp. The equivalent of about 41 bp (75%) of this DNA denatured above 55 °C, whereas 80% of protein-free DNA of this length denatured by 55 °C. Thus at least 32 bp of DNA were stabilized by binding H2a and H2b. Only about 16–18 bp (29–33%) of the DNA in SN4u denatured in the high T_m transition (i.e., above 65 °C). The denaturation behavior of SN4u can be rationalized in terms of potential electrostatic interactions. The total amount of stabilized DNA is aproximately equal to the total positive charges in an H2a–H2b pair (54 Lys + Arg), while the amount of highly stable DNA corresponds well with the number of Lys + Arg residues (ca. 27) concentrated in the highly basic amino-terminal tails. [This does not prove that the most stable DNA is entirely bound by basic tails, however. The basic tails are defined here as the amino-terminal sequences that do not participate in structure formation as detected by NMR studies (Moss et al., 1976; Böhm et al., 1977).]

The subnucleosome containing H3–H4 pairs (SN6u) was much more stable than SN4u. Although SN6u contained 20-bp longer DNA, on the average, than SN4u, approximately 86% (65 bp) was more stable than protein-free DNA. Furthermore, about two-thirds of the total DNA, corresponding to 48–50 bp, denatured in a highly cooperative transition (BW = 9 °C) with a T_m nearly identical with that of nucleosome cores. Only about 16 DNA bp could be stabilized by electrostatic interactions with basic tails of an H3–H4 pair, and only 28 bp by the total basic residues in the pair (56 Lys + Arg).

Thermal denaturation of SN5u was similar to, but not identical with, that of SN6u. The major transition (T_m = 73 °C) was only moderately cooperative (BW = 18 °C). The equivalent of about 42 bp denatured between 65 and 90 °C.

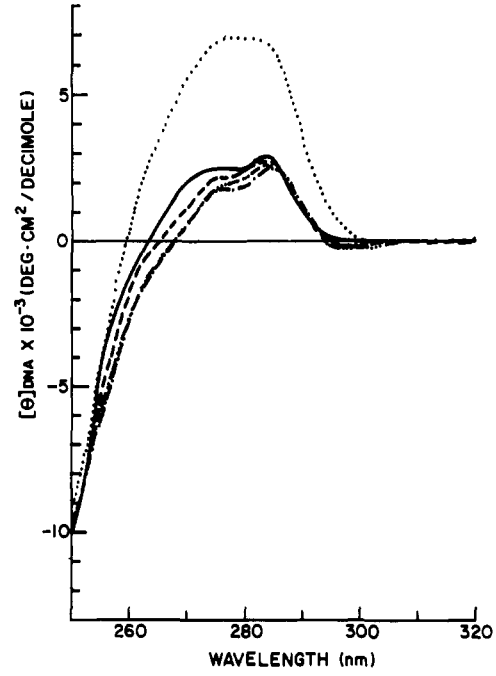

FIGURE 3: Near-UV circular dichroism spectra of SN7u (–···–···), MNCu (–·–·), MN1u (– – –), approximately 65 bp length DNA from SN5u (···), and a whole digest (ca. 18% acid soluble) (—). Molecular ellipticities expressed per mole of DNA phosphate. Spectra shown are the averages for three preparations (precision = ±5%).

Since H1 contains about 65 basic residues (Cole, 1977; MacLeod et al., 1977), only ca. 32 of these 42 bp could be stabilized electrostatically. Another 13 bp was partly stabilized, i.e., denatured between 55 and 65 °C.

Circular dichroism spectra are sensitive indicators of secondary structures in both proteins and nucleic acids. Specifically, the spectra of nucleohistones from 250 to 320 nm reflect only the DNA conformation since histones possess few aromatic residues. Histones and DNA contribute below 250 nm, but peptide bond contributions typically dominate because of the high α-helix contents of histones.

Near-UV spectra (250–320 nm) of intact and partial cores were identical within error (Figure 3). Features of both spectra agreed well with those typically reported for nucleosome core preparations—two unresolved positive peaks at 274 and 284 nm that were greatly reduced in intensity compared to protein-free DNA and a weak negative band at 294–295 nm [e.g., see Sahasrabuddhe & Van Holde (1974); Olins et al., 1977]. The reduced 270–290-nm CD intensity of DNA in nucleosomes and in high concentrations of salt has been attributed by some to an increase in the winding angle of DNA relative to the B conformation (Hanlon et al., 1975; Baase & Johnson, 1979; Chan et al., 1979). Baase & Johnson's (1979) studies of PM2 DNA suggest that the CD of DNA drops from $\Delta\epsilon$(275 nm) = 2.4 to $\Delta\epsilon$(275 nm) = 1.5 [(θ) = 7915–4947 deg·cm^2/dmol of phosphate] for a change in average rotation per base pair of about 0.5°. Further small increases in the rotation angle cause more dramatic decreases in $\Delta\epsilon$(275 nm). On the basis of this interpretation we calculated a change in winding angle of +0.68° per base pair, relative to the B conformation, for DNA in partial and intact cores. This compares well with the value of +0.71° calculated by Baase & Johnson (1979) based on CD spectra of cores reported by Cowman & Fasman (1978). [We note that Cowman & Fasman (1978) have attributed the reduction in circular dichroism of DNA in cores to tertiary structure effects, i.e., the close proximity of DNA coils, rather than secondary structure changes. This interpretation seems unlikely since the CD of

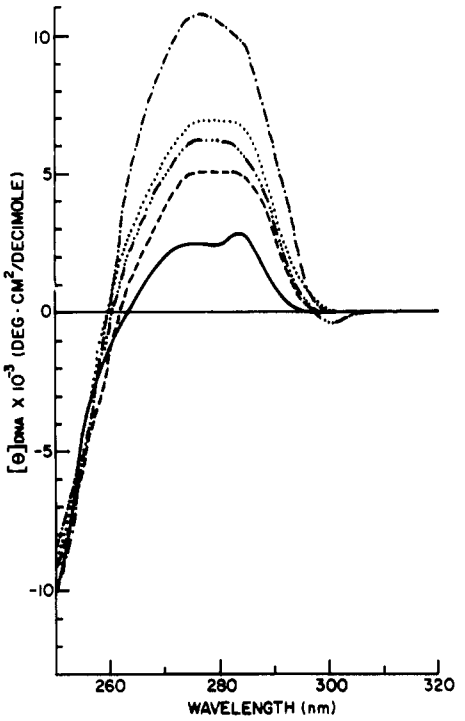

FIGURE 4: Near-UV circular dichroism spectra of SN6u (- - -), SN5u (-·-·), SN4u (-···-···), 65-bp DNA (···), and a whole digest (—), as in Figure 3.

DNA in subnucleosomes is also significantly reduced (see below), although they contain insufficient length DNA to form more than one loop of the dimensions suggested for DNA in cores.]

The CD (275 nm) of chromatosomes was about 24% greater than that of cores. A 51% increase in intensity is expected if the additional 25 bp of DNA in the chromatosome were in the B conformation, and the conformation of core was unaffected by the presence of H5.[2] The dichroism of chromatosomes is most simply interpreted to indicate that the conformation of the additional 25 bp is also overwound compared to B-form DNA, due to interactions with H5 or core histones. Assuming that the CD of core DNA in chromatosomes is the same as that of isolated cores, $(\theta)(275\text{ nm}) = 4600$ deg·cm^2/dmol of phosphate for the extra 25 bp of DNA. The suggested change in winding angle is about 0.59° from the B form, only slightly less than that found for the core DNA. Similar data for nucleosomes and chromatosomes containing H1 were reported by Cowman & Fasman (1978) and Simpson (1978).

The near-UV CD spectra of subnucleosomes were distinctively different (Figure 4). Spectra of subnucleosomes containing H3–H4 pairs (SN6u) and H2a–H2b pairs (SN4u) were intermediate in intensity between the DNA and core spectra. These spectra cannot be compared directly because of differences in histone/DNA ratios (Table I), but qualitatively it is clear that H3–H4 pairs more efficiently reduce the CD of DNA than H2a–H2b pairs. Calculations of the percent reduction in intensity at 275 nm per unit of histone per unit

[2] Calculated from the equation

$$\%\text{ increase} = \left(\frac{146}{170} + \frac{25(\theta)_{DNA}}{170(\theta)_{MNC}} - 1\right) \times 100$$

The value of (θ) for DNA at 275 nm was taken from the reference spectral data for DNA at low ionic strengths given by Hanlon et al. (1975).

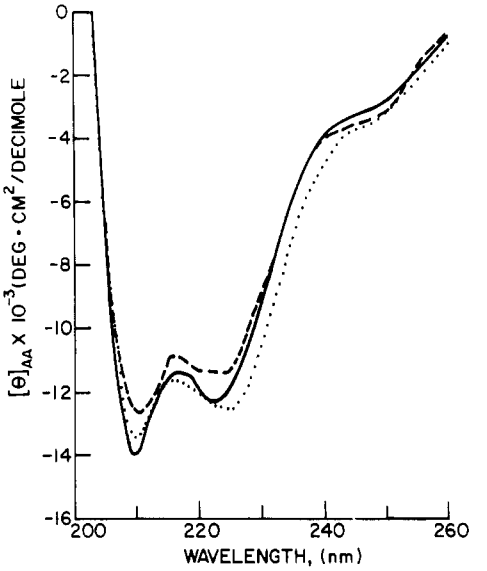

FIGURE 5: Far-UV circular dichroism spectra of MN1u (···), MNCu (—), and SN7u (- - -). Molecular ellipticities expressed per mole of amino acid residues (uncorrected for DNA contributions). Spectra shown are the average for three preparations (precision = ±10%).

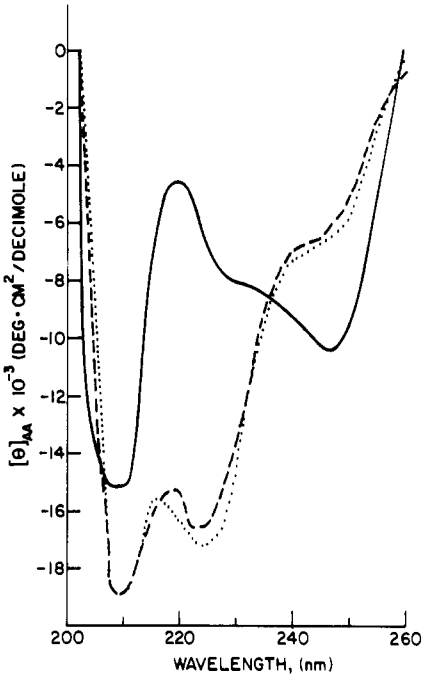

FIGURE 6: Far-UV circular dichroism spectra of SN6u (- - -), SN5u (—), and SN4u (···), as in Figure 5.

of DNA (weight to weight ratio) indicated that H3–H4 pairs in SN6u were appproximately twice as effective as H2a–H2b pairs in SN4u in reducing the DNA spectrum.

Treatment of these histone-induced changes in DNA circular dichroism in terms of a two-state model is of interest since most DNA in these subnucleosomes denatured in two distinct phases. With the assumption that DNA in a subnucleosome exists in two conformations, either the B form or the form of nucleosome core DNA, the fraction of corelike DNA is readily calculated.[3] This fraction is 54%, or about

[3] The fraction, X, of DNA in subnucleosomes in a conformation like that in cores was calculated from molecular ellipticities at 275 nm according to the equation

$$(\theta)_{obsd} = X(\theta)_{MNC} + (1 - X)(\theta)_{DNA}$$

41 of the 75 bp in SN6u, and 35%, or about 19 of the 54 bp in SN4u. These values agree reasonably well with the amount of highly stable DNA in SN6u and SN4u.

H1 bound to 60–70 bp length DNA in SN5u had a totally different effect on the DNA spectrum. The band intensity increased, relative to B-form DNA (25% at 275 nm), and the peak maximum was slightly blue shifted. These spectral changes resemble those observed when high concentrations of ethanol or other solvents that may induce transitions to the A form are added to DNA, but are much smaller than expected for full transition to the A form (Ivanov et al., 1973).

Far-UV CD spectra of chromatosomes, nucleosome cores, partial cores (SN7u), and two of the small subnucleosomes (SN6u and SN4u) were very similar (Figures 5 and 6). All were dominated by intense negative bands at 222 and 208 nm characteristic of α-helical polypeptides. The spectrum obtained for cores was in excellent agreement with spectra reported by Olins et al. (1977) and Weischet et al. (1978). These spectra cannot be precisely compared because of different DNA contents, but the contributions due to DNA are small [$(\theta) < 1 \times 10^3$ deg·cm^2/dmol of amino acid from 205 to 230 nm]. Because the DNA spectrum is a minor part of the total spectrum in each case and is relatively insensitive to DNA conformation (Hanlon et al., 1975), the histone contribution to the spectrum can be calculated with reasonable certainty by subtracting the spectrum of B-form DNA. Difference spectra for chromatosomes, intact cores, and partial cores were identical within precision. Helix contents calculated by using the reference spectra of Greenfield & Fasman (1969) or of Chen et al. (1974) yielded similar estimates of 47 ± 5%, in good agreement with previous CD and laser Raman studies of cores (Thomas et al., 1977; Weischet et al., 1978). No comparison data for chromatosomes are available. Helix contents calculated similarly for subnucleosomes containing H2a–H2b pairs and H3–H4 pairs were nearly identical (50 ± 5%). (The experimental precision in this region was ±10%. Uncertainties arising from the method of calculating helix contents were within these limits. Small differences noted between these particles and nucleosome cores were within experimental error and are of doubtful significance.)

In comparison to spectra of other subnucleosomes, the spectrum of SN5u was dominated less by α-helical peptide contributions. Nonetheless, the large negative band near 210 nm, where DNA contributes negligibly, is typical of proteins with moderate helix contents. Calculations according to Greenfield & Fasman (1969) and Chen et al. (1974) yielded similar estimates of 30 ± 5% helix and essentially 0% β sheet.

Reversibility of Urea Effects of Chromatin Structure. The possibility that 3 M urea causes irreversible changes in histone or chromatin conformation was tested, in part, by comparing the circular dichroism spectra and thermal denaturation of partly digested chromatin (15% acid soluble) before and after exposure to 3 M urea. One aliquot was kept in suitable sample buffer, and another was dialyzed against electrophoresis buffer containing 3 M urea for 2 days and then against sample buffer. No differences were noted in the above properties of the two samples (data not shown). All samples studied appeared very stable. No changes in properties were noted after storage for over a month of 4 °C in the presence of protease inhibitors.

DNase I Digestion of Intact and Partial Nucleosome Cores. DNase I selectively cleaves DNA in native nucleosome cores at sites spaced at about 10.4-nucleotide intervals on each strand. The relative positions of these cleavages seem most directly related to the inherent periodicity of the DNA helix, while the selectivity and relative rates of cleavage may reflect

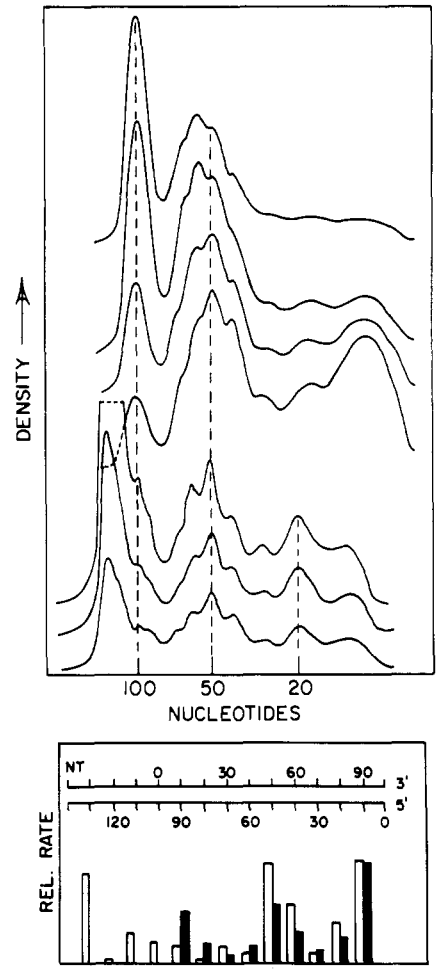

FIGURE 7: (Top) Densitometer scans of autoradiograms of DNA from DNase I digestion of MNC and SN7u prelabeled on the 5′ ends with [^{32}P]phosphate. Electrophoresis was on denaturing gels. The extents of electrophoresis on the two gels used were slightly different. Scans (top to bottom) are from SN7u digested for 20, 40, 80, and 160 s and from MNC digested for 20, 50, and 120 s, respectively. Plots of log (band number) vs. mobility for all scans were linear. (Bottom) Histograms of the relative rates of DNAse I cleavages at 10.4-nucleotide intervals (represented here and above as 10-nucleotide intervals) within nucleosome cores (open bars) and SN7u (filled bars). The upper representation of the cutting sites is aligned with respect to the histogram so that the rate of cleavage 10 nucleotides from the core end on the lower strand is represented at the far right. Numbering on the upper strand refers to SN7u with 100 bp (actually 105 bp) length DNA. The relative cutting rates shown for SN7u are simple averages; e.g., the rate of cutting at sites 30 nucleotides from the ends of SN7u is the average of the rates of cutting at sites 30 and 70 nucleotides from the core ends. Since both these rates are low, SN7u is also cut with low frequency at this site.

protection by bound histones [reviewed by McGhee & Felsenfeld (1980)]. If loss of H2a, H2b, and associated DNA from one core end created a histone-free gap or otherwise perturbed the folding of the remaining core fragment, then the pattern of DNase I digestion of partial cores should be significantly different from that of the core. This possibility was tested by DNase I digestion of intact and partial cores labeled on the 5′ ends with ^{32}P. Upon electrophoresis on denaturing gels, DNA from both samples yielded similar ladders of labeled bands (Figure 7). The ladder obtained from partial cores was somewhat more diffuse than that of cores. This diffuseness was probably due to the greater dispersion of the parent DNA lengths in SN7u and the longer exposure time required for autoradiography. Of most significance is the observation that both samples were relatively depleted in 80-, 70-, and 30-nucleotide fragments and enriched in 50-

nucleotide fragments, in agreement with other studies of DNase I cutting of cores (Simpson & Whitlock, 1976; Lutter, 1978).

In principle the distribution of labeled DNA sizes that should be obtained from partial cores if the structure was not perturbed could be predicted from the apparent rates of cleavages at specific sites in intact cores (Lutter, 1978). This analysis could not be performed precisely because of difficulties in preparing partial cores with well defined ends from whole chromatin digests. We therefore compared the relative rate constants determined for cutting at sites 10.4 nucleotides from the 5′ ends of cores to the relative rates predicted for cutting the equivalent sites of partial cores containing 105 bp length DNA, the most abundant length in our preparations (Figure 7). This comparison shows that sites 30, 70, and 80 nucleotides from the ends of both species should be cut with low frequency, as observed. Thus sites that are relatively inaccessible to DNase I in intact cores remained inaccessible after removal of H2a and H2b and associated DNA from one end.

Discussion

Conformational Subdomains of Nucleosome Cores. Only minor differences were noted in the circular dichroism, denaturation, DNase I susceptibility, and sedimentation[4] of partial and intact cores. The absence of major effects of the loss of an H2a–H2b pair and associated DNA on these core properties shows that H2a–H2b pairs do not make contacts with the remaining core DNA or histones that are critical to the integrity of the remaining core structure. Integrity in this context refers to preservation of a compact shape, native DNA and histone conformations, and strength of histone–DNA interactions as reflected by stability to heat or nuclease. This conclusion is also consistent with the finding that nucleosome cores cleaved 30 bp from the ends readily lose this length of DNA and associated H2a and H2b (Nelson et al., 1977, 1982) and with studies showing that the core ends unfold at low ionic strengths (Gordon et al., 1978, 1979; Wu et al., 1979).

Under the assumption that no major, conpensating conformational changes occur when the terminal H2a + H2b and 40–50 bp are lost,[5] the similarities of the CD spectra of partial and intact cores imply that the conformation of the terminal 40–50 bp of DNA in the intact core must be similar to the conformation of the remaining core DNA. In addition, the helix contents of H2a–H2b pairs in the intact core must be similar to the helix contents of H3–H4 pairs. The latter conclusion is substantiated by the identities of the far-UV circular dichroism spectra of the smaller subnucleosomes containing only H2a + H2b (SN4u) or H3 + H4 (SN6u). These results indicate that homotypic pairs of histones within nucleosome cores define relatively independent polypeptide conformational domains. That is, the helical secondary structure of a histone pair, which involves nearly 50% of the chains, is not significantly dependent on the proximity of other histone pairs in the folded nucleosome core structure.

Comparisons of the near-UV circular dichroism and thermal denaturation of subnucleosomes and cores further suggest that homotypic histone pairs *and the DNA they tightly bind* define quasi-independent conformational domains in cores. As noted above, the core DNA conformation and stability are little affected by loss of an H2a–H2b pair and associated DNA. Thermal denaturation data showed that H3–H4 pairs and H2a–H2b pairs can stabilize 48–50 and 16–18 DNA base pairs, respectively; i.e., two sets of homotypic pairs could stabilize a total of 128–136 DNA bp. Similarly, treatment of the CD data in terms of a two-state model showed that H3–H4 pairs and H2a–H2b pairs could alter the CD of about 41 and 19 bp of DNA, respectively, to that of DNA in the intact core. That is, two sets of homotypic pairs could account for the CD spectrum of 120 of the 146 bp of DNA in cores. Thus the stability and overwinding of DNA in intact cores can be reproduced almost entirely by summing the local, independent effects of homotypic histone pairs on the conformation of DNA.

The organization of nucleosome cores into semi-independent conformational domains could offer several distinct advantages for nucleosome assembly, unfolding, and refolding during replication and transcription. These processes could occur in stepwise, rather than concerted fashion, if most of the intrinsic stability and conformational properties of the core were determined within subdomains [see also Weintraub et al. (1976)]. Since the structures of these subdomains are dictated primarily by histone–histone and histone–DNA interactions of dimers, the energy required to complete and fine tune the core folding would correspond to the free energy of forming histone octamers from dimers. The energy available for these processes appears more than sufficient to provide the extra stability required (Isenberg, 1979).

Relative Roles of Homotypic Histone Pairs in Nucleosome Cores. The abilities of H3 and H4, but not H2a and H2b, to partially organize nucleosome cores is well established [reviewed by McGhee & Felsenfeld (1980)]. During in vitro assembly of nucleosome cores, core length DNA can be initially bound and partly folded by H3–H4 tetramers. Folding is completed by binding H2a–H2b pairs (Klevan et al., 1978). H3 + H4, but not H2a + H2b, induce supercoiling of circular DNA's (Bina-Stein & Simpson, 1977) and may fold core length DNA into one superhelical turn (Klevan et al., 1978). Equimolar H3 + H4 protect approximately 70–80-bp regions of DNA against micrococcal nuclease, whereas much smaller DNA regions are protected by H2a + H2b (Camerini-Oteri et al., 1976).

The properties of SN6u and SN4u clearly are consistent with the different roles suggested for the homotypic H3–H4 and H2a–H2b pairs. In addition they suggest an operational basis for these differences. A single H2a–H2b pair in SN4u strongly stabilizes and may alter the winding angle of only 16–19 DNA base pairs, while an H3–H4 pair in SN6u strongly stabilizes and alters the winding angle of 40–50 DNA base pairs. Previously we have suggested that the H3–H4 tetramer occupies the central 80 bp of core DNA, based on studies of the origins of subnucleosomes (Nelson et al., 1982) and the locations of histone–DNA cross-links identified by Mirzabekov and co-workers (Shick et al., 1980; Belyavsky et al., 1980). The properties of SN6u show that two H3–H4 pairs can tightly bind almost precisely this amount of DNA and thereby organize the critical central DNA loop in cores. The properties of SN4u are consistent with the proposal that H2a–H2b pairs primarily stabilize and wind about 10–20 bp of DNA near each core terminus and complete the folding, probably by interacting with H3 and H4.

The disproportionate stabilization of DNA by H3–H4 pairs cannot be explained simply in terms of electrostatic effects. As noted previously, there are only a total of 56 Lys + Arg

[4] Previously we have shown that SN7u sediments only slightly slower than nucleosome cores ($s_{20,w}$ = 9 S) (Nelson et al., 1982).

[5] SN7u results from cleavages 40–50 bp from the ends of cores containing 146 bp length DNA. The lengths of DNA in SN7u isolated for these studies were approximately 10 bp longer because the average core length is >146 bp after moderate digestion of chromatin (Nelson et al., 1982).

residues in an H3–H4 pair; hence, at least 20 bp in SN6u must be strongly bound and stabilized by nonelectrostatic interactions. Proximity effects cannot be readily invoked since the electrostatic potentials of H3–H4 and H2a–H2b pairs are nearly identical, yet H2a and H2b strongly stabilize only slightly more DNA than expected from amino-terminal tail binding.

Contributions of nonelectrostatic interactions to the conformational stability of chromatin have been recognized for some time. The order of elution of histones from DNA by increasing concentrations of simple salts is not directly correlated with basicity, since H1 is removed at much lower salt concentrations than less basic core histones (Ohlenbusch et al., 1967). Ionic detergents or urea greatly facilitate the removal of H3 and H4, which otherwise bind most tightly (Senshu, 1971; Smart & Bonner, 1971; Bartley & Chalkley, 1972, 1973; Hayashi & Ohba, 1974).

Consideration of the origins of subnucleosomes and the extra stabilization of DNA in SN6u directly suggest that H3 and H4 partly interact with DNA through some combination of hydrophobic effects and hydrogen bonding. We have observed that nucleosome cores cleaved at sites about 30 bp from the ends spontaneously lose an H2a–H2b pair and an associated small DNA fragment, but cores cleaved approximately 40 and 50 bp from the ends do not dissociate into subnucleosomes unless 2–3 M urea is added (Nelson et al., 1982). The latter observation indicates that H3 and H4 bind strongly to DNA 30–50 bp from the ends of (146 bp) cores through interactions that are sensitive to urea. The extra 20 bp in SN6u that are stabilized nonelectrostatically can be reasonably associated with these urea-sensitive sites since urea predominantly disrupts hydrophobic effects and hydrogen bonding and has little effect on electrostatic interactions.

The properties of H1 complexes with short DNA (SN5u) are interesting in several respects. The increase in the positive, near-UV circular dichroism band of DNA is curious since complexes of H1 or H5 with large DNA cause either no CD change, at low ionic strengths (0.01), or a significant *decrease* in intensity at near physiological ionic strengths (0.15) (Olins, 1969; Fasman et al., 1970). It is difficult to interpret this increase unequivocally since solutions of this subnucleosome were mildly turbid, indicating aggregation. Simply interpreted, the CD increase suggests a modest tendency of H1 to unwind DNA. Such a tendency would be consistent with the preference of H1 for supercoiled over relaxed circular DNA (Vogel & Singer, 1975; 1976), but H1 seems to have little ability to introduce superhelical turns in DNA (Bina-Stein & Singer, 1977). In any case the local effects of H1 on DNA conformation are quite different from those of core histones.

The aggregation of these small H1–DNA complexes when urea is removed is noteworthy. H1 alone does not aggregate, but H1 complexes with large DNA can assume very compact configurations [e.g., see Glotov et al. (1978); Matthews & Bradbury, 1978], and H1 is involved in condensing chromatin [reviewed by McGhee & Felsenfeld (1980)]. Aggregation of SN5u could occur either by rearrangement of H1 to form bridges between DNA fragments or by intermolecular association of globular H1 domains. Arguments have been made for and against contributions of both mechanisms to the higher order folding of chromatin by H1 (Bradbury et al., 1973; Hartman et al., 1977; Glotov et al., 1978; Aviles et al., 1978). An increase in the helix content of H1 may accompany DNA binding upon aggregation, since the far-UV CD spectrum of SN5u suggested a helix content of about 30%. In addition, a helix content for H5 approaching that of core histones was suggested by the similarities of the far-UV spectra of chromatosomes and cores. H1 and H5 in the absence of DNA appear to be 16% or less helical, depending on the ionic conditions (Crane-Robinson et al., 1976; Smerdon & Isenberg, 1976). More detailed studies of complexes of H1 with short DNA may be useful for distinguishing the intrinsic association properties and conformation of DNA-bound H1.

H1 is very effective in increasing the thermal stability of both short and long DNA [e.g., see Ansevin & Brown (1971)]. Like H3–H4 pairs, and unlike H2a–H2b pairs, H1 can stabilize more DNA than is predicted purely from electrostatic considerations. Thus both basic ends of H1 and perhaps the globular region must interact strongly with DNA. Such multiple valency is consistent with the cross-linking role proposed for H1.

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