Homogeneous Reconstituted Oligonucleosomes, Evidence for Salt-Dependent Folding in the Absence of Histone H1†

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ABSTRACT: Using the method of salt dialysis, we have reconstituted histone octamers onto DNA templates consisting of 12 tandem repeats, each containing a fragment of the sea urchin 5S rRNA gene [Simpson, R. T., Thoma, F., & Brubaker, J. M. (1985) *Cell 42*, 799–808]. In these templates, each sea urchin repeat contains a sequence for preferred nucleosome positioning. Sedimentation velocity and sedimentation equilibrium studies in the analytical ultracentrifuge indicate that at molar histone/DNA ratios of 1.0–1.1 extremely homogeneous preparations of fully loaded oligonucleosomes (12 nucleosomes/template) can be regularly obtained. Digestion of the oligonucleosomes with micrococcal nuclease, followed by restriction mapping of purified nucleosome-bound DNA sequences, yields a complicated but consistent pattern of nucleosome positioning. Roughly 50% of the nucleosomes appear to be phased at positions 1–146 of each repeat, while the remainder of the nucleosomes occupy a number of other minor discrete positions along the template that differ by multiples of 10 bp. From sedimentation velocity studies of the oligonucleosomes in 0–0.2 M NaCl, we observe a reversible increase in mean sedimentation coefficient by almost 30%, accompanied by development of heterogeneity in sedimentation. These results, in combination with theoretical predictions, indicate that linear stretches of chromatin in the absence of lysine-rich histones exist in solution in a salt-dependent equilibrium between an extended (low salt) conformation and one or more folded (high salt) structures. In addition, by 100 mM NaCl, salt-dependent dissociation of histone octamers from these linear oligonucleosomes is observed.

Linear arrays of nucleosomes comprise the primary level of chromatin organization. While there is little question that linear arrays of nucleosomes can be compacted into a three-dimensional fiber of about 30 nm in diameter, the molecular mechanism of compaction is poorly understood. It has been observed by electron microscopy (EM)[1] that in low ionic strengths (2 mM < I < 20 mM) chromatin strands depleted of lysine-rich histones (e.g., H1/H5) appear as an extended "beads-on-a-string" structure (Thoma et al., 1979), whereas H1-containing chromatin adopts a zigzag-like conformation (Thoma et al., 1979; Worcel et al., 1981). At > 60 mM NaCl, H1-containing chromatin further compacts into the 30-nm fiber, while strands of H1-depleted chromatin tend to form poorly-defined clumps on the EM grid. Thus, these EM results suggest that both binding of lysine-rich histones *and* increased ionic strength are necessary to cause extended arrays of nucleosomes to form 30-nm fibers. It becomes of interest, then, to investigate the conformations which can be adopted in solution by both H1-containing and H1-depleted chromatin at physiological salt concentrations. However, while there have been extensive investigations of histone H1 interaction with nucleosomes and chromatin, there have been few studies of the effects of salt alone on chromatin folding. Although a salt-dependent change in the hydrodynamic properties of bulk H1-depleted chromatin has been observed previously (Butler & Thomas, 1980; Gale & Smerdon, 1988), it has been difficult to interpret these results due to sample heterogeneity. As a result, fundamental questions presently remain regarding the effects of salt on chromatin conformation, both in the absence and in the presence of lysine-rich histones.

The largest barrier to unambiguous studies of the structural properties of chromatin in solution has been the unavailability of a homogeneous preparation of chromatin. Chromatin fragments isolated from endogenous sources vary in size (perhaps the most important limitation), linker DNA lengths, DNA sequence composition, and probably local protein composition as well. Chromatin reconstituted in vitro from random sequence DNA suffers from most of the same deficiencies, as well as a tendency to form close-packed oligosomes (Steinmetz et al., 1978; Ruiz-Carrillo et al., 1979). It is therefore significant that a potential means to obtain homogeneous chromatin has been developed recently by Simpson, who has engineered DNA templates consisting of tandem repeats of a DNA sequence which contains a nucleosome positioning signal (Simpson & Stafford, 1983). Upon reconstitution with histone octamers, these constructs should in principle yield defined-length oligonucleosomes comprised of precisely positioned nucleosomes separated by constant linker lengths. Simpson et al. (1985) have previously reported the reconstitution of such structures in small amounts and characterized their properties using electron microscopy and agarose gel electrophoresis.

In this paper, milligram quantities of the tandemly repeated templates engineered by Simpson have been reconstituted by salt dialysis and used to initiate systematic studies of chromatin folding in solution. In this regard, we have focused initially on the effect of NaCl per se on the structure of linear oligonucleosomes; no lysine-rich histones are present. Our results demonstrate that these linear arrays of nucleosomes are ex-

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[1] Abbreviations: EM, electron microscopy; TE, 10 mM Tris-HCl and 0.25 mM Na_2EDTA, pH 7.8 (23 °C); s, sedimentation coefficient; $s_{20,w}$, sedimentation coefficient at 20 °C in H_2O; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; bp, base pair; kbp, kilobase pair; PAGE, polyacrylamide gel electrophoresis.

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tended in the absence of salt but reversibly condense into a (nonsolenoidal) folded structure in the presence of even small amounts of NaCl. Furthermore, the extended and folded states apparently exist in rapid equilibrium in moderate salt.

Experimental Procedures

Materials. White Leghorn roosters were obtained from the Oregon State University poultry farm. Restriction endonucleases were obtained from either Bethesda Research Labs or New England Biolabs and used as described below. Micrococcal nuclease was purchased from Worthington Biochemical and resuspended to 45 000 units/mL in double-distilled H_2O prior to use. Ultragel A2 was obtained from IBF Biotechnics (Savage, MD). Carboxymethyl-Sephadex was obtained from Pharmacia. All reagents were analytical grade.

Preparation of Histone Octamers. Purified trimmed nucleosome monomers were prepared from chicken erythrocytes as described in detail (method II) by Yager et al. (1989) and used as the source of histone octamers. Briefly, the nuclei prepared from 200 mL of blood were digested for 5 min at 37 °C with micrococcal nuclease (14 units/mg of DNA), and long chromatin was pelleted by centrifugation at 6900*g* for 20 min. Chromatin was resuspended in 10 mM Tris-HCl, 0.25 mM EDTA, and 0.35 M NaCl, pH 8.0, and histone H1/H5 was removed by incubation of chromatin with 30 μg/mL carboxymethyl-Sephadex for 3 h at 4 °C, followed by centrifugation at 7700*g* for 30 min and dialysis of the supernatant into 15 mM Tris-HCl, 15 mM NaCl, 60 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, and 0.34 M sucrose, pH 7.5. Stripped chromatin was digested for 5 min at 37 °C with micrococcal nuclease (5 units/μg of DNA), and the resulting nucleosome monomers and dimers were concentrated with an Amicon XM-50. Nucleosome monomers were then chromatographed on a Sephacryl S-300 column, eluted in 5 mM Tris-HCl, 0.25 mM EDTA, 20 mM NaCl, and 2 mM 2-mercaptoethanol, pH 7.5, and concentrated to >30 mg/mL with the Amicon XM-50.

Concentrated nucleosome monomers were brought to 2.2 M NaCl and 0.1 M potassium phosphate, pH 6.7, and chromatographed on a hydroxylapatite column equilibrated with the same buffer as described by Simon and Felsenfeld (1979). Individual fractions were collected and electrophoresed on a 0.5-mm discontinuous (6%/15%) SDS–polyacrylamide gel (Laemmli, 1970) to determine histone content. The octamers used in these studies were eluted in a single peak fraction at 2.6 mg/mL and stored at 4 °C in the presence of 0.2 mM PMSF. These octamers contained equimolar amounts of the four core histones, but <1% H1/H5, as judged by scanning laser densitometry of 0.5-mm discontinuous SDS gels stained with Coomassie blue. Octamer concentration was determined from measurements of A_{230} (Stein, 1979).

Preparation of Template DNA. All DNA templates originated from plasmid p5S172-12, p5S190-12, or p5S207-12, which have been constructed and introduced into *Escherichia coli* HB101 by Simpson et al. (1985). Bacteria were grown for 12–18 h in the presence of 10 μg/mL chloramphenicol (Frenkel & Bremer, 1986), and plasmids were isolated by the alkaline lysis method (Birnboim & Doly, 1979; Micard et al., 1985). In some cases, plasmids were further purified by subsequent banding in CsCl/ethidium bromide gradients (Maniatis et al., 1982). Purified p5S plasmids were digested with 1.5–2.0 units of *Hha*I/μg of DNA for 8–12 h at 37 °C (which liberates the oligonucleosome template), ethanol precipitated, and resuspended in 10 mM Tris-HCl and 0.25 mM Na_2EDTA, pH 7.8 (TE). Template DNA was then purified as described (Hansen & Rickett, 1989). Between 1.0 and 1.5 mg of total plasmid digest (in 0.25–0.3 mL of TE) was chromatographed on a 115-mL Ultragel A2 column, 1.0-mL fractions were collected, and 20-μL aliquots were electrophoresed on a 1.5% agarose gel (Tris–acetate). Template-containing fractions (usually 33–45) were pooled, and DNA yield was quantitated by determination of A_{260}. The template DNA was then precipitated with 2.5 volumes of 95% ethanol and stored at −20 °C until use.

Oligonucleosome Reconstitution. Immediately prior to reconstitution, precipitated template DNA was centrifuged for 30 min at 12000*g*, resuspended to approximately 1 mg/mL in TE, and quantitated by determination of the A_{260} of multiple template dilutions. Reconstitutions were carried out as described by Tatchell and van Holde (1977). Template DNA was made 2.0 M with NaCl and mixed with histone octamers. The final DNA concentration was always 40–50 μg/mL, and the histone octamer concentration was 1.0–1.1 mol of octamer/mol of DNA *repeat*. Samples were then dialyzed against 0.5–1.0 L of TE/NaCl as follows: 1.5 M NaCl, 4 h; 1.0 M NaCl, 4 h; 0.75 M NaCl, 3 h; 0.50 M NaCl, 3 h. The final dialysis was always overnight (>10 h) into TE buffer. After reconstitution, the oligonucleosomes were concentrated to 0.5–1.0 mg/mL in a Centricon-30 (Amicon) and stored at 4 °C prior to use.

Isolation of Mononucleosome DNA Sequences. Reconstituted oligonucleosomes in 1 mM $CaCl_2$ were digested for various times at 37 °C with micrococcal nuclease (0.06 unit/μg of DNA). Samples were dissociated by incubation with 0.1% SDS for 20 min at 37 °C, and DNA was electrophoresed (10 V/cm) on a 5.5% [1:29 bis(acrylamide):acrylamide] polyacrylamide gel (Tris–borate). Gel slices containing mononucleosome DNA were removed, and DNA was purified essentially as described by Maxam and Gilbert (1980). Gel slices were mashed to a paste and incubated overnight at 37 °C in 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS, pH 8.0. Samples were centrifuged for 5 min at 17000*g*, supernatants were passed through a 0.45-μm Nalgene filter, and DNA was precipitated with 3 volumes of 95% ethanol at −80 °C. The DNA was resuspended in 300 mM sodium acetate, precipitated again with 95% ethanol, and resuspended in sterile double-distilled H_2O prior to digestion with restriction enzymes (see below).

Analytical Sedimentation. All sedimentation studies were carried out on a Beckman Model E analytical ultracentrifuge, equipped with temperature control and scanner optics. All sedimentation velocity experiments utilized 12-mm double-sector cells in a four-hole AN-F rotor. Temperatures were near 20 °C, regulated to better than ±0.1 °C. For sedimentation velocity studies, solutions with A_{265} = 0.6–0.8 were used, and data were corrected to $s_{20,w}$ in the usual manner, with an estimated value of 0.65 for partial specific volume (Eisenberg & Felsenfeld, 1981; Ausio et al., 1984) and values of buffer density and viscosity from standard tables. For these experiments a rotor speed of 22 000 rpm was employed. Sedimentation equilibrium experiments utilized either AN-F or AN-J rotors, with 12-mm double-sector cells. Runs were initially overspeeded by about 30% for a few hours and then shifted to the final speed, which ranged from 1800 to 2200 rpm. Repeated scans were taken until no change could be observed. In all sedimentation experiments involving added NaCl, the samples were prepared as follows: 10–20 μL of concentrated oligonucleosomes (0.5–1.0 mg/mL) was mixed with 430–440 μL of a solution of NaCl in TE buffer to yield a final NaCl concentration as desired. In this manner, the oligonucleosomes were never exposed to a concentration of

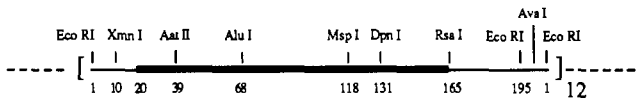

FIGURE 1: Structure of the 207-12 oligonucleosome template. Each template contains 12 tandem repeats of a 195-bp fragment containing the sea urchin 5S rRNA gene. In the cases of the 172-12 and 190-12 templates, the length of the sea urchin fragment is 160 and 178 bp, respectively. Each repeat is connected to the next by 12 bp of DNA derived from plasmid pARA during construction. In addition, after *Hha*I digestion of the p5S plasmid sources, the full templates retain 6 bp of DNA at the 3′ end and 32 bp of DNA at the 5′ end originally present in the pAT153 vector. The shaded box indicates the preferred nucleosome position observed upon reconstitution with the monomeric repeat (sequences 20–165). If nucleosomes assembled onto these templates are positioned uniformly, the 172-12, 190-12, and 207-12 oligonucleosomes will contain 27, 45, and 62 bp of linker DNA, respectively.

NaCl greater than 10% above the final salt concentration achieved after mixing.

Results

Structure and Large-Scale Isolation of Oligonucleosome Template DNA. The composition of the DNA templates used in our studies is shown in Figure 1. As engineered by Simpson et al. (1985), these templates consist of 12 tandem repeats of fragments of the sea urchin 5S rRNA gene. Each repeat is connected to the next by 12 bp of pARA-derived DNA, yielding total repeat lengths of 172, 190, and 207 bp in the 172-12, 190-12, and 207-12 templates, respectively [see Simpson et al. (1985) for original assignment of nomenclature]. A number of restriction sites are present within the sea urchin DNA repeat, which itself can be regenerated by digestion of the intact 2.5-kbp template with *Eco*RI. Significantly, previous studies have indicated that the sea urchin DNA fragment repeated in these constructs contains a strong phasing signal that results in a precisely positioned nucleosome at sequences 20–165 after reconstitution (Simpson & Stafford, 1983; FitzGerald & Simpson, 1985; Richmond et al., 1988; Moyer et al., 1989). Thus, reconstitution of histone octamers onto these linear DNA templates should in principle yield a homogeneous oligonucleosome sample in which each nucleosome is separated from the next by 27, 45, or 62 bp of linker DNA in the 172-12, 190-12, and 207-12 templates, respectively.

In order to obtain the amounts of oligonucleosomes necessary for physicochemical studies, we have developed a simple protocol for purification of very large quantities of the oligonucleosome DNA template from the numerous smaller fragments that result when the p5S plasmids are digested with *Hha*I. Using exclusion chromatography on Ultragel A2, we have routinely purified 0.5 mg of template DNA in one step with >90% yield (Hansen & Rickett, 1989). This has allowed repeated reconstitutions of the milligram amounts of oligonucleosomes used in the work described below.

Analytical Sedimentation of Reconstituted 207-12 Oligonucleosomes in TE Buffer. A rigorous determination of the molecular homogeneity of the reconstituted oligonucleosomes can be obtained from sedimentation velocity and sedimentation equilibrium studies in the analytical ultracentrifuge. The integral distribution of sedimentation coefficients of 207-12 oligonucleosomes obtained from the analysis of a typical sedimentation velocity experiment by the method of van Holde and Weischet (1978) is shown in Figure 2A. In this example, about 80% of the 207-12 oligonucleosomes in TE buffer (reconstituted at 1.1 mol of octamer/mol of DNA repeat) sediment at 29 S, with the remainder sedimenting at a very slightly lower value. While this analysis indicates that these

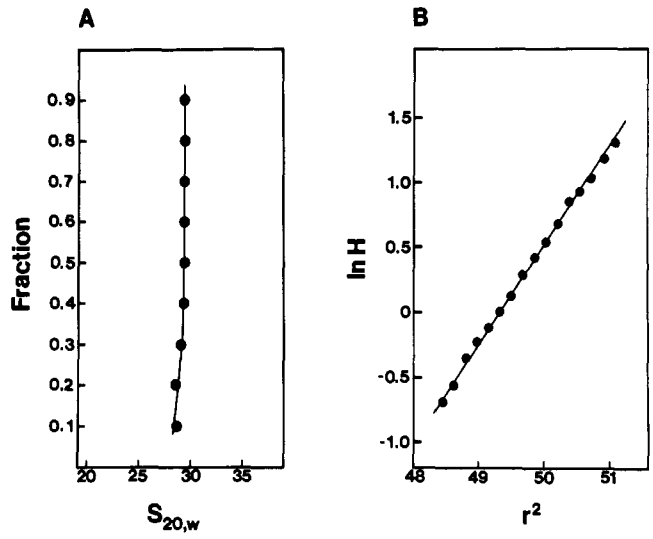

FIGURE 2: Sedimentation of 207-12 oligonucleosomes in TE buffer. (A) Sedimentation velocity. 207-12 oligonucleosomes reconstituted at a molar histone/DNA = 1.1 were sedimented in the analytical ultracentrifuge as described under Experimental Procedures. Panel A illustrates the integral distribution of s obtained after analysis of the boundaries by the method of van Holde and Weischet (1978). The ordinate measures the fraction of material with $s_{20,w}$ less than or equal to the value given on the abscissa. (B) Sedimentation equilibrium. Shown is a plot of ln H vs r^2 for some material sedimented in (A). H is the pen displacement on the scanner trace (cm) and was linearly correlated with oligonucleosome concentration. r indicates the distance from the center of the rotor (cm). The molecular mass calculated from the slope of the linear regression (solid line) is 2.92 × 10^6 daltons.

oligonucleosomes are extremely homogeneous with respect to sedimentation coefficient, it gives no information on the number of nucleosomes/template on the 29S material. In order to answer this question, we have determined the molecular weight of oligonucleosome preparations using sedimentation equilibrium. A plot of ln H versus r^2 for the sample used for Figure 2A is shown in Figure 2B. The graph is linear, confirming the nearly perfect homogeneity of the sample, and yields an oligonucleosome molecular mass of 2.92 × 10^6 daltons. The molecular mass expected for the 207-12 template containing 12 histone octamers is 2.96 × 10^6 daltons. Thus, the combined results of the sedimentation velocity and equilibrium studies indicate that these reconstituted oligonucleosomes represent the most homogeneous samples of chromatin strands obtained to this date for structural studies of chromatin conformation in solution. While we have observed that samples reconstituted at a molar histone/DNA ratio of 1.0–1.1 regularly yield highly homogeneous preparations of 29S oligonucleosomes, it must be cautioned that reconstitution of the oligonucleosomes at lower molar ratios leads to more heterogeneous samples, undersaturated with nucleosomes, whereas reconstitution at higher molar ratios yields particles and aggregates supersaturated with histone octamers.[2]

Determination of Nucleosome Positioning. In order to map the gross positions of reconstituted nucleosomes, as well as to confirm the average number of nucleosomes on each template as determined from sedimentation studies (Figure 2), we digested the 207-12 oligonucleosomes with *Eco*RI, which cuts at the junctions of each sea urchin repeat within the tandemly repeated template DNA (Figure 1). In principle, if every single repeat contained a bound nucleosome (100% template reconstitution) and if all *Eco*RI sites lie in linker regions, digestion should yield a single band corresponding to nu-

[2] J. C. Hansen and K. E. van Holde, unpublished results.

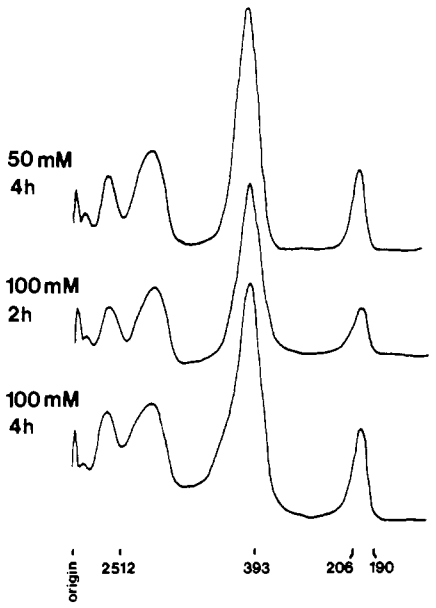

FIGURE 3: *Eco*RI digestion of 207-12 oligonucleosomes. 207-12 templates reconstituted at a molar histone/DNA ratio = 1.0 were digested with *Eco*RI (10 units/μg of DNA) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, and either 50 or 100 mM NaCl for the indicated times at 38 °C. Digests were then electrophoresed on a native 4.0% [1:20 bis(acrylamide):acrylamide] polyacrylamide gel. The gel was stained with 1 μg/mL ethidium bromide and photographed under ultraviolet illumination. Shown are densitometer tracings obtained from the photograph negative. Numbers at the bottom of the figure indicate migration of p5S207-12/*Hha*I DNA standards. Quantitation of these results (including correction for nonlinearity of the film) indicates that the percent of the total sample that migrated as free DNA (197 bp), mononucleosomes (425 bp), dinucleosomes (1300 bp), trinucleosomes (3300 bp), and tetranucleosomes (6200 bp), respectively, was as follows: 50 mM NaCl, 4 h, 4/44/23/17/6; 100 mM NaCl, 2 h, 5/47/20/19/5; 100 mM NaCl, 4 h, 4/48/22/17/5. In each case, 4–6% of the total material remained at the origin.

cleosome monomers when the digest is electrophoresed on a native polyacrylamide gel. However, if repeats are free of nucleosomes, they will migrate at 195 bp (the *Eco*RI repeat length). Furthermore, if some nucleosomes span or block *Eco*RI binding site(s), one will observe nucleosome multimers due to nucleosome protection of the *Eco*RI binding site(s) [see Morse (1989)].

The pattern we observe when 29S 207-12 oligonucleosomes are digested with excess *Eco*RI as a function of time and NaCl concentration is shown in Figure 3. While a large fraction of the digest migrates as nucleosome monomers, in all cases one also observes significant amounts of partially digested oligonucleosomes, as well as a very small amount of 195-bp free DNA. Furthermore, the *amount* of 195-bp DNA liberated by *Eco*RI is constant in each instance. There is no evidence for 207-bp DNA, indicating that in these nucleosome-free repeats both *Eco*RI sites present are being cleaved by the enzyme. Quantitation of band intensities of many such experiments with 29S oligonucleosomes [corrected for the 2.5-fold-reduced fluorescence of nucleosomes as compared to that of free DNA (McMurray & van Holde, 1986)] indicates that ≤5% of the total number of repeats are devoid of nucleosomes, confirming the results obtained from sedimentation analysis (Figure 2). Similar digestion patterns have also been obtained with *Ava*I (which cuts between the two *Eco*RI sites).

In all cases tested (n = 32), digestion of 172-12, 190-12, and 207-12 oligonucleosomes with an excess of either *Eco*RI or *Ava*I (see Figure 1 for map) fails to convert all of the oligonucleosomes into nucleosome monomers, instead yielding

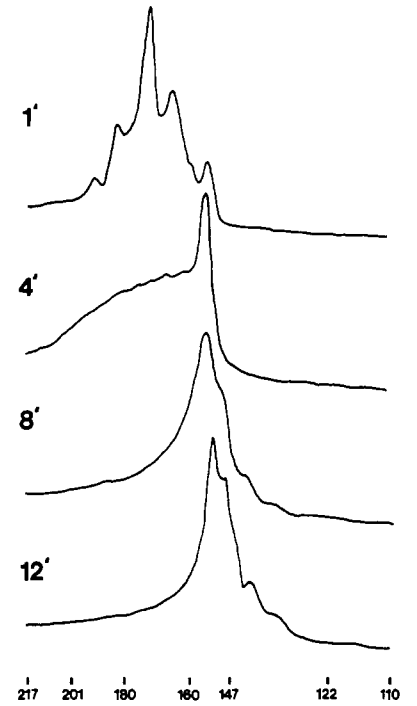

FIGURE 4: Time course of digestion of 207-12 oligonucleosomes with micrococcal nuclease. 207-12 oligonucleosomes in 1 mM $CaCl_2$ and 2.5 mM NaCl were digested with micrococcal nuclease (0.06 unit/μg), for various times at 37 °C, samples were dissociated with 0.2% SDS (20 min, 37 °C), and DNA was electrophoresed on a native 6.0% polyacrylamide gel. The gel was stained with 1 μg/mL ethidium bromide and visualized under UV illumination. Shown for each time point is the densitometer tracing obtained from the photograph negative.

about 50% of the material as nucleosome dimers, trimers, and tetramers. Essentially the same result is observed over a 100-fold range of *Eco*RI concentrations and after 20-h digestions at 37 °C (data not shown). In contrast, under the same conditions, the free template DNA is completely digested into either 195-bp (*Eco*RI) or 207-bp fragments (*Ava*I), indicating that incomplete oligonucleosome digestion is not due simply to partially active enzymes. Instead, these results indicate that either (1) restriction enzyme digestion of these oligonucleosomes is considerably less efficient than that of the free DNA or (2) up to 50% of the total restriction sites are blocked by histone octamers in such a way as to be inaccessible to *Ava*I and *Eco*RI during digestion.

In order to determine the precise positioning of histone octamers on the reconstituted DNA template, we digested the 207-12 oligonucleosomes into trimmed nucleosome monomers with micrococcal nuclease, purified the nucleosome-bound DNA, and digested the DNA with restriction enzymes. Figure 4 illustrates a time course of micrococcal nuclease digestion of 207-12 oligonucleosomes at 2.5 mM NaCl. Under these conditions, digestion of oligonucleosomes into a series of nucleosome monomers occurs very rapidly (1 min). Discrete bands are observed initially at 188, 182, 171, 163, and 153 bp. During the next few minutes, the higher molecular weight monomers are trimmed almost exclusively to the 153-bp species. By 12 min of digestion, some 146-bp DNA and subnucleosome bands also become apparent. For the purpose of the nucleosome positioning experiments described below, oligonucleosomes were usually digested for 6 min, followed by excision and purification of the discrete 153-bp band as described under Experimental Procedures.

The purified 207-12 nucleosome bound DNA sequences were then digested with seven different restriction enzymes

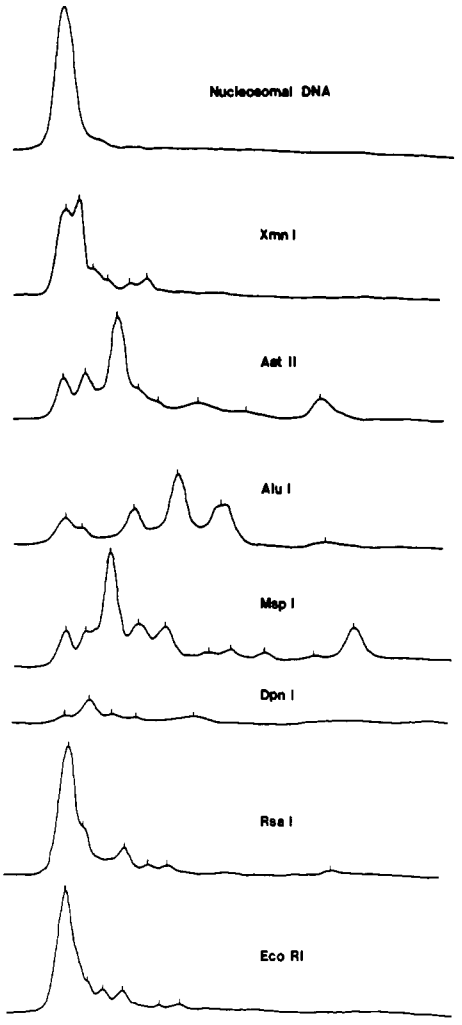

FIGURE 5: Restriction mapping of nucleosome-bound DNA sequences. Nucleosomal DNA was isolated as described under Experimental Procedures and incubated with the indicated restriction enzymes for 20 h at 37 °C. All digestions occurred at 10–20 units of enzyme/μg of DNA. Restriction digests were electrophoresed on a native 6.0% polyacrylamide gel. The gel was stained with 1 μg/mL ethidium bromide and photographed under UV illumination. Shown for each digest is the densitometer tracing obtained from the photograph negative.

spanning the sea urchin DNA repeat. Results are shown in Figure 5. There are two extreme results that might be expected from this type of experiment. If the nucleosomes were completely randomly distributed along the DNA templates, one would expect to see a smear of DNA in the electrophoresis patterns from all seven digests. If, on the other hand, all the nucleosomes were occupying a single sequence position (100% phased), one should see, in each case, two characteristic bands for those enzymes that cut within the nucleosome-bound DNA and no further digestion by those enzymes that cut in the linker DNA. Using mononucleosome DNA derived from the 207-12 oligonucleosomes, we find that approximately 50% of the DNA is digested into major band(s) by *Xmn*I, *Aat*II, *Alu*I, *Msp*I, and *Dpn*I, whereas an equivalent amount of DNA was left undigested by *Rsa*I and *Eco*RI. Quantitation of the band mobilities (Table I) indicates in all cases that the preferred nucleosome position on the 207-12 template occurs at the sequence 1–146, in contrast to the preferred positioning at the sequence 20–165 observed when the repeat-length DNA fragment is reconstituted. In addition to the major band(s) observed, a number of *discrete* minor bands also appear in all digests. In most cases these minor bands differ in size by multiples of 10 bp (Table I). Interestingly, one of these minor

Table I: Quantitation of Restriction Digests of Mononucleosome DNA

enzyme	cleavage site(s)	fragments observed (bp)[a]
*Xmn*I	10	154, 143, 134, 125, 112, 102
*Aat*II	39	155, 138, 116, 76, 60, 40
*Alu*I	68	153, 140, 108, 85, 68, 38
*Msp*I	118	150, 136, 129, 118, 104, 91, 71, 64, 53, 31
*Dpn*I	131	148, 132, 116, 105, 76
*Rsa*I	165	150, 141, 113, 101, 91, 38
*Eco*RI	1, 195	147, 131, 120, 110, 92, 82

[a] Sizes were calculated from the midpoints of the peaks shown in Figure 5 and a standard curve based on the migration of the *Msp*I/pBR322 fragments. Greater than 95% of the nucleosomal DNA shown in Figure 5 ran at 153 bp. Underlined fragments represent the major band(s) present in each digest. In the case of *Aat*II, *Alu*I, and *Msp*I, the combined size of the major fragment(s) observed was within 1 bp of the size of the undigested mononucleosome DNA present in the same lane. In two cases (*Xmn*I and *Dpn*I), only one major band was observed. However, in all five cases one of the major bands equaled the length of either the cleavage position (*Alu*I, *Msp*I, *Dpn*I, *Aat*II) or the undigested DNA minus the cleavage position (*Xmn*I), indicating that the beginning of the major nucleosome position maps to the first sequence of the sea urchin repeat.

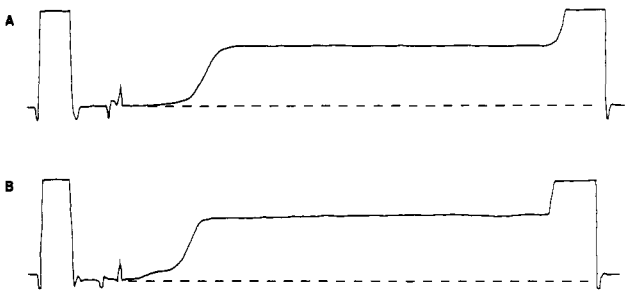

FIGURE 6: Histone octamers dissociate from linear oligonucleosomes in moderate salt. Indicated are scans obtained after sedimentation of 207-12 oligonucleosomes in 40 mM NaCl (A) and 100 mM NaCl (B) for 40 min at 22 000 rpm. Dashed lines indicate the base lines (A_{265} = 0).

preferred positions maps to the sequence 20–165. Most of the other positions are consistent with a 5′ nucleosome border encroaching into the "linker" DNA at 10-bp intervals (e.g., position 185 of one repeat to position 125 of the adjacent repeat). The observation that about 50% of the isolated nucleosome-bound DNA is cleaved by *Eco*RI (Figure 5) indicates that about half of these enzyme sites are occupied by histone octamers after reconstitution, a conclusion also suggested by the incomplete oligonucleosome digestion by *Eco*RI (Figure 3). While these somewhat unexpected results indicate that the nucleosomes reconstituted onto the 207-12 template are not positioned as expected from earlier results with monomeric reconstitutes, it is important to note that the observed positioning is nonetheless sufficient to yield homogeneous reconstitutes with 12 nucleosomes/template (Figure 2).

Analytical Sedimentation of 207-12 Oligonucleosomes in Moderate Salt. When the ionic strength is increased by adding NaCl to TE buffer, two effects on oligonucleosome structure are observed. First, there is a slow partial dissociation of histone octamers at NaCl concentrations ≥ 100 mM, as evidenced by the appearance of ~15% of the oligonucleosomes in a slower boundary (Figure 6). Second, the average sedimentation coefficient of the fast oligonucleosome boundary increases dramatically between 0 and 200 mM NaCl (Figure 7). A salt-dependent increase in average sedimentation velocity of bulk H1-depleted oligonucleosomes has been reported previously (Butler & Thomas, 1980; Gale & Smerdon, 1988). As is the case with the data of Butler and Thomas, a replot of the data in Figure 7 as log $s_{20,w}$ versus log [NaCl] is linear

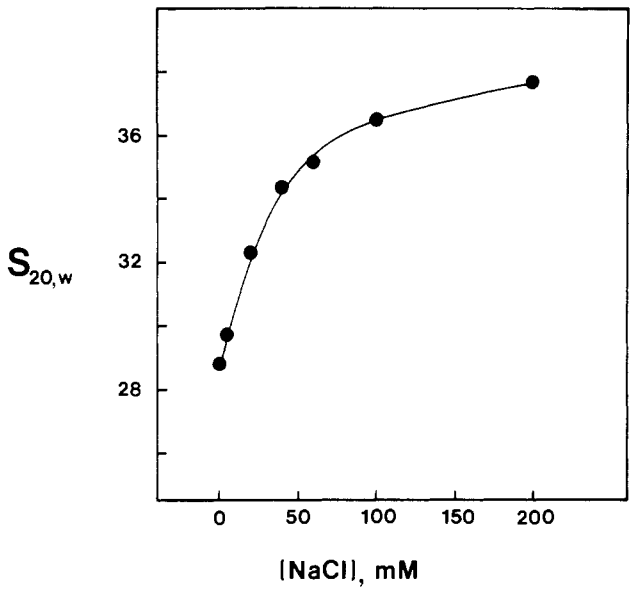

FIGURE 7: Effect of NaCl on the average sedimentation velocity of 207-12 oligonucleosomes. Oligonucleosomes in various salt concentrations were prepared and sedimented as described under Experimental Procedures. In all cases, data were obtained within 60–180 min after mixing with NaCl. Mean sedimentation coefficients were determined by measuring the velocity of the boundary midpoints.

between 5 and 100 mM NaCl. Sedimentation equilibrium studies of 207-12 oligonucleosomes in 300 mM NaCl (data not shown) give no evidence for an increase in molecular mass. Instead, the molecular mass value found for material over the lower two-thirds of the solution column was 2.93×10^6 daltons, in excellent agreement with the value obtained in TE. Near the meniscus, a significant decrease in the slope of the ln H vs r^2 graph was observed, a consequence of partial nucleosome dissociation. The results of the sedimentation equilibrium experiments demonstrate that the increase in oligonucleosome $s_{20,w}$ we observe at elevated NaCl concentrations is *not* due to particle aggregation but instead is due to some type of oligonucleosome folding with an accompanying decrease in oligonucleosome frictional coefficient.

The integral distribution analyses of 207-12 oligonucleosomes sedimented in 40 mM NaCl, 100 mM NaCl, and 40 mM Tris–acetate are shown in Figure 8. Somewhat surprisingly, the oligonucleosomes in 40 and 100 mM NaCl exhibit a broad distribution of sedimentation coefficients, despite the fact that the boundaries superficially appear sharp. Although no evidence is obtained by this method for species >42 S, the precise upper limiting value of s is difficult to judge due to the nucleosome dissociation that begins to occur by 100 mM NaCl. In contrast to results obtained in NaCl, the distribution of s in 40 mM Tris–acetate (agarose gel electrophoresis buffer) is very similar to that obtained in TE buffer. Even though there is a broad distribution of s in moderate salt, we have observed that if oligonucleosomes are incubated in 50 mM NaCl and then subsequently diluted into TE buffer, they sediment with the same velocity (within 3%) as the original material in TE (data not shown), indicating that the salt-dependent oligonucleosome folding is reversible.

In order to determine if the salt-dependent increase in $s_{20,w}$ is unique to the 207-12 oligonucleosomes, we also performed sedimentation velocity experiments of 172-12 and 190-12 oligonucleosomes in TE buffer ± 0.2 M NaCl. As is observed for the 207-12 oligonucleosomes, both 172-12 and 190-12 oligonucleosomes exhibited an increase of about 30% in mean $s_{20,w}$ when the NaCl concentration was raised from 0 to 0.2 M (Table II). This indicates that the process of oligonucleosome folding in salt is a general one, occurring for oligonucleosomes with average linker lengths ranging from 27 to 62 bp.

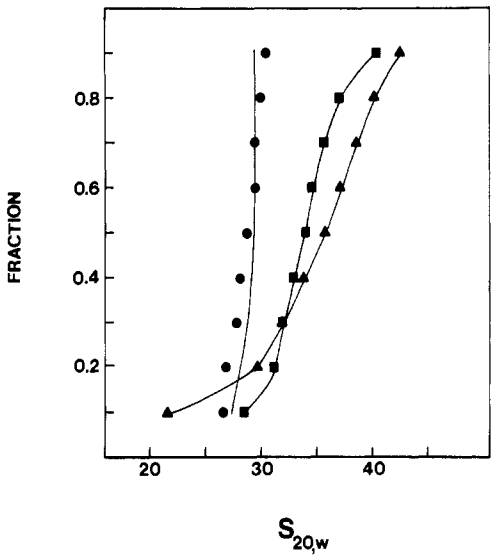

FIGURE 8: Integral distribution of 207-12 oligonucleosomes in NaCl. The boundaries of 207-12 oligonucleosomes sedimented in 40 mM Tris–acetate "electrophoresis" buffer (●), 40 mM NaCl (■), and 100 mM NaCl (▲) were analyzed by the method of van Holde and Weischet (1978). The solid line indicates the starting distribution of 207-12 oligonucleosomes obtained in TE buffer.

Table II: Salt-Dependent Increase in Oligonucleosome $s_{20,w}$ Is Independent of Linker DNA Length

reconstitute	average linker length[a]	NaCl $s_{20,w}$/TE $s_{20,w}$[b]
172-12	27	1.35
190-12	45	1.26
207-12	62	1.31

[a] Values were calculated by assuming one bound nucleosome per repeat (i.e., equals nucleosome repeat length of −145). [b] Values were calculated from the mean sedimentation coefficients of the fast boundaries of identical samples sedimented in TE and 0.2 M NaCl.

Discussion

An essential condition for unambiguous study of the structures and conformational changes of macromolecules in solution is sample homogeneity. For example, the ability to prepare large amounts of nearly homogeneous preparations of nucleosome core particles has led to over 10 years of fruitful research (van Holde, 1988), climaxing with the crystallization of phased nucleosomes (Richmond et al., 1988), nucleosome core particles (Richmond et al., 1984), and the histone octamer (Burlingame et al., 1984). In direct contrast, the inability to obtain homogeneous preparations of chromatin (either with or without H1) has inhibited unambiguous structural characterization of both the "primary" and higher-order structures of arrays of nucleosomes in solution. Using a scaled-up reconstituted oligonucleosome system developed by Simpson et al. (1985), the studies reported here have overcome this problem and, as such, represent the first detailed analysis of the structural features of a homogeneous preparation of chromatin in solution.

The sedimentation studies of 207-12 oligonucleosomes in TE buffer (Figure 2) indicate that, in very low ionic strength, the oligonucleosomes are homogeneous not only with respect to size (molecular mass) but also with respect to conformation (frictional coefficient). Taking advantage of this structural homogeneity, we have calculated the expected sedimentation coefficients of various oligonucleosome conformations using

Table III: Prediction of Sedimentation Coefficients for Various Models of Oligonucleosome Conformations

model	predicted $s_{20,w}$[a]
(I) extended	
1.0 DNA turns/nucleosome	18.0
1.75 DNA turns/nucleosome	28.5
2.0 DNA turns/nucleosome	28.5
(II) folded	
contacting 90° zigzag	39.0
open helix	42.0[b]
contacting helix (solenoid)	51.5

[a] See supplementary material for details of calculations. [b] Data interpolated from Shaw and Schmitz (1979).

the Kirkwood formalism (Table III). The calculations assumed the oligomers to be represented by rigid arrays of beads, each bead corresponding to a nucleosome with a sedimentation coefficient of 11 S. Details of the computations are availabile as supplementary material. A comparison of the $s_{20,w}$ of the 207-12 oligonucleosomes in TE (29 S) with that predicted for a number of extended and folded structures yields a clear conclusion: The structure in low salt is consistent only with a particle exhibiting considerable extension. This general conclusion is consistent with electron microscopic studies [e.g., Thoma et al. (1979)]. However, our data pertain to the solution state and show, by the homogeneity in s, that a wide range of conformations is not available under these conditions.

While our results indicate that the 207-12 oligonucleosomes are extended in TE buffer, the situation in moderate salt is considerably more complicated. As has been reported previously for bulk H1-stripped chromatin (Butler & Thomas, 1980; Gale & Smerdon, 1988), exposure of extended 207-12 oligonucleosomes to 200 mM NaCl results in about a 30% increase in *mean* oligonucleosome $s_{20,w}$ and a similar change in s is observed for the 172-12 and 190-12 oligonucleosomes as well. However, a more rigorous analysis of the 207-12 oligonucleosome boundaries indicates that, rather than a single faster migrating oligonucleosome species being detected, one in fact observes a broad distribution of s present in both 40 and 100 mM NaCl (Figure 8). In both salt concentrations, the distribution of s in the fast boundary ranges from 29 S (the sedimentation coefficient of the oligonucleosomes in TE) to approximately 40 S, with considerably more material sedimenting at >35 S in 100 mM NaCl than in 40 mM NaCl (thus the apparent increase in mean oligonucleosome $s_{20,w}$ with increasing salt). Since there is no increase in oligonucleosome molecular mass in 300 mM NaCl, sedimentation coefficients greater than 29 S must reflect a decrease in frictional coefficient, and therefore a more condensed oligonucleosome structure. Together with the observation that oligonucleosome folding is reversible, these results suggest that the oligonucleosomes in moderate salt exist in equilibrium between various conformational states. There would appear to be two limiting conformations: the 29S extended conformation and a condensed structure of ≥40 S. In this view, the distribution of oligonucleosome sedimentation coefficients between the two limiting s values arises from a series of rapidly equilibrating intermediates consisting of partially folded regions and partially extended regions. The presence of increasing amounts of NaCl favors the folded state (thus increasing the amount of material > 35 S), but elevated NaCl alone is insufficient to stabilize all oligonucleosome particles in the limiting folded conformation.

It is of interest that the upper value of oligonucleosome s observed in moderate NaCl is comparable to that predicted for both contacting 90° "zigzag" and *open* helical structures (Table III) but is considerably less than that expected for a 30-nm solenoidal particle (Table III). Thus, while a chromatin fiber composed of core histones alone can achieve significant folding in moderate salt, there is no evidence for formation of the 30-nm higher order structure in the absence of lysine-rich histones. From these cumulative results, we propose the following explanation for the observed necessity of both NaCl and lysine-rich histones for the promotion of a 30-nm fiber. The primary effect of NaCl is to screen DNA charge, thus increasing linker DNA flexibility and allowing closer nucleosome–nucleosome approach. A folded zigzag or open-helical structure is favored by the geometry of nucleosomes but is prevented in low salt by electrostatic repulsion. The presence of lysine-rich histones either serves to constrain the exit angle of DNA from the nucleosome or promotes close nucleosome contacts, resulting in additional compaction into the 30-nm structure. In our view, the limiting condensed structure observed for H1-depleted oligonucleosomes in moderate salt represents a folding intermediate which must form before H1/H5 can promote the 30-nm chromatin fiber. It should also be noted that, in addition to the putative role in 30-nm filament formation, it also seems likely that the folded oligonucleosome structure observed in "physiological" salt may influence the mechanism of nuclear processes (e.g., transcription, replication, and repair) involving linear arrays of nucleosomes in the absence of H1/H5.

Besides yielding important information regarding chromatin folding, these studies have also addressed the mechanism of nucleosome positioning on the sea urchin 5S rRNA sequence. We were surprised to find that while most of the nucleosomes appear to occupy defined positions along the 207-12 DNA template, the major 207-12 phasing sequence is not the same as that observed for the monomeric DNA repeat after reconstitution (Simpson & Stafford, 1983; FitzGerald & Simpson, 1985; Richmond et al., 1988; Moyer et al., 1989). In the case of the 207-12 template, the preferred nucleosome position appears to be shifted 20 bp, to sequences 1–146 of the sea urchin repeat. In addition, there appears to be six to eight additional distinct nucleosome positions on the oligonucleosome template. While unexpected, the pattern of oligonucleosome positioning found here is not unrelated to the positioning observed previously for the monomeric repeat. Both the 1–146 positions and most of the minor positions found in the oligonucleosome retain the original DNA strand–histone orientation, since positioning shifts have occurred as multiples of 10 bp. Furthermore, residues 20–146 are bound to the histone core in both the 1–146 and 20–165 positionings. Considering that residues 146–165 have been shown to be relatively unimportant for nucleosome positioning (FitzGerald & Simpson, 1985), it is likely that the same "phasing signal" is involved in preferred nucleosome positioning on both the monomeric and tandemly repeated templates. In this respect, even those nucleosomes that span the repeat junction will occupy at least sequences 20–80 of one of the repeats. Taken together, our results suggest that nucleosome positioning on the 207-12 template results from distribution between a number of discrete *preferred* positions, only one of which (the 1–146 position) possesses a significantly greater affinity for the histone octamer than the others. It should be noted in this context that the results obtained here with a tandemly repeated sequence (one major phasing position plus a number of subsidiary ones) are reminiscent of results obtained for nucleosome position on tandemly repeated satellite DNAs in vivo (Zhang et al., 1983; Zhang & Hörz, 1984; Bock et al., 1984).

Nevertheless, why should different nucleosome positions be preferred on the *same* sequence of DNA, when reconstituted

as a monomeric or tandemly repeated structure? A reasonable answer can be obtained assuming that (1) positions 1–146 are intrinsically the most favored nucleosome position and (2) the most stable nucleosome structure in low salt involved interaction of 165 bp of DNA with the histone core, yielding a particle containing two complete turns of DNA. Evidence for such a 165-bp nucleosomal structure has been presented previously (Todd & Garrard, 1977; Weischet et al., 1979; Albright et al., 1980) and is consistent with the extended structure adopted by oligonucleosomes in very low salt. Given these assumptions, we postulate that the positioning of nucleosomes on the sequence 1–146 on the *monomeric* repeat is compromised by the fact that this does not allow interaction with a full 165 bp, even though the fragment is 195 bp in length. As a result, histone octamers bind to a less favored position (the sequence 20–165) that *can* form a 165-bp structure. If this is the case, the stability gained from interaction of an additional 10 bp of DNA must be greater than the decrease of stability resulting from interaction with a less favored sequence position. Regardless of the actual causes of shifted, multiple phasings, these results imply that the determinants of nucleosome positioning may be more subtle than hitherto believed, particularly in the case of tandemly repeated templates.

In conclusion, we find that the oligonucleosomes constructed in these studies represent structurally homogeneous models for study of chromatin conformation in solution. These initial experiments have yielded surprising information regarding the nature of nucleosome positioning and present evidence that the structure of linear stretches of chromatin in the absence of lysine-rich histones is much more dynamic than currently believed. Perhaps most importantly, the availability of large quantities of these homogeneous model systems will allow for a much better understanding of the solution-state structure and properties of chromatin in the future.

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Supplementary Material Available

A description of the calculations and detailed models used to predict the oligonucleosome sedimentation coefficients indicated in Table III (4 pages). Ordering information is given on any current masthead page.

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