# Subnucleosomes and Their Relationships to the Arrangement of Histone Binding Sites along Nucleosome Deoxyribonucleic Acid†

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ABSTRACT: Micrococcal nuclease cleaves within nucleosomes at sites spaced about 10.4 base pairs (bp) apart. Cleavages at sites equivalent to 30–35 bp from the ends of 146-bp cores cause spontaneous loss of an H2a–H2b pair associated with 30–40 bp length DNA. Cleavages at certain other sites do not affect the nucleosome integrity unless a solvent perturbant such as urea is added. Chromatin moderately digested with micrococcal nuclease, when fractionated by sedimentation or electrophoresis in the presence of 3 M urea, yielded four previously unobserved subnucleosomes with the following histone/DNA compositions: $(H3)_2(H4)_2(H2a)(H2b)$/95–115 bp; (H3)(H4)/70–80-bp DNA; (H2a)(H2b)/50–60-bp DNA; and (H1)/60–70-bp DNA. All but the latter subnucleosome were also obtained upon DNase I digestion of purified nucleosome cores labeled on the 5′ ends with $^{32}P$. Only subnucleosomes that retained H2a and H2b also retained labeled ends. These results show that H2a and H2b are paired on the terminal 30–40 bp of core DNA, as suggested from analyses of histone–DNA cross-link products by Mirzabekov and coworkers [Mirzabekov, A. D., Schick, V. V., Belyavsky, A. V., & Bavykin, S. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4184–4188]. Considerations of the origins and compositions of subnucleosomes and of cross-linking data suggest an expanded model for the locations of histone binding sites along nucleosome core DNA. The principal features of this model are (i) strong electrostatic binding sites of H2a and H2b occur at positions approximately 20–30 bp from the core ends, (ii) strong electrostatic binding sites of H3 and H4 occur primarily on the central 40 bp of core DNA, (iii) strong nonelectrostatic, urea-sensitive binding sites of H3 and H4 occur at positions approximately 30–50 bp from the core ends, and (iv) urea-sensitive binding sites of H2a or H2b may occur on the terminal 10–20 bp of core DNA.

Nuclease attack of chromatin is restricted by the organization of DNA and histones into nucleosomes.[1] Micrococcal nuclease preferentially cleaves the linkers between nucleosome cores and degrades the linker DNA from the ends. In addition, micrococcal nuclease and other nucleases attack within the core at sites spaced about 10.2–10.4 bp[2] apart. The specificities of the latter cleavages reflect the native environment and geometry of DNA in nucleosomes and are used as criteria of the fidelity of reconstituted chromatin and nucleosomes [for reviews see Kornberg (1977), Felsenfeld (1978), Rill (1979), and McGhee & Felsenfeld (1980a)].

Although DNA fragments produced by internal cleavages of nucleosomes have been examined extensively, the corresponding nucleohistone fragments or "subnucleosomes" have received relatively little attention. In principle the compositions of these fragments should provide information about the order of histones along nucleosome DNA in a manner conceptually analogous to conventional sequencing methods. Furthermore, since these fragments represent subdomains of nucleosomes, their physicochemical properties may reveal features of nucleosome constituents that are not accessible from studies of the complete structure or other combinations of the parts.

We describe here the isolation and compositions of six major histone-containing subnucleosomes derived from chicken erythrocyte nuclei digested with micrococcal nuclease. A problem encountered in these studies arose from the fact that histones make multiple contacts within nucleosomes. Some cleavages are "nonproductive" because histone–DNA or histone–histone interactions in nucleosomes bridge the DNA discontinuity, preventing spontaneous dissociation into subnucleosomes. Only two major, stable, subnucleosomes containing core histones were isolated from chromatin digests in the absence of solvent perturbants. Modest concentrations of urea (2–3 M), which are sufficient to partially unfold intact nucleosome cores without affecting histone secondary structure (Olins et al., 1977), dissociated internally cleaved nucleosomes and permitted the isolation of four additional subnucleosomes. Considerations of the compositions and origins of these subnucleosomes confirm other indications that H2a and H2b bind mainly to the terminal 30–40 bp of nucleosome cores (Shick et al., 1980; Belyavsky et al., 1980) and suggest additional features of the interactions of histones with specific regions of nucleosome DNA.[3]

## Materials and Methods

*Isolation and Digestion of Erythroid Nuclei.* Blood was obtained by heart puncture with a syringe containing 0.1 volume of 50 mM $Na_2EDTA$, 0.23 M NaCl, and 1 mM PMSF (pH 7.0). Cells were washed with the same solution by repeated suspension and centrifugation until the upper "buffer coat" of white cells was removed. The final cell

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[1] Nucleosomes are defined here as the total chromatin repeat units containing about 212 base pair length DNA (in erythrocytes) and associated histones, including H1 or H5.

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

[3] A preliminary report of this work was presented at the Cold Springs Harbor Symposia on Quantitative Biology, 1977.

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suspension was used immediately or made 25% in glycerol and stored at −20 °C.

Cells were lysed by mild agitation in TKMC buffer containing 0.25 M sucrose, 0.5% Triton X-100, and 0.1 mM PMSF (final pH 7.5 at 20 °C). (TKMC buffer contains 50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, and 1 mM $CaCl_2$ and is adjusted to pH 7.5 with cacodylic acid.) Nuclei were recovered by low-speed centrifugation, washed twice with the above solution, and sedimented through dense sucrose as described by Rill et al. (1978). Recovered nuclei were washed twice with 0.25 M sucrose in TKMC buffer containing 0.1 mM PMSF and stored at −20 °C, if necessary, in the same solution containing 25% glycerol. Nuclei were usually digested with micrococcal nuclease (Worthington, 1 or 2 units/50 μg of DNA at 37 °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 cacodylic acid, adjusted to pH 6.5 with solid Tris. We have also examined products of nuclei isolated and digested in lower ionic strength solutions, as described by Sollner-Webb & Felsenfeld (1975), and of nuclei isolated in higher ionic strength solutions containing spermine and spermidine and lacking divalent cations, as described by Hewish & Burgoyne (1973) and Noll (1974). Digestion was terminated in all cases by addition of 0.1 M $Na_2EDTA$ to yield a final EDTA concentration in slight excess of the divalent ion concentration. All methods yielded the same nucleosome and subnucleosome species.

*Density gradient centrifugation* of digests was performed on linear 5–20% sucrose gradients as described previously (Rill et al., 1975) using the International SB-283 rotor at 40 000 rpm or the SB-110 rotor at 25 000 rpm. Prior to centrifugation digested nuclei were dialyzed into 10 mM Tris-HCl (pH 7.5), 1 mM $Na_2EDTA$, and 0.1 mM PMSF (containing 0–3 M urea, as required).

*Electrophoresis of nucleoproteins* was performed on 6, 8, or 10% polyacrylamide slab or tube gels, as required; 10 mM Tris and 2 mM $Na_2EDTA$, adjusted to pH 7.6 at 20 °C with Mops, were used for both the tray and gel buffers. Bromothymol blue ($pK_a$ = 6.8) was included in the lower tray to monitor pH changes. Tube gels were overlayered with butanol until polymerization was complete, then butanol was replaced with tray buffer, and the gels were allowed to cure overnight. All gels were preelectrophoresed at least 2 h at 130 V before use.

Electrophoresis of nucleoproteins in the presence of urea was performed as above except that various concentrations of urea were added to the running gel buffer as noted under Results. Preparative electrophoresis was performed on 1.2 × 17 cm cylindrical gels of 8% polyacrylamide [acrylamide/bis(acrylamide), 80:1]. Prior to electrophoresis all samples were dialyzed against 10 mM Tris–Mops, 2 mM EDTA, 0.0025% bromothymol blue, and an appropriate concentration of urea (usually 3 M; final pH 7.6) and then centrifuged at 10 000 rpm for 10 min. Oligonucleosome larger than tetramers were removed from samples used for preparative electrophoresis by a step gradient centrifugation procedure. Stock 10 M urea solutions were deionized immediately prior to use with analytical grade mixed-bed resin (AG 501-X8, 20–50 mesh, Bio-Rad Laboratories). Urea was not included in the tray buffers of urea gels, but gels were overlayered with buffered urea solutions during preelectrophoresis to avoid dilution of urea in the gels. Samples containing 10% glycerol were then layered under the urea solution by using a syringe. Gels were stained with ethidium bromide (1 μg/mL) and photographed under black light illumination or were scanned at 260 nm by using a Gilford spectrophotometer with gel scanning accessory.

*Electrophoresis of histones* on acid–urea gels was performed as described by Panyim & Chalkley (1969).

Electrophoresis on gels containing $NaDodSO_4$ was performed as described by Laemmli (1970) by using 18% polyacrylamide separating gels [acrylamide/bis(acrylamide), 200:1] and 3% stacking gels. Slab gels were 0.3 × 20 × 20 cm, and tube gels were 5 mm × 15 cm. So that histones could be electrophoresed directly from slices of preparative nucleoprotein gels, portions of previously frozen slices (3 × 7 × 7 mm) were incubated for 90 min at room temperature in 10 mM Tris, 0.1 mM EDTA, and 0.1 mM PMSF (pH 7.5) to remove Mops (which interfered with stacking) and then at 50 °C for 30 min in 50 μL of 1.1% $NaDodSO_4$, 10 mM Tris, 5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, and 0.005% bromphenol blue (pH 7.0). Strips from slab gel separations of nucleoproteins were treated as above for second-dimension analyses. Slices or strips were then firmly pressed into slab gel wells. Samples from gradient fractions were made 1% in $NaDodSO_4$ and dialyzed against the above sample buffer prior to electrophoresis. Gels were stained in 0.25% Coomassie blue in 5:4:1 water/methanol/acetic acid and destained in the same solution containing a small amount of dye ($A_{580}$ = 0.1). Gels were scanned at 580 nm by using a Gelman DCD-16 gel scanner.

*Electrophoresis of double-stranded DNA* was performed on 6% or 12% slab gels (0.3 × 20 × 20 cm) as described previously (Rill et al., 1978) except that 0.1% $NaDodSO_4$ was included in the tray and gel buffers in cases where nucleoproteins were applied to the gels. Inclusion in the system of a stacking gel of 3% polyacrylamide [acrylamide/bis(acrylamide), 30:1] containing 80 mM histidine, 0.1 mM EDTA, 0.1% $NaDodSO_4$, and 4 mM Tris (pH 5.0) was found to provide narrower bands when preparative gel slices were applied to the gels. Gel slices were incubated at 37 °C for 1 h in 10 mM Tris-HCl and 0.1 mM EDTA (pH 7.5), blotted to remove moisture, and then incubated at 37 °C for 1 h in 1.1% $NaDodSO_4$, 10 mM Tris-HCl, 5 mM EDTA, and 0.005% bromphenol blue (pH 7.0). After electrophoresis gels were stained with ethidium bromide (2 mg/L), soaked in 50% methanol to remove $NaDodSO_4$ and excess ethidium bromide (Todd & Garrard, 1977), and then restored to their original dimensions by soaking in distilled water. Restriction endonuclease *Hae*III fragments of φX174 RF DNA included as markers were purchased from Bethesda Research Laboratories, Inc.

*Electrophoresis of single-stranded DNA* was performed on 12% polyacrylamide slab gels containing 7 M urea as described by Maniatis et al. (1975). Gels were stained in Stains-all (Dahlberg et al., 1969).

*Determination of the histone/DNA stoichiometries of nucleoproteins* was done in three ways: (1) Equal aliquots ($A_{260}$ units) of nucleosomes, cores, and subnucleosomes were electrophoresed on $NaDodSO_4$ gels, and the staining intensities of histones in subnucleosomes were compared to those of nucleosomes and cores. (2) Nucleoproteins were electrophoresed, and gels were scanned at 260 nm to quantitate DNA, then stained with Coomassie blue, and scanned at 580 nm to quantitate total protein. (3) Nucleosomes, cores, and subnucleosomes were isolated by preparative electrophoresis and analyzed by using the Bio-Rad protein assay. The assay was calibrated with standard solutions of each of the purified chicken histones prepared by chromatography on Bio-Gel P-10. Histone concentrations were determined from absorbancies at 275.5 nm, using the extinction coefficients reported by

D'Anna & Isenberg (1974). Histone/DNA stoichiometries were calculated from the determined protein concentrations, the relative concentrations of histone indicated by quantitative gel analyses, and the 260-nm absorbancies of the samples. Highest accuracy is expected from the latter method, but all three methods yielded equivalent results.

*End Labeling and Redigestion of Nucleosome Cores.* Nucleosome cores were obtained from chromatin depleted of H1 and H5 by the method of Bolund & Johns (1973) and digested to ca. 15% acid solubility with micrococcal nuclease (1 unit/50 $\mu$g of DNA in 10 mM Tris-HCl and 1 mM $CaCl_2$, pH 8.0, at 37 °C). Cores were isolated in three separate experiments by (1) gel filtration of Bio-Gel A5m, (2) sucrose density gradient centrifugation, and (3) preparative electrophoresis in 3 M urea. Preparation 3 contained the sharpest distribution of ca. 146-bp DNA, and least amounts of contaminating subnucleosomes, but was slightly contaminated with nucleosomes due to incomplete removal of H5. The presence of nucleosomes does not affect the conclusions drawn.

Cores were labeled on the 5′ ends with [$\gamma$-$^{32}$P]ATP (New England Nuclear; 10–40 Ci/mmol) and polynucleotide kinase (Miles; 1.5 × $10^4$ units/mg) as described by Simpson & Whitlock (1976), freed of excess $^{32}$P by density gradient centrifugation, concentrated by ultrafiltration, and dialyzed against 10 mM Tris-HCl and 0.1 mM EDTA (pH 8.0). Purified cores were further digested with DNase I (Worthington; 0.5 $\mu$g/50 $\mu$g of DNA) at 37 °C after addition of $MgCl_2$. Trial digests were done with unlabeled cores treated as above to determine the appropriate digestion times and the amount of $MgCl_2$ required to provide an excess over EDTA. Labeled samples were digested under the indicated conditions, and three or four aliquots were removed at times corresponding to slight, moderate, and extensive digestion. After addition of EDTA, samples were electrophoresed as described above to resolve DNA and nucleoproteins. Gels were stained with ethidium bromide, photographed, and then dried and autoradiographed by using Kodak X-OMat XR-5 film. Autoradiograms were scanned with a Joyce-Loebl densitometer.

For determination of the lengths of DNA in subnucleosomes, DNA was isolated by the method of Maxam & Gilbert (1977) from gel slices containing cores and subnucleosomes and then electrophoresed and autoradiographed as above.

*Isolation of Native, Tritium-Labeled, Mouse Myeloma Histones.* Mouse myeloma cells (kindly provided by Dr. William Marzluff, Florida State University) were grown in a suspension culture on Dulbecco's modified Eagle's medium with reduced lysine (10% normal) and 10% horse serum. Cells were labeled with 500 $\mu$Ci of [$^3$H]lysine (New England Nuclear; 25 Ci/mmol) for 16 h, during which time the cell number doubled to ca. $10^6$/mL. Nuclei isolated as described by Jackson et al. (1979) were lysed with 0.35 M NaCl, 2 mM EDTA, 0.1 mM PMSF, and 10 mM Tris-HCl (pH 7.5) and washed 3 times with the same solution to remove most cytoplasmic and nonhistone proteins. Histones were extracted by dialyzing washed chromatin against the above buffer with the NaCl concentration increased to 4 M, followed by centrifugation at 140000$g$ for 24 h to remove DNA. Core histones were obtained by the same procedure except that the chromatin was first depleted of H1 by sedimentation through the above solution containing 0.6 M NaCl into a 2.0 M sucrose pillow containing 0.62 M NaCl.

*Test for Histone Exchange Using Labeled Histones.* Labeled myeloma histones were concentrated and dialyzed against the buffer used for nuclear digestion, and then ca. 50-$\mu$g portions were added to aliquots of digested chicken erythrocyte nuclei (ca. 3 mg of histone equivalents) that were previously dialyzed against digestion buffer or the buffer used to electrophorese nucleoproteins (with or without 3 M urea). All mixtures were further dialyzed extensively against electrophoresis buffer (with or without urea). Samples in urea were sedimented on linear, 5–20% sucrose gradients containing 3 M urea for 26 h at 40000 rpm (4 °C); samples without urea were sedimented as above on gradients without urea for 14 h. Gradient fractions were analyzed for histone by using the Bio-Rad protein assay, with commercial calf thymus histone (Sigma) as a standard. Aliquots (100 $\mu$L) were diluted with 5 mL of $H_2O$, added to 10 mL of Triton/toluene (1:2) containing 4 g/L Omnifluor (New England Nuclear), and counted on the Beckman LS70000 liquid scintillation counter. Specific activities of fractions containing no detectable protein were calculated based on the sensitivity limit of the assay.

*Cross-Linking of Histones in Nucleosome Cores and Subnucleosomes with Lomant's Reagent.* Nucleosome cores containing 146-bp DNA and minimal internal cleavages were isolated by density gradient centrifugation from chromatin depleted of H1 and H5 (Bolund & Johns, 1973) and digested with micrococcal nuclease to 15% acid solubility. Subnucleosomes were isolated from whole chromatin digests by density gradient centrifugation. Prior to cross-linking, samples were dialyzed exhaustively against 25 mM triethanolamine–HCl (pH 8.0) and then diluted to a concentration of 0.1 mg/mL protein (determined by the Bio-Rad dye binding assay). Aliquots were reacted with varying concentrations of Lomant's reagent (Pierce Chemicals) for 1 h at room temperature. Reactions were terminated by adding an equal volume of 50% $Cl_3CCOOH$ or 0.1 volume of 0.1 M Tris-HCl (pH 8.0). Pure histones, prepared by chromatography of whole acid-extracted histones on a 1.5 × 170 cm column of Bio-Gel P-10 (200–400 mesh; Bio-Rad Laboratories) eluted with 10 mM HCl, were cross-linked by the same procedure.

The degree of cross-linking was assessed initially on 5% polyacrylamide gels containing $NaDodSO_4$ (Weber & Osborn, 1969). Histone dimer compositions were determined by two-dimensional electrophoresis on 30 cm, 18% gels containing $NaDodSO_4$ as described by Thomas & Kornberg (1978).

*Cross-Linking of Histones in Nucleosome Cores with MMBI.* Nucleosome cores (0.2 mg of DNA equivalents/mL) in 50 mM boric acid, 1 mM $Na_2EDTA$, and 0.1 mM PMSF (adjusted to final pH 9.0 with NaOH and made 3% in mercaptoethanol just prior to use) were reacted with varying concentrations of methyl 4-mercaptobutyrimidate hydrochloride (Pierce Chemical Co.) for 1 h at ambient temperature. Reaction was terminated by addition of a slight excess of glycine and cooling. Samples were dialyzed exhaustively against 10 mM Tris, 1 mM $Na_2EDTA$, and 0.1 mM PMSF (pH 8.0) to remove mercaptoethanol and then cross-linked by oxidation with hydrogen peroxide (40 mM, 30 min at room temperature). Remaining free thiols were blocked by reaction with iodoacetamide (17 mM, in the dark for 30 min). Cross-linked products were analyzed by electrophoresis as described above. Core histones were fully cross-linked to octamers by using 3–5 mg of MMBI/mg of histone.

## Results

*Subnucleosomes Observed in the Absence of Urea.* Chromatin fragments generated by digestion to increasing extents with micrococcal nuclease were analyzed electrophoretically to identify major subnucleosomes and observe their production rates relative to nucleosomes. The primary small products of very limited digestion (<5% acid solubility) were two nu-

Table I: Summary of DNA Lengths in Nucleosomes and Subnucleosomes

| particle[a] | source[b] | acid soluble[c] (%) | DNA lengths (bp)[d] | | | | | |
|---|---|---|---|---|---|---|---|---|
| MN3 | WD | 21 | 220 ± 20 | | 141 ± 15 | | 73 ± 10 | |
| MN2 | MN | 11 | 216 ± 34 | | 148 ± 15 | | 76 ± 16 | |
| MN2 | WD | 14 | 213 ± 33 | | 147 ± 18 | | 75 ± 23 | |
| MN2 | WD | 21 | 192 ± 22 | | 131 ± 17 | | 65 ± 13 | |
| MN1 | MN | 11 | 215 ± 35 | | 144 ± 26 | | 74 ± 23 | |
| MN1 | WD | 14 | 214 ± 35 | | 139 ± 26 | | 76 ± 24 | |
| MN1 | WD | 21 | 192 ± 22 | | 129 ± 20 | | 65 ± 13 | |
| MNC | MN | 11 | | 168 ± 29 | | 108 ± 12 | 68 ± 17 | |
| MNC | WD | 14 | | 169 ± 28 | | 100 ± 11 | 71 ± 18 | |
| MNC | WD | 21 | | 162 ± 18 | | 108 ± 17 | 63 ± 12 | |
| SN7 | MN | 11 | | | 127 ± 10 | | 63 ± 12 | |
| SN7 | WD | 14 | | | 120 ± 17 | | 63 ± 13 | |
| SN7 | WD | 21 | | | 116 ± 16 | | 60 | |
| SN4(U) | SN | 11 | | | | | | 48 ± 10, 25 ± 4 |
| SN4(L) | SN | 11 | | | | | | 40 ± 9 |
| SN4 | WD | 14 | | | | | | 45 ± 10 |
| SN4(U) | WD | 21 | | | | | | 33 ± 5 |
| SN4(L) | WD | 21 | | | | | | 38 ± 6 |

[a] Particles were obtained from slices of bands from preparative gels. U and L signify upper and lower halves of a band. [b] Source denotes the sample subjected to preparative electrophoresis: whole digest (WD), monomer gradient fraction (MN), or subnucleosome gradient fraction (SN). [c] Extent of digestion of nuclei. [d] Variations indicated are the bandwidths. The precision of the method is at least ±5%.

cleosomes (MN1 and MN2), nucleosome cores (MNC), and three subnucleosomes (SN6, SN7, and SN8) (Figure 1). Upon further digestion a third nucleosome appeared (MN3), the yields of subnucleosomes SN7, SN4, and SN1 were enhanced, and other subnucleosomes virtually disappeared. Second-dimension electrophoresis confirmed that MN2, MN1, and MNC were nucleosomes containing H1 (MN2), nucleosomes containing H5 (MN1), and nucleosome cores (MNC), as described by Bakayeva & Bakayev (1978). MN3 contained core histones, H1, and a few nonhistone proteins, mainly one that remained near the top of 15% gels containing $NaDodSO_4$ (not shown).

SN4 and SN7 were characterized in detail since only these subnucleosomes contained histones and persisted during the period of chromatin digestion when yields of nucleosomes and cores were optimal and therefore could represent major products of internal cleavages of nucleosomes. (SN1 contained no detectable protein and very short DNA fragments). Second-dimension electrophoresis showed that SN7 contained no H1 or H5 and approximately half as much H2a and H2b as H3 and H4. Quantitative comparisons of electrophoretic patterns of histones from nucleosome cores and SN7 isolated by preparative electrophoresis confirmed this 2:1 stoichiometry (Figure 2). The average length of DNA in SN7 varied with the extent of chromatin digestion, decreasing from 127 to 116 bp when digestion was increased from 11% to 21% acid solubility. The average lengths of DNA recovered in nucleosome cores decreased concurrently from 168–169 bp to 162 bp (Table I; see also Figure 5 below).

Two-dimensional electrophoresis showed that SN4 contained only H2a and H2b in essentially equimolar amounts (Figure 3). Lengths of DNA recovered from SN4 by preparative electrophoresis decreased from 40–50 bp after moderate digestion (11% acid solubility) to 30–40 bp after 21% digestion (Table I). No DNA larger than 35 bp was recovered from SN4 isolated after 30% digestion, when the core length was reduced almost entirely to 146 bp (Figure 3). Semiquantitative comparisons of the ratio of total protein staining to absorbance at 260 nm indicated that the protein/DNA ratio of nucleosome cores and SN4 were nearly equal, i.e., that SN4 contained two histones per DNA fragment. This assignment was confirmed by cross-linking SN4 and nucleosome cores under identical

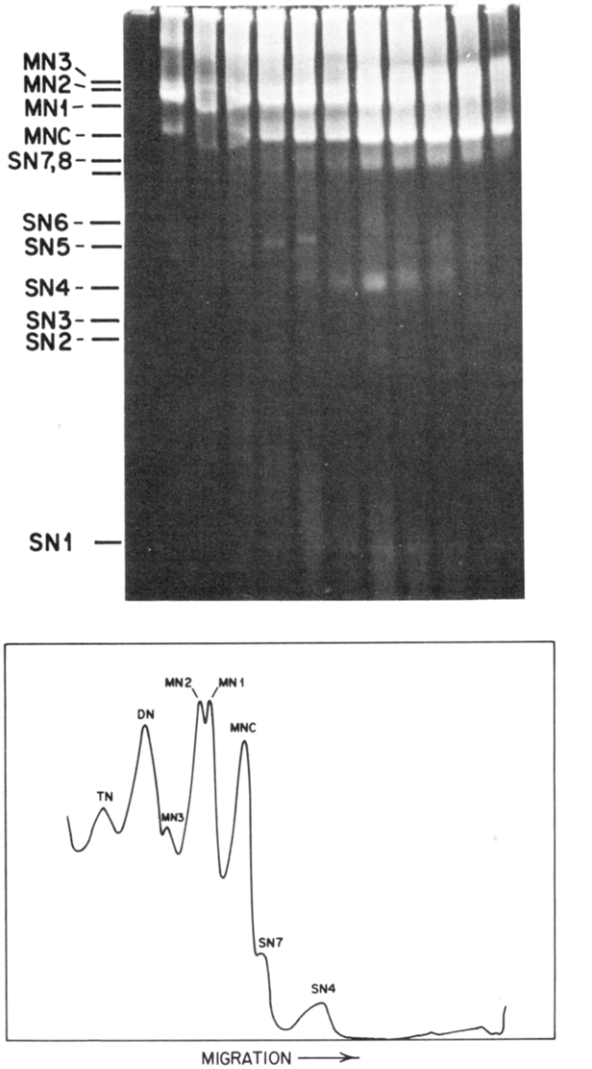


FIGURE 1: (Top) Electrophoresis of nucleoproteins from chromatin digests on a 15-cm, 10% polyacrylamide slab gel. Bromphenol blue was electrophoresed to the bottom. Samples were digested to 0, 0.6, 1.3, 2.4, 4.0, 5.8, 8.4, 11, 16, 22, 28, and 35% acid solubility (left to right). (Bottom) Densitometer scan of nucleoproteins from chromatin digested to 11% acid solubility and electrophoresed on a 15-cm, 6% polyacrylamide tube gel.

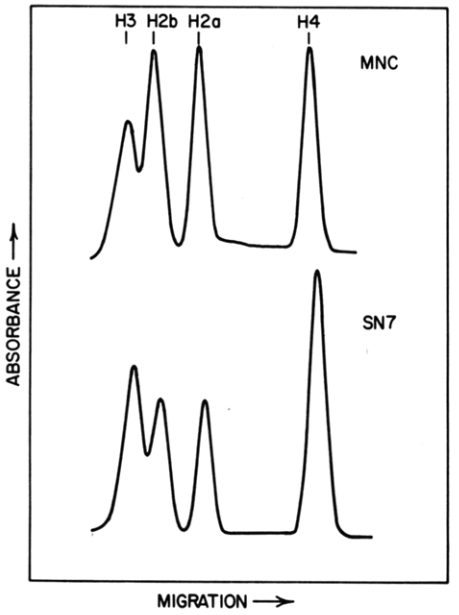


FIGURE 2: Quantitation of histones in subnucleosome SN7. Nucleosome cores (MNC) and SN7 were isolated by preparative electrophoresis. Four aliquots of each, spanning a 4-fold concentration range, were electrophoresed directly on 30-cm, 18% gels containing $NaDodSO_4$. Gels were stained with amido black. Integration of gel scans showed that the staining ratio (H2a + H2b)/(H3 + H4) for SN7 (0.53 ± 0.05) was exactly half that of nucleosome cores (1.05 ± 0.07).

conditions with increasing concentrations of Lomant's reagent. SN4 yielded only dimers under conditions that polymerized virtually all nucleosome histones to octamer or larger species that remained near the top of 18% gels (Figure 4). Cross-linking of purified individual histones under the same conditions yielded ladders of oligomer bands (not shown). Partial cross-linking of SN4 did not affect the ratio of H2a and H2b monomers and yielded a single major dimer band with the same mobility as the major dimer of partially cross-linked nucleosomes (Figure 4). Two-dimensional electrophoresis (before and after reversal of the cross-links) on long gels confirmed that the dimer from SN4 migrated with the same mobility as the H2a–H2b dimer from nucleosomes (Figure 4). From these results, the similar degree of disappearance of H2a and H2b upon cross-linking, and the comigration of equal amounts of H2a and H2b with DNA in subnucleosomes, we conclude that SN4 contained H2a–H2b pairs.

*Productive and Nonproductive Cleavages of Nucleosomes.* SN4 and SN7 contained only a subset of DNA fragments shorter than the intact nucleosome core length that is found in total digests. Most of the remaining subnucleosome length fragments coelectrophoresed and cosedimented with nucleosomes and nucleosome cores. For example, each class of nucleosome (MN1, MN2, and MN3) and nucleosome cores isolated after digestion to 11, 14, and 21% digestion contained small DNA fragments ranging down to 50 bp in length (Figure 5). Oligonucleosomes also contained considerable amounts of small DNA (not shown). Since the separation of nucleoproteins is highly dependent on DNA length, most of these small fragments must have originated from internal cleavages of nucleosome DNA that were nonproductive, i.e., did not cause dissociation into the respective nucleohistone fragments (this origin is confirmed below).

Although nucleosomes and nucleosome cores contained a broad range of DNA fragment lengths, each species *lacked* a characteristic, narrow range of sizes. These absent sizes must correspond either to sites in nucleosomes and cores that are

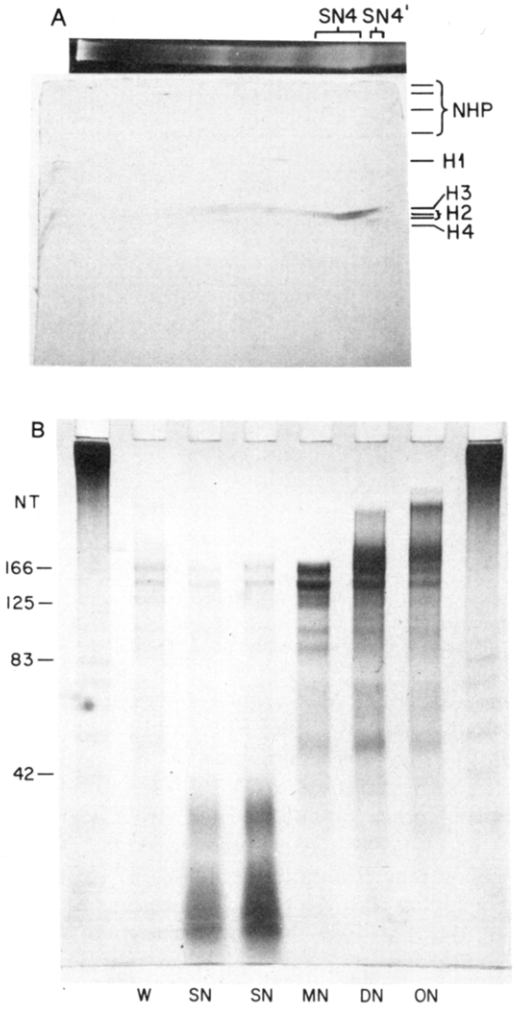


FIGURE 3: (A) Two-dimensional electrophoresis of proteins from subnucleosomes (principally SN4). Subnucleosomes from chromatin digested to 11% acid solubility were isolated by sedimentation on sucrose gradients (2–7S fractions) and then electrophoresed on a 15-cm, 10% slab gel. Proteins were electrophoresed on a second-dimension, 18% gel containing $NaDodSO_4$. (Whole chromatin was applied at the far left.) (B) Electrophoretic comparisons of DNA fragments in subnucleosomes and nucleosomes from extensively digested chromatin. Subnucleosomes (SN, 2–8 S), nucleosomes (MN, 9–13 S), nucleosomes (MN, 9–13 S), and dinucleosomes (DN, 14–18 S) were isolated by density gradient centrifugation. DNA was electrophoresed on a 20-cm denaturing gel alongside marker fragments from a DNase I digest of chromatin (far right and left). Staining was with Stains-all.

not cleaved or to sites that are cleaved productively to yield subnucleosomes. For example, MN2 and MN1 isolated after 11–21% digestion lacked ca. 160–180 bp and 90–110 bp length fragments (Figure 5 and Table I). The absence of the larger fragments is presumably the consequence of productive cleavages of nucleosome spacers that yield cores. Nucleosome cores generally lacked fragments 115–135 and < 50 base pairs long. The correspondence of these missing lengths with lengths of DNA found in SN7 and SN4 implies that these subnucleosomes result from productive cleavages 30–40 bp from the ends of cores.

*Separation of Subnucleosomes in the Presence of Urea.* The effects of increasing concentrations of urea on separations of chromatin digests were examined to determine if this perturbant would dissociate internally cleaved nucleosomes into subnucleosomes without causing histone rearrangement. Addition of urea to sucrose gradients or polyacrylamide gels at concentrations up to 3 M caused large increases in the

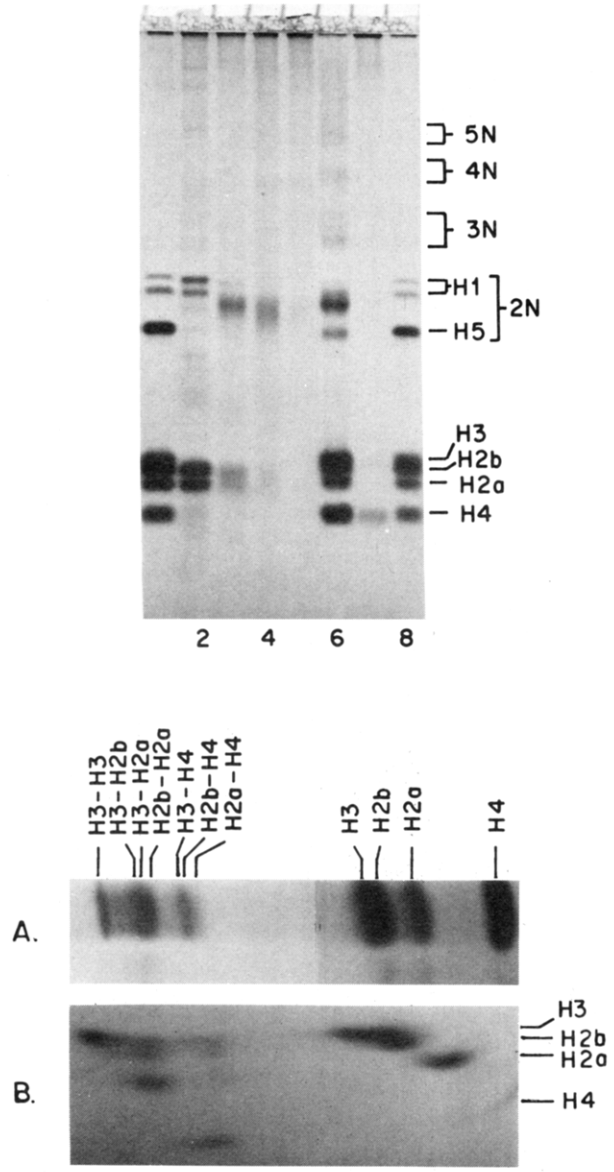


FIGURE 4: (Top) Cross-linking of histones in SN4 and nucleosomes. Subnucleosomes and nucleosomes (mainly cores) isolated by gradient centrifugation were cross-linked with Lomant's reagent and then electrophoresed on a 20-cm, 15% gel containing $NaDodSO_4$. (Lanes 1 and 8) Whole digest, un-cross-linked; (lanes 2–5) subnucleosomes cross-linked with 0, 0.8, 2.4, and 4.8 mg of Lomant's reagent/mg of histone for 1 h at pH 8.0; (lanes 6–7) nucleosomes cross-linked with 0.8 and 4.8 mg of Lomant's reagent/mg of histone as above. (Bottom) Analyses of histone dimers from nucleosome cores. Chromatin from a 20% acid-soluble digest was cross-linked with 1.2 mg of Lomant's reagent/mg of histone as above, and then subnucleosomes and nucleosomes were isolated by density gradient centrifugation. Histone dimers from nucleosomes (frame A, top lane) and subnucleosomes (frame A, lower lane) were resolved by electrophoresis on a 30-cm, 15% gel containing $NaDodSO_4$. A slice from the region of the gel containing dimers from nucleosomes was treated with mercaptoethanol to reverse the cross-links, and then un-cross-linked histones were electrophoresed on a 30-cm, 18% gel (frame B). (Second-dimension electrophoresis of the dimer from SN4 confirmed that SN4 contained only H2a–H2b dimers, but the bands were too faint to reproduce photographically.)

amounts of particles with smaller sedimentation coefficients and higher electrophoretic mobilities than those of nucleosome cores (Figure 6). Urea concentrations >3 M caused loss of electrophoretic resolution, probably due to histone conformational changes described by Olins et al. (1977). Samples dialyzed against 3 or 6 M urea, and then against 0 or 3 M urea, yielded electrophoretic patterns on gels containing the

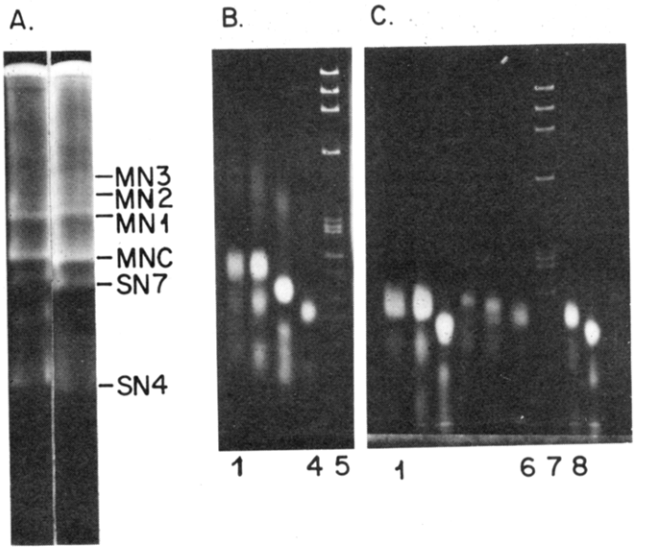


FIGURE 5: (A) Preparative electrophoresis of chromatin digested to 11% (left) and 21% (right) acid solubility. (B) Analyses of DNA in species MN2, MN1, MNC, and SN7 (lanes 1–4) from preparative gel separations of chromatin digested to 11% acid solubility. Marker *Hae*III fragments of $\phi$X174 RF DNA are in lane 5. Samples were electrophoresed on a 15-cm, 6% nondenaturing gel containing $NaDodSO_4$. (C) Analyses of DNA from MN2, MN1, and MNC of chromatin digested to 14% acid solubility (lanes 1–3) and MN3 (lanes 4 and 5), MN2, MN1, and MNC (lanes 6, 8, and 9) of chromatin digested to 21% acid solubility. Electrophoresis and marker (lane 7) as in (B).

Table II: Compositions of Nucleohistones Isolated by Preparative Electrophoresis of Chromatin Digested with Micrococcal Nuclease

| nucleo-histone[a] | histones[b] | $L$, DNA (bp)[c] | yield[d] |
|---|---|---|---|
| MN2 | $(1)(3,4)_2(2a,2b)_2$ | 180–200 | |
| MN1 | $(5)(3,4)_2(2a,2b)_2$ | 180–200 | 1.17 |
| MNC | $(3,4)_2(2a,2b)_2$ | 145–170 | 1.0 |
| SN7 | $(3,4)_2(2a,2b)_1$ | 115–125 | 0.2 |
| SN7u | $(3,4)_2(2a,2b)_1$ | 95–115 | 0.62 |
| SN6u | $(3,4)_1$ | 70–80 | 0.18 |
| SN5u | (1) | 60–70 | 1.06 |
| SN4u | $(2a,2b)_1$ | 50–60 | 0.47 |
| SN4 | $(2a,2b)_1$ | 30–40 | 0.2 |

[a] Listed in order of increasing electrophoretic mobility. [b] Stoichiometries were determined by the Bio-Rad dye binding assay using purified histones as standards. Values are rounded to the nearest integer. The precision of the method was ±10%. [c] DNA lengths varied slightly with extent of digestion. Lengths listed were obtained after moderate digestion (15–18% acid-soluble oligonucleotides). [d] Relative molar yields of the particles after preparative electrophoresis of a moderately digested sample, estimated from $A_{260nm}$.

final concentrations of urea that were almost identical with patterns of samples not exposed to the higher urea concentrations (not shown), suggesting that effects of urea were largely reversible.

*Compositions of Nucleosomes and Subnucleosomes Isolated in 3 M Urea.* Electrophoresis in 3 M urea resolved two major species of nucleosomes (MN2u and MN1u), nucleosome cores (MNCu), and four major subnucleosomes (SN4u, SN5u, SN6u, and SN7u) from chromatin digested under all conditions investigated (see Materials and Methods). Essentially homogeneous samples of each species were obtained by preparative electrophoresis (Figure 7). Nucleosomes and nucleosome cores isolated in the presence of urea were identical in composition with those isolated without urea except for the absence of DNA fragments smaller than the parent length, demonstrating that dissociation of internally cleaved species

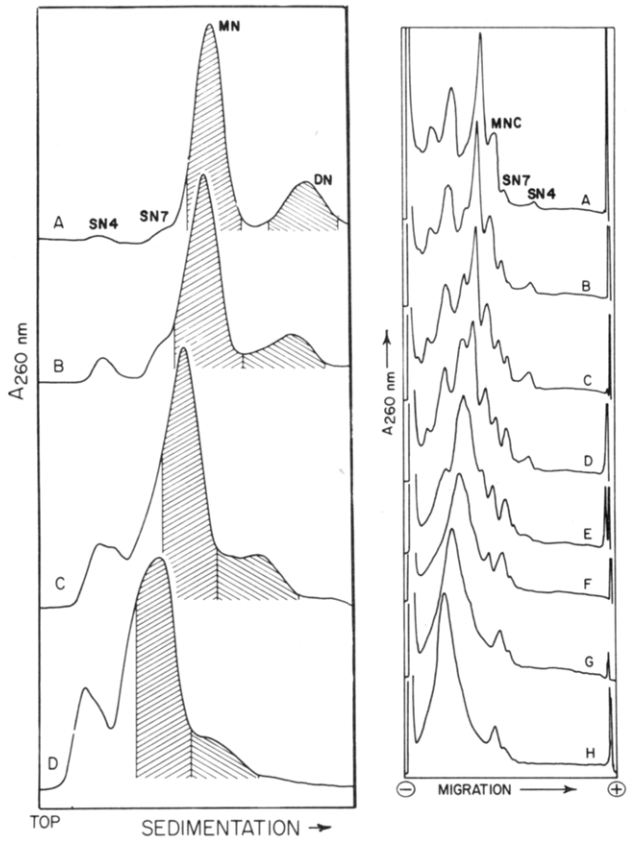


FIGURE 6: Effects of increasing concentrations of urea on the sedimentation (left) and electrophoretic patterns (right) of nucleoproteins from chicken erythrocyte nuclei digested with micrococcal nuclease. Dialyzable oligonucleotides were removed prior to fractionation. A 30% acid-soluble digest was sedimented for 12 h at 40 000 rpm on linear 5–20% sucrose gradients containing uniform concentrations of 0, 1, 2, and 3 M urea (A–D). A 10% acid-soluble digest was electrophoresed on 11-cm, 6% polyacrylamide gels containing 0, 1, 2, 3, 4, 5, 6, and 7 M urea (A–H). Bromphenol blue was electrophoresed to the bottom of each gel. Gels were scanned at 260 nm after soaking 1 h in distilled water.

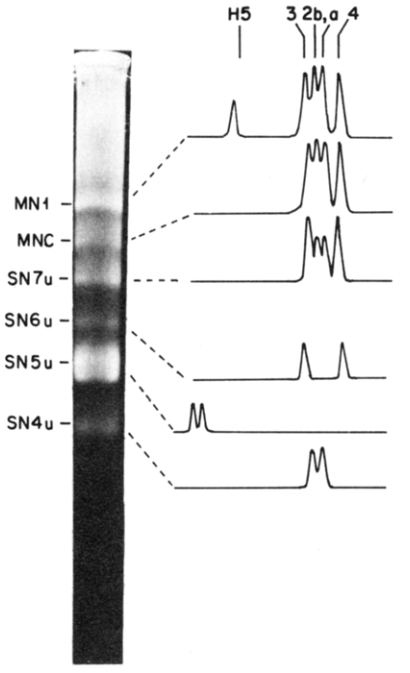


FIGURE 7: Histone compositions of small nucleoproteins from digested chromatin (18% acid soluble) separated by preparative electrophoresis on an 8% gel containing 3 M urea. Particles were electroeluted from gel slices and electrophoresed on 20-cm, 18% gels containing Na-DodSO$_4$.

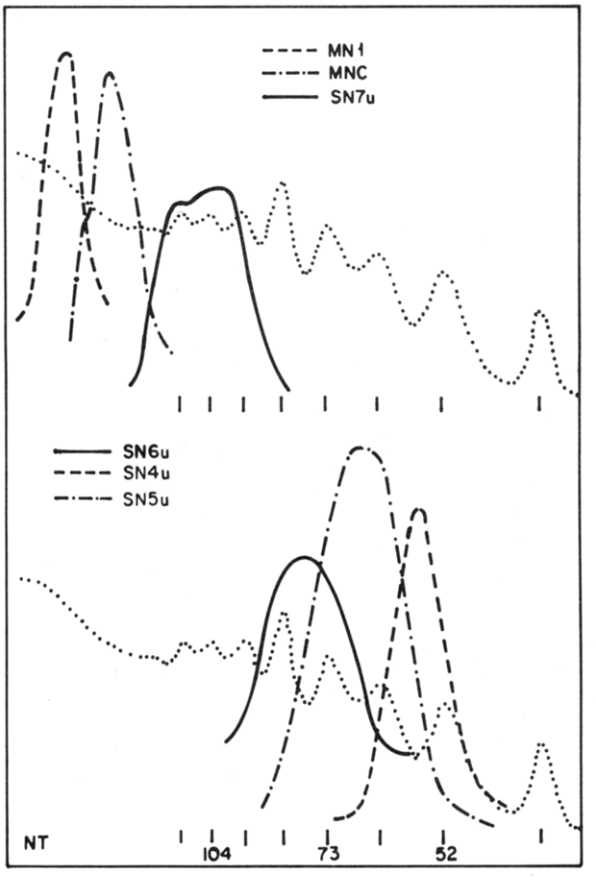


FIGURE 8: Analyses of the DNA lengths in small nucleoproteins separated by preparative electrophoresis in urea (same samples as in Figure 7). Deproteinized DNA was electrophoresed under denaturing conditions. Marker fragments ($N \times 10.4$ nucleotides) obtained from a DNase I digest of chromatin were electrophoresed beside each sample. Gels were stained with Stains-all and then scanned.

was complete in 3 M urea (Figures 7 and 8). Each subnucleosome contained a subset of histones associated with a unique range of DNA lengths (Figures 7 and 8). SN7u and SN4u were isolated in nearly equimolar amounts and were identical in histone composition with SN7 and SN4, respectively, but differed in DNA length (Table II). After moderate digestion (15–20% acid solubility) DNA in SN7u was about 15 bp shorter than in SN7, while DNA in SN4u was about 15 bp longer than in SN4. SN5u contained a single H1 (a and b) per 55–75 bp length DNA, and SN6u contained one copy each of H3 and H4 per 65-85 bp length DNA. The molar yield of the former particle was close to that of nucleosome cores, but the yield of SN6u was only about one-third that of SN7u (Table II).

*Origins of Subnucleosomes.* Although internal cleavages of all nucleosomes seem to occur at similar sites [e.g., see Felsenfeld (1978) and McGhee & Felsenfeld (1980a)], subnucleosomes could arise from special classes of nucleosomes or nonnucleosomal parts of chromatin. This possibility was tested in two ways: First, digested chromatin was electrophoresed in one dimension in the absence of urea and then in a second dimension on gels containing or lacking 3 M urea. Nearly all nucleohistones appeared as single spots in the second-dimension gel lacking urea, but subnucleosomes were observed across the entire second-dimension gel containing urea (Figure 9). Thus all subpopulations of mono- and oligonucleosomes were internally cleaved and yielded electrophoretically identical subnucleosomes. So that the chemical identities of these subnucleosomes could be established, MN1,

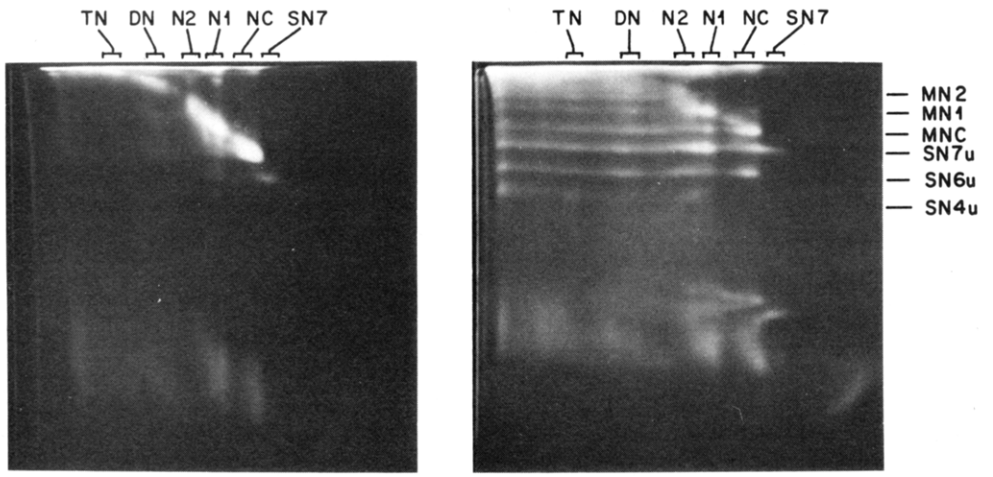


FIGURE 9: Origins of subnucleosomes. Chromatin digested to ca. 15% acid solubility was electrophoresed on a 6% gel lacking urea, and then lanes were transferred and electrophoresed on 8% gels lacking (left) or containing (right) 3 M urea. Staining was with ethidium bromide.

MN2, and MNC were isolated by preparative electrophoresis in the absence of urea, and then subnucleosomes were isolated from each of these species by electrophoresis on second-dimension preparative gels containing urea. Histone and DNA contents of all subnucleosomes were essentially identical with those listed in Table II.

A second set of experiments showed that the same subnucleosomes, except SN5u, could be obtained directly from nucleosome cores and that SN4u originated from the end(s) of cores. Cores containing 146-bp DNA were isolated by preparative electrophoresis in urea, labeled on the 5′ ends with $^{32}PO_4$, and then digested with DNase I.[4] DNA isolated from one aliquot of each digest was electrophoresed on a denaturing gel, and nucleohistones from another aliquot were electrophoresed on a gel containing urea (Figure 10). The patterns of end-labeled DNA fragments were consistent with other studies of the digestion of native cores by DNase I (Simpson & Whitlock, 1976; Lutter, 1978). The electrophoretic patterns and histone contents of subnucleosomes obtained with DNase I were identical with those obtained with micrococcal nuclease (Figure 11). Autoradiography of the nucleohistone gel showed that digestion caused the appearance of end-labeled SN4u, but no additional label appeared in SN6u. Two other core preparations yielded the same results.[5] DNA from SN4u, but not SN6u, therefore was derived from the end(s) of cores.

End-labeled DNA isolated from SN4u was predominantly 50 nucleotides long after moderate DNase I digestion and 40 + 50 nucleotides long after more extensive digestion. End-labeled DNA from SN7u was of complementary length, 95–105 nucleotides. Thus nonproductive DNase I cleavages occurred 40 and 50 nucleotides from the core end. Virtually no subnucleosomes were observed when digested cores were electrophoresed in the absence of urea, presumably because of the low frequency of cleavages, 30 nucleotides from the ends, that are needed to produce SN4 (Simpson & Whitlock, 1976; Lutter, 1978).

*Stability of Subnucleosomes.* The possibility of histone rearrangement during digestion and isolation must be excluded to interpret the compositions of subnucleosomes in terms of the structure of nucleosomes. Histones do not dissociate from whole chromatin or isolated nucleosomes in low ionic strength solutions containing up to 6 M urea (Bartley & Chalkley, 1972, 1973; Jackson & Chalkley, 1974; Olins et al., 1977). Subnucleosomes could differ in this respect if histone–DNA contacts were lost after nuclease digestion. The general stabilities of subnucleosomes were assessed by dialyzing digested chromatin into buffered 3 M urea, then into TKMC buffer lacking urea, and again into buffered 3 M urea. A second chromatin sample was allowed to stand 2 weeks in buffered 3 M urea. Both samples yielded the same subnucleosomes as a control digest not exposed to urea until electrophoresis. Samples of individual subnucleosomes isolated for physical studies were stable for at least a month at 4 °C (A. J. Mencke and R. L. Rill, unpublished observations).

The possibility of histone equilibration among subnucleosomes was tested directly by adding small amounts of $^{14}C$-labeled native histones to an excess of digested chromatin prior to fractionation. Since nucleosomes bind extra histones (Voordouw & Eisenberg, 1978), some incorporation of labeled histones into nucleohistones was expected. If only simple binding occurred, the specific activity of any unbound histones would remain considerably higher than the specific activity of histones in subnucleosomes and nucleosomes. If histones of the chromatin digest exchanged among DNA fragments, then labeled histones would participate in the exchange equilibrium, and the specific activity of unbound histones would be the same as the specific activity of histones in subnucleosomes and nucleosomes.

When chromatin digests plus labeled histones were sedimented on sucrose gradients containing or lacking 3 M urea, a significant fraction of labeled histones remained near the meniscus. No protein was detected in meniscus fractions; thus a minimum specific activity was calculated based on the sensitivity limit of the assay. The minimum specific activity at the meniscus was 5–8 times that of subnucleosome fractions and 10–20 times that of nucleosome fractions (Figure 12), indicating that histones were not in exchange equilibrium among subnucleosomes in the presence or absence of urea but that extra histones bound more readily to subnucleosomes than nucleosomes. This result is not suprising since the histone/DNA ratio of subnucleosomes is about half that of nucleosomes.

The ability to obtain subnucleosomes directly from cores was used to exclude possible histone rearrangement during nuclease digestion. Histones in cores were cross-linked to octamers by using methyl 4-mercaptobutyrimidate, and then

[4] Similar experiments attempted with micrococcal nuclease were not successful due to a rapid removal of the end label, as observed in other laboratories.

[5] All three preparations were contaminated to a small degree (<5%) with SN6u. In no case did additional label appear in SN6u after DNase I digestion (usually label in this region decreased), although significant amounts of label appeared in SN4u.

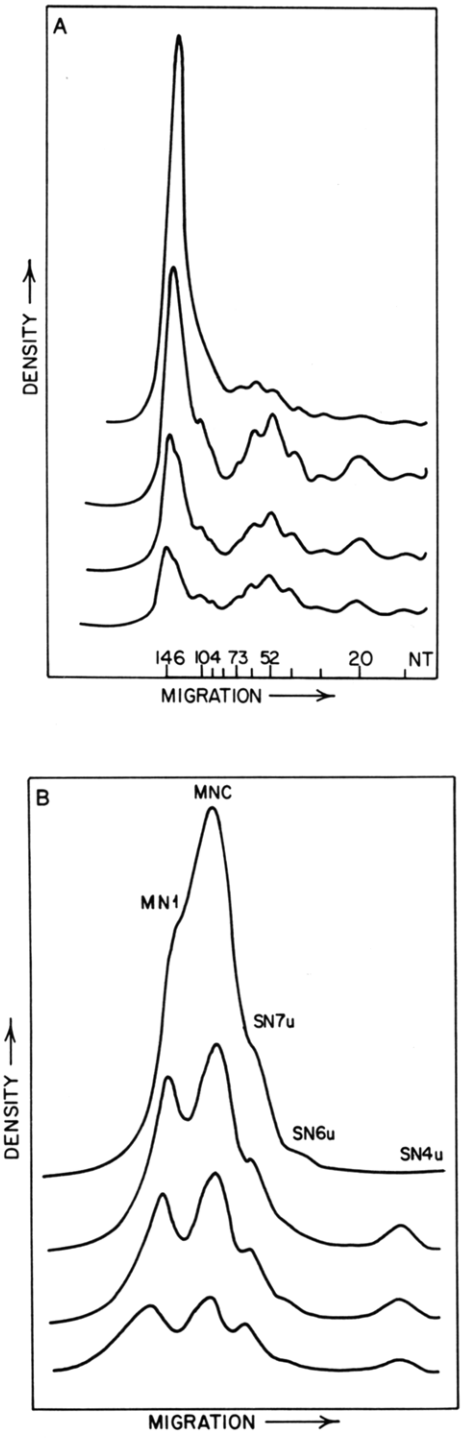


FIGURE 10: (A) Distributions of DNase I cleavages from the ends of nucleosome cores. Cores were labeled on the 5′ ends with $^{32}P$ and digested for 0, 20, 50 and 120 s (top to bottom) with DNase I. Isolated DNA was electrophoresed under denaturing conditions and autoradiographed by using preflashed film. The film was scanned with a Joyce-Loebl recording densitometer. (B) Distributions of labeled ends among subnucleosomes generated by DNase I digestion of nucleosome cores. Cores were labeled on the 5′ ends with $^{32}P$, digested with DNase I for 0, 20, 50, and 120 s [top to bottom, same samples as in (A)], and electrophoresed on an 8% gel containing 3 M urea. Densitometer scans of the resulting autoradiograms are shown. Peaks were assigned based on analysis shown in Figure 11.

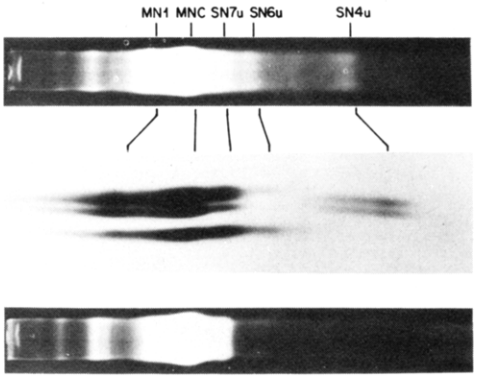


FIGURE 11: Electrophoretic patterns and histone compositions of subnucleosomes from nucleosome cores digested with DNase I. (Top) Nucleosome cores digested for 50 s with DNase I and electrophoresed in 3 M urea; (center) second-dimension electrophoresis of core histones from nucleosomes and subnucleosomes separated in the top gel (note that this preparation was partially contaminated with 11S particles containing H5 and ca. 170-bp DNA; the H5 region of the gel is not shown); (bottom) electrophoresis of initial, uncut cores on gel containing urea.

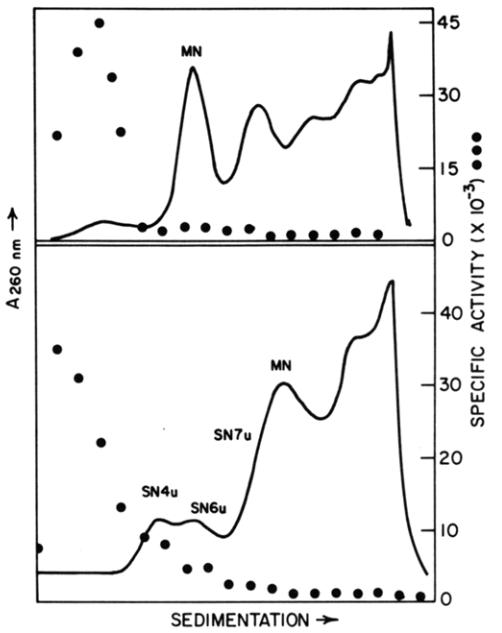


FIGURE 12: Test for histone exchange among subnucleosomes using histones labeled with [$^3H$]lysine. Labeled mouse myeloma histones were added to a large excess of erythrocyte chromatin digested with micrococcal nuclease, and mixtures were dialyzed exhaustively against gradient buffer lacking (top) or containing (bottom) 3 M urea and then centrifuged on linear 5–20% sucrose gradients lacking urea (top) or gradients containing 3 M urea (bottom). Count levels in all peak fractions were at least 10× background levels. Protein-specific activities were determined based on the Bio-Rad dye-binding assay. The specific activity distribution was not significantly altered when samples were dialyzed against nuclear digestion buffer prior to the above treatment (not shown).

cores were digested with DNase I. Electrophoresis on gels containing 3 M urea after the cross-links were reversed yielded the same subnucleosomes as an un-cross-linked control sample (Figure 13). Histone compositions of these subnucleosomes were confirmed by second-dimension electrophoresis. When cross-links were not reversed prior to electrophoresis, only intact cores were observed (Figure 13); thus subnucleosomes appearing after reversal of the cross-links originated from fully cross-linked cores.

## Discussion

The abilities of micrococcal nuclease and DNase I to cleave specific sites within nucleosomes are well recognized. We have shown that nucleohistone fragments, or subnucleosomes, resulting from these cleavages can be isolated under conditions that do not cause histone rearrangement. Subnucleosomes

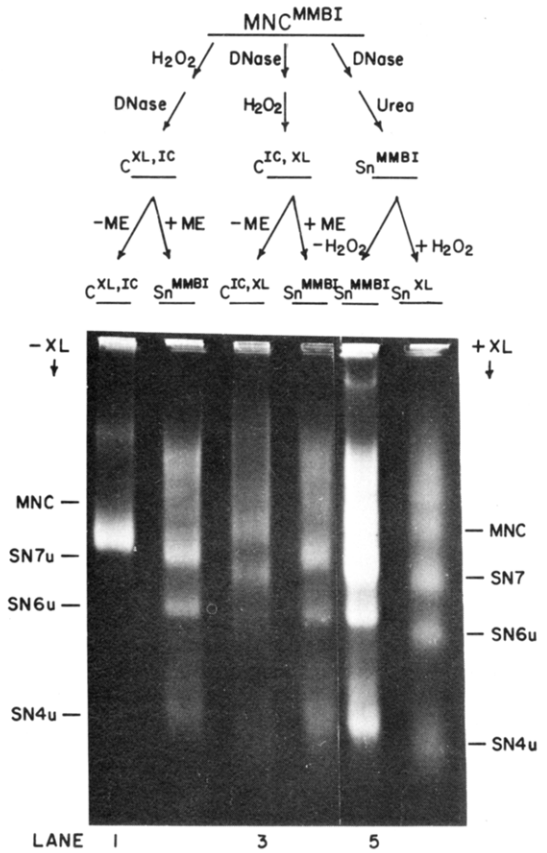


FIGURE 13: Test for histone exchange using cross-linked nucleosome cores. Purified nucleosome cores were reacted with methyl mercaptobutyrimidate (MMBI) to the extent required to fully cross-link core histone to octamers after oxidation (4 mg of MMBI/mg of total histone). Activated, un-cross-linked cores, when treated with DNase I and electrophoresed in 3 M urea, yielded the typical pattern of subnucleosomes (lane 5). Cross-linking of subnucleosomes in 3 M urea prior to electrophoresis did not change the general pattern but did slightly increase the mobilities of all particles (lane 6). Cross-linking of cores prior to DNase digestion prevented dissociation into subnucleosomes in 3 M urea (lane 1), but the normal pattern of subnucleosomes was obtained from these cores when cross-links were reversed with mercaptoethanol (ME) prior to electrophoresis in urea (lane 2). Cores treated with DNase I prior to cross-linking partially dissociated to subnucleosomes in the absence of urea (lane 3), but these cores yielded the typical subnucleosome pattern when treated with mercaptoethanol and electrophoresed in urea (lane 4; compare with lanes 2 and 5). [Note that all particles electrophoresed in cross-linked form (lanes 1, 3, and 6) migrated slightly faster than the corresponding un-cross-linked particles.] All samples were treated identically with DNase and peroxide, except the order of addition, and electrophoresed on an 8%, 15-cm slab gel. Equivalent amounts were loaded on lanes 1–4.

isolated at any extent of digestion of erythrocyte chromatin were a significant fraction of nucleohistones of mononucleosome size or smaller. For example, the amount of DNA recovered in total subnucleosomes was about half that recovered in nucleosome cores after digestion with micrococcal nuclease to ca. 15–20% acid solubility. This value was estimated from the yields after preparative electrophoresis (Table II) and from integrations of gel scans (e.g., see Figure 6). Furthermore, virtually all DNA fragments shorter than the core length were recovered in the major subnucleosomes described. Since considerable evidence indicates that the locations of micrococcal nuclease and DNase I attack are intimately related to the general nucleosome structure [e.g., see McGhee & Felsenfeld (1980a)], we believe that the compositions of these subnucleosomes are representative of true subdomains of nucleosomes. The possibility that histone modifications or other subtle differences between nucleosomes affect the rates of internal cleavages cannot be ruled out but would not affect the conclusions drawn below.

The effects of internal cleavages on the integrity of nucleosomes or cores depend upon the strengths of histone–DNA or histone–histone interactions that bridge the DNA discontinuity. If these interactions are weak under the conditions used to fractionate digested chromatin, nucleosomes will spontaneously dissociate into subnucleosomes. The compositions of subnucleosomes can therefore be interpreted in terms of the location of tightest binding of specific histones under these conditions.

Subnucleosomes SN4 and SN7 were complementary in histone and DNA composition, as were SN4u and SN7u; i.e., the histone compositions and total DNA lengths in these pairs were equivalent to nucleosome cores isolated from the same digests. The molar yields of SN4 and SN7 were nearly equal, as were the yields of SN4u and SN7u. Histones H2a and H2b were found to be paired in SN4, and DNase I digestion of end-labeled nucleosome cores yielded labeled SN4u containing equal amounts of H2a and H2b. Together these data demonstrate that H2a and H2b are paired on at least one end of nucleosome cores. Several lines of evidence indicate that nucleosome cores and core histone octamers are symmetric (Klug et al., 1980; McGhee & Felsenfeld, 1980a); hence, we assume that H2a and H2b are paired on both core ends.

The length of core DNA tightly covered by H2a and H2b can be estimated from the lengths of DNA recovered in SN4 and SN7. After moderate digestion (11–15% acid solubility), when nucleosome core DNA was mainly 160–170 bp long, DNA in SN4 and SN7 was 40–50 and 120–130 bp long, respectively. More extensive digestion reduced the average DNA length in cores to nearly 146 bp and the average lengths in SN4 and SN7 to approximately 30–35 and 115–120 bp, respectively. SN4 and SN7, therefore, must have originated from cleavages approximately 30 bp from the ends of 145-bp 146-bp and 40–50 bp from the ends of 160–170-bp cores. The spontaneous appearance of SN4 and SN7 when digested chromatin was fractionated in low ionic strength media indicates that H2a and H2b bind most tightly to the terminal 30 bp of cores under these conditions and bind weakly, if at all, to other core DNA or histones. Likewise, no other histones can bind strongly to the terminal 30 bp of cores at low ionic strengths.

In contrast, the dissociation of cores cleaved at sites more than 30 bp from the ends occurred only in the presence of 2–3 M urea. This effect is consistent with observations that moderate concentrations of urea (2–4 M) noncooperatively swell and increase the asymmetry of cores, without affecting the secondary structure of core histones or dissociating histones from DNA (Olins et al., 1977). Olins and co-workers suggested two plausible models for the swollen conformation of the core. In one, the histone quaternary structure is preserved, and swelling is attributed to partial uncoiling of DNA due to extensive loss of histone–DNA interactions. In the second model, swelling is attributed mainly to loss of histone tertiary and quaternary structure, with retention of secondary structure and histone–DNA interactions. This first model cannot be strictly correct since we did not observe histone-free subnucleosome length DNA in 3 M urea. The low histone/DNA ratios of SN4u and SN6u indicate that an intermediate model is most appropriate, i.e., that both histone–histone and histone–DNA interactions are broken in 3 M urea.

The locations of urea-sensitive histone–DNA interactions are suggested by the compositions of subnucleosomes isolated only in urea. Examination of the variations in DNA lengths

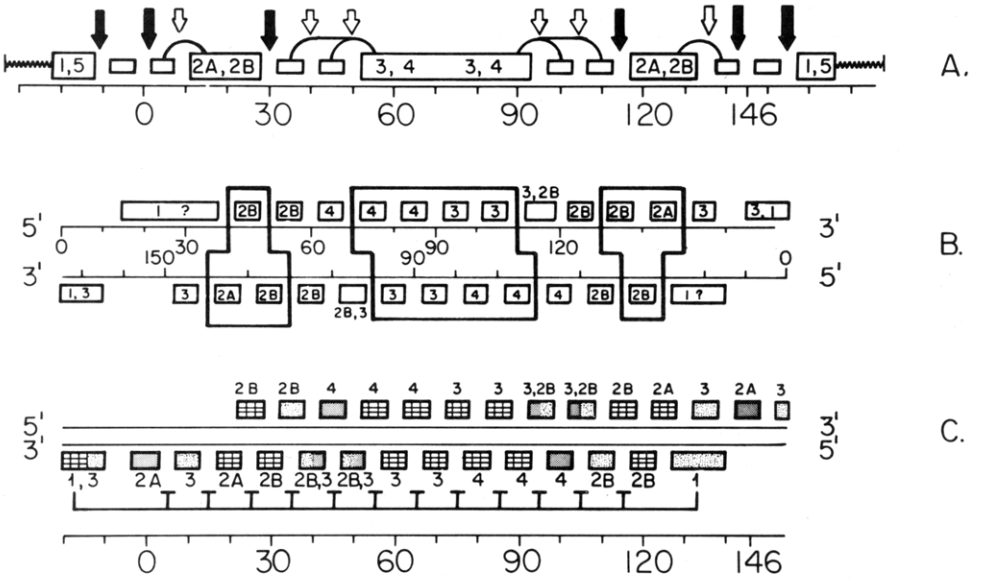


FIGURE 14: Models of the linear arrangement of histones along nucleosome DNA. (A) The relative dispositions of the major binding zones of homotypic histones on nucleosome DNA deduced from analyses of subnucleosomes. Major binding zones are indicated by large blocks and minor, urea-sensitive binding sites by small blocks connected to the appropriate histone groups. The order of histones indicated within blocks is unspecified. Approximate locations of productive cleavages within or flanking the core are denoted with solid arrows; locations of nonproductive, urea-sensitive cleavage sites are denoted with open, smaller arrows. (Note that the positions of micrococcal nuclease and DNase I are similar but are not necessarily coincident.) The nucleosome spacer, denoted by zigzag lines, is taken to be relatively uniformly accessible to micrococcal nuclease and probably is of species-dependent length. (B) The arrangement of specific histone contact points along DNA in I–nucleosomes proposed by Belyavsky et al. (1980) based on histone–DNA cross-link products. (C) Expanded model of the arrangement of histone contacts along nucleosome DNA based on subnucleosome and cross-linking data. Proposed strongly electrostatic interaction sites, presumed to correspond to highly basic histone tails, are indicated by cross-hatched blocks. Urea-sensitive, probably hydrophobic interaction sites are indicated by darkly speckled blocks. Sites of weak interactions broken at low ionic strengths are indicated by lightly speckled blocks. Horizontal bars in the lower portion of the figure denote sites of H1 contacts proposed by Belyavsky et al. (1980). Numbering is with respect to the ends of cores containing 146 bp length DNA, as in (A).

recovered in SN4u and SN7u with extent of micrococcal nuclease digestion of chromatin or DNase I digestion of cores indicates that these subnucleosomes originated from nonproductive cleavages approximately 40 and 50 bp from the ends of 146-bp cores. If we assume that all cleavages 30 bp from the core ends are productive and that the core structure is symmetric, then H3 and H4 must bind to core DNA approximately 30–50 bp from the ends through urea-sensitive interactions.

This assignment for H3 and H4 provides a rationale for the otherwise puzzling appearance of SN6u. There is no obvious mechanism of generation of this subnucleosome, which contained only one H3–H4 pair per 70–80 bp length DNA. SN6u probably arose from two internal cleavages of cores since it was isolated in low yields and lacked label when isolated from end-labeled cores digested with DNase I. The most plausible possibility is a combination of cleavages about 30 bp from one end and 50 bp from the other. In the presence of urea the H2a–H2b pairs on each end would be lost, along with the stabilizing interactions between one of the two remaining H3–H4 pairs and the DNA region 30–50 bp from the end. Loss of the latter interactions, possibly combined with the effects of urea and low ionic strength on other histone–histone or histone–DNA interactions, could account for the dissociation of one H3–H4 pair from the residual ca. 75-bp DNA fragment.

SN5u, the last major subnucleosome found, contained a single H1 associated with 60–70 bp length DNA. The nucleosome repeat length in chicken erythrocytes is about 212 bp (Morris, 1976); thus the linker length (if defined as the repeat length less the stable core length of 146 bp) is identical with the DNA length in SN5u. This identity implies that SN5u originated from nucleosomes that were cleaved internally at sites near the ends of 146-bp cores but were held intact by urea-sensitive interactions. Since reduction of the DNA length of nucleosomes to 146 bp invariably causes loss of H1 in the absence of urea, these interactions are most likely due to H2a or H2b, rather than H1.

The assignment of H2a and H2b binding sites to the ends of core DNA agrees with the linear arrangement of histones along nucleosome DNA determined by Mirzabekov and coworkers from analyses of DNA–histone cross-links formed after reaction with dimethyl sulfate (Mirzabekov et al., 1978; Shick et al., 1980; Belyavsky et al., 1980). Considerations of the origins of subnucleosomes suggest that the histone–DNA interactions defined by Belyavsky et al. can be further characterized in terms of three categories of histone–DNA interactions as follows (see also Figure 14).

(1) Histone interactions located 32–38 (H2b), 92–98 (H2b), 102–108 (H2b), and 132–138 (H3) nucleotides from the 5′ ends are classified as weak since cleavages 30–35 bp from the ends of cores caused dissociation into SN4 and SN7 at low ionic strengths.

(2) Interactions located 92–98 (H3) and 42–48 (H4) nucleotides from the 5′ ends are classified as strong but urea sensitive because urea was required to dissociate cores cleaved 50 bp from the ends to yield SN4u and SN7u. Additional strong, urea-sensitive interactions may exist at two other locations. One, assigned to H2a or H2b, is located near the core terminus to explain the appearance of SN5u in urea. Another, assigned to H3 or H4, is located 30–40 bp from the end since cores cleaved approximately 40 bp from the ends only dissociated in urea. These interactions are not reflected by histone–DNA cross-link products and could involve histone sequences lacking lysine residues in appropriate positions for cross-linking. Since urea primarily alters hydrophobic effects and has little influence on charge–charge interactions, these urea-sensitive interactions are most likely to involve the rel-

atively nonbasic, carboxy-terminal sequence regions of core histones.

(3) The remaining cross-linking sites located 22–28 (H2b), 52–58 (H4), 72–78 (H3), 112–118 (H2b), and 122–128 (H2a) nucleotides from the 5′ ends are assigned to strong electrostatic interactions that are expected for the basic, amino-terminal tails of core histones.

These suggestions can be compared to established features of nucleosome cores. Cores thermally denature in two phases. The first phase corresponds to unfolding and subsequent strand separation of 20–27 bp on each core end (Weischet et al., 1978; Simpson, 1979; McGhee & Felsenfeld, 1980b), indicating that interactions responsible for folding this terminal DNA are weak. In addition, nucleosome cores partly unfold at low ionic strengths, presumably due to increased charge repulsions (Gordon et al., 1978, 1979; Wu et al., 1979). Martinson and co-workers have shown that interactions between the carboxy-terminal sequences of H2b and H4 are broken by this partial unfolding (Martinson et al., 1979a,b; Martinson & True, 1979). These findings are consistent with the spontaneous dissociation of cores cleaved ca. 30 bp from the ends and with the proposed binding of carboxy-terminal regions of H2b and H4 to positions 32–38 and 42–48 nucleotides, respectively, from the 5′ ends of the core.

Studies of the ionic strength dependence of the first step of the biphasic thermal denaturation of cores indicate that only 16 ± 6 of the terminal 54 nucleotides (27 bp) on each core end interact electrostatically with histones (McGhee & Felsenfeld, 1980b), in agreement with the proposed strong electrostatic binding of H2a and H2b to 18 of 60 nucleotides (30 bp) on each terminus. These electrostatic interactions represent only about half of the total Lys + Arg residues in H2a + H2b tails. Proton NMR studies have indicated that a significant fraction of the lysine side chains of H2a and H2b are not bound to DNA in isolated nucleosome cores (Cary et al., 1978).

The suggestion that H3–H4 tetramers interact with almost all core DNA except the principal H2a–H2b binding sites is particularly interesting. Although the dominant role of the H3–H4 tetramer in organizing nucleosomes is well recognized [e.g., see reviews by Felsenfeld (1978), Rill (1979), and McGhee & Felsenfeld (1980a)], little is known about the nature and extent of tetramer interactions with core DNA. Amino-terminal tails of H3 and H4 together contain about 30–34 positive residues (counting histidines); hence, the basic tails of an H3–H4 tetramer could effectively neutralize the phosphates on the central 40 bp of core DNA. Assignment of H3–H4 amino-terminal tails to this region is supported by the observation that the site ca. 60 nucleotides from the 5′ end, which is highly resistant to DNase I cleavage in normal cores, is readily attacked in cores containing hyperacetylated histones (Simpson, 1978). Binding of relatively nonbasic carboxy-terminal H3 and H4 sequences to DNA approximately 30–50 bp from the ends is attractive because these interactions, combined with strong histone–histone interactions in the H3–H4 tetramer, could be sufficient to stabilize the central coil of core DNA. Whitlock & Stein (1978) have shown that trypsinized core histones lacking their basic, amino-terminal tails can combine with 146-bp DNA to form compact particles with many properties resembling those of native cores.

Cataloging of interactions of specific histones with core DNA as suggested by studies of subnucleosomes is therefore consistent with many known features of nucleosome cores. These suggestions provide a working basis for future studies of histone–DNA interactions and can be tested by identifying specific histone–DNA cross-linking products.

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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% $\alpha$ 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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