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Nucleosome Reconstitution: Effect of DNA Length on Nucleosome Structure†

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ABSTRACT: Core histones (H2A, H2B, H3, and H4) are reconstituted by salt gradient dialysis with DNA molecules ranging in length from 177 bp down to 50 bp. While reconstituted particles containing 125 bp are very similar to native particles, those particles containing a single piece of shorter DNA tend to aggregate. The aggregation depends on the ionic strength and DNA length. The DNA placement on the histone core is not random as determined by pancreatic DNase I digestions of particles containing ^{32}P 5′-end-labeled DNA. Rather, it is found that all DNA molecules, up to 161 bp in length, reassociate with core histones in such a way as to produce defined patterns of DNase I cutting with respect to the 5′ ends. Particles were made that contained two pieces of 65-bp DNA. These particles are very similar to native particles under most conditions but tended to dissociate at very low ionic strength. It is suggested that this dissociation results in the production of two half-nucleosomes (hemisomes).

As was shown in an earlier paper (Tatchell & Van Holde, 1977), nucleosomes can be reconstituted from isolated DNA and histones, reproducing most or all of the structural features found in vivo [see Felsenfeld (1978) for review]. Using physical and enzymatic probes as criteria for reconsititution, we have shown that nucleosomal core particles reconstituted from 144-bp DNA and histones are very similar, if not identical, to the native particle. In this paper we describe reconstitutions using core histones together with DNA molecules both shorter and longer that usually associated with the core particle. The resultant products have been characterized by a number of techniques.

The core histones in eukaryotic chromatin protect 140–170-bp DNA fragments from hydrolysis by micrococcal nuclease but there is evidence that DNA–histone interactions are not uniform along the DNA (Simpson & Whitlock, 1976; Mirzabekov et al., 1978; Weischet et al., 1978). Pancreatic DNase I digestion of core particles containing DNA ends labeled with ^{32}P indicates that some positions along the DNA are highly protected while others are much more susceptible

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0006-2960/79/0418-2871$01.00/0

to hydrolysis (Simpson & Whitlock, 1976; Lutter, 1978). Mirzabekov et al. (1978) have mapped the sites of histone–DNA interaction by protein–DNA cross-linking and found that the 5′ end of the DNA in the core particle is devoid of major cross-links. Thermal denaturation studies of core particles indicate about 40 bp out of the 144-bp DNA are less resistant to thermal denaturation (Weischet et al., 1978). The results presented here further define the positions of histone–DNA interaction and provide a method of studying conformational changes in nucleosomes and association reactions between such particles.

While nucleosomes can be reconstituted onto DNA from many sources, eukaryotic and procaryotic alike, the possibility still exists for some sequence specificity of histone binding. Ponder & Crawford (1977) have observed a partial phasing of nucleosomes on the polyoma virus chromosome that could be the result of sequence recognition by histones. Reconstitution studies with specific sequences are important to understanding the mode of DNA–histone interaction, but care must be taken in generalizing from reconstitutions with sequence specific DNAs to reconstitutions with random sequence DNA. Therefore, we have sought to study DNA length dependence on the reconstitution process using random sequence DNA molecules prepared by a micrococcal nuclease "limit digestion" of nuclei.

The DNA in chromatin or whole nuclei can be hydrolyzed by micrococcal nuclease until about 50% of the DNA is acid soluble (Clark & Felsenfeld, 1971). At this point, a digestion plateau is reached since additional enzyme or digestion time does not increase the amount of acid-soluble DNA. The DNA at this limit is found to be a series of short double-stranded fragments, most of which lie between 160 bp and 30 bp (Axel et al., 1974). Although micrococcal nuclease has a slight preference for A–T-rich DNA (Roberts et al., 1962), no sequences in chromatin have been shown to be preferentially cleaved by micrococcal nuclease (Lacy & Axel, 1975). Fractionation of this limit digest into different DNA sizes should, therefore, provide reasonably homogeneous DNA samples that resemble the entire genome in sequence complexity. We have studied the reconstitution of salt-extracted histones with such DNA fragments.

Materials and Methods

Isolation of Core Particles. Nuclei were isolated from chicken erythrocytes according to the method of Shaw et al. (1976) with the modifications described by Tatchell & Van Holde (1977). Core particles and core histones were prepared from lysed nuclei that were depleted of histone H1 and H5 by 0.65 M NaCl washes as described previously (Tatchell & Van Holde, 1977).

DNA Extraction. DNA was extracted by using a modified Marmur technique (Britten et al., 1974), precipitated with 2.5 vol of ethanol, and redissolved in 0.1 ionic strength electrophoresis buffer. DNA that was to be used for preparative electrophoresis and reconstitution was extracted an additional three times with ether prior to the ethanol precipitation.

Analytical Gel Electrophoresis. The DNA was electrophoresed on polyacrylamide slab gels (0.2 × 20 × 20 cm). The DNA was analyzed under nondenaturing conditions on 10% gels according to Loening (1967). Eight percent polyacrylamide–urea gels, used for examining denatured DNA, were run by following the procedure of Maniatis et al. (1975). PM2–*Hae*III restriction fragments were used to calibrate the DNA sizes (Tatchell & Van Holde, 1978). Gels were stained in electrophoresis buffer containing 1 μg/mL ethidium bromide for 1 h and then photographed under ultraviolet light by using a Polaroid MP4 camera and type 55 P/N (Polaroid) film.

In cases where ^{32}P-labeled DNA was electrophoresed, 25–30 μg of DNA carrying up to 10^5 cpm of ^{32}P was loaded on each well so that the gel could be both stained with ethidium bromide and then autoradiographed. Gels to be autoradiographed were soaked in 5% trichloroacetic acid, after ethidium bromide staining and photography, for 15 min at 4 °C to fix the DNA in the gel. Excess Cl_3CCOOH was removed by two 1-min rinses in cold water followed by one 5-min rinse. The gel was then covered with plastic wrap and autoradiographed by using Kodak X-omat R X-ray film and a Kodak intensifying screen. Polaroid type 55 negatives of the ethidium bromide stained gel and the autoradiograms were scanned with a Joyce Lobel densitometer.

Protein Characterization. Proteins were electrophoresed on sodium dodecyl sulfate–15% polyacrylamide slab gels with 6% polyacrylamide stacking gels by using the buffer system of Laemmli (1971) as described in Tatchell & Van Holde (1977). Slab gels of dimensions 0.5 × 80 × 100 mm were run with the apparatus designed by Matsudaira & Burgess (1978). Lowry protein analyses were run according to the procedure of Hartree (1972).

Isolation of DNA Fragments by Preparative Gel Electrophoresis. Chicken erythrocyte nuclei were digested with 200 units/mL micrococcal nuclease for 4–8 h at 37 °C in 10 mM Tris–cacodylate, pH 7.2, 1 mM $CaCl_2$. A limit of digestion is reached as about 50% of the DNA becomes acid soluble (Clark & Felsenfeld, 1974) so there is no danger of overdigesting the nuclei. The DNA from this limit digest was purified and fractionated into size classes by preparative gel electrophoresis. Double-stranded DNA fractions were obtained from 180 bp to 50 bp in roughly 10-bp intervals.

Two methods of preparative electrophoresis were used to fractionate the "limit" DNA digest. In the first, a large 10% polyacrylamide slab gel (0.6 × 20 × 20 cm) was loaded with 15–20 mg of DNA, electrophoresed, and then sliced into horizontal strips. The gel was stained briefly in ethidium bromide to observe any curvature in the banding pattern. The DNA from each slice was eluted from the gel by the procedure of Maxam & Gilbert (1977). A 1.0–1.5-mL gel slice was placed in a 5-mL Pipetman (Gilson) pipet tip that had been plugged with a piece of siliconized glass wool and heat sealed at the tip. The gel slice was ground to a paste in elution buffer (0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1% $NaDodSO_4$, 0.1 mM Na_2EDTA). Another milliliter of elution buffer was added and mixed with the paste. The pipet tip was covered with parafilm and incubated overnight at 37 °C. The sealed pipet tip was then cut, allowing the elution buffer and solubilized DNA to flow through the glass wool and out the tip. The pipet tip was washed with an additional 1 mL of elution buffer and the DNA contained in the combined elutants was precipitated with 2.5 volumes of ethanol. The precipitated DNA was redissolved in buffer, extracted with isoamyl alcohol to remove any residual ethidium bromide, and dialyzed extensively against lysis buffer. Any small pieces of polyacrylamide not filtered out by the glass wool were removed by centrifugation prior to dialysis.

The second technique for preparative fractionation required electrophoresing DNA through a large preparative gel and eluting the DNA from the bottom of the gel as it electrophoresed off. Two devices were used: an apparatus containing a 1.7-cm cylindrical gel (Savant Instrument Co.) and an apparatus containing a 7.7-cm cylindrical gel constructed according to Hagen (1979). The small apparatus could handle 1–2 mg of DNA, while 20–25 mg was loaded on the large

7.7-cm model. In each case, an 8-cm-long 10% polyacrylamide (20:1 acrylamide:bisacrylamide) gel was electrophoresed at 40–50 V at room temperature with recirculation of buffer between reservoirs. The elution buffer flow rate was 0.1–0.3 mL/min. The slower flow rate gave less homogeneous fractions, while faster flow rates gave more homogeneous fractions but at a lower concentration of DNA. In most cases, adjacent 2-mL fractions were pooled in order to obtain enough DNA of one size class but not without some loss in size homogeneity.

Reconstitution Methods. All reconstitution experiments were done in 10 mM Tris–cacodylic acid, pH 7.2, 0.7 mM Na_2EDTA (CP buffer) plus additional NaCl as designated. Core histone and DNA were reconstituted by mixing the components in dialysis bags in 2 M NaCl and then lowering the ionic strength by step dialysis as described previously (Tatchell & Van Holde, 1977). In recent experiments, we have replaced the dialysis steps by an exponential salt gradient using a constant volume mixing chamber. The ionic strength was lowered in the mixing chamber from 2 M NaCl to 0.6 NaCl, and then the mixture was taken to 10 mM Tris in one final dialysis step. The reconstituted core particles, now in CP buffer, were sometimes dialyzed to various ionic strengths before further analysis.

Sedimentation Experiments. All sedimentation experiments were performed with a Beckman Model E analytical ultracentrifuge equipped with scanner optics as described elsewhere (Tatchell & Van Holde, 1977). Samples were studied at absorbances of 0.3 to 1.0. In order to determine the true heterogeneity of various reconstituted preparations, we have calculated the integral distribution of sedimentation coefficients ($G(S)$) for most runs. This method, described in detail by Van Holde & Weischet (1978), effectively removes broadening in the boundary due to diffusion.[1]

Thermal Denaturation. Core particles and reconstituted particles were thermally denatured in 1.0 mM cacodylic acid, pH 7.2, as described by Weischet et al. (1978).

Digestion of Particles with Pancreatic DNase I. Native and reconstituted particles, at a concentration of 50 μg/mL DNA, were digested with 100 units/mL of pancreatic DNase I (Worthington) at 37 °C in 10 mM Tris–cacodylate, pH 7.2, 0.7 mM Ca^{2+}. Digestions were terminated by making the digestions 10 mM in Na_2EDTA and cooling on ice.

End-Labeling Core Particles and DNA with ^{32}P. T4 polynucleotide kinase was prepared from T4 lysates and purified by DEAE-cellulose chromatography. Core particle preparations were labeled with ^{32}P at the 5′ ends of the DNA as described by Simpson & Whitlock (1976) to a specific activity of 10^5 to 10^6 cpm/μg of DNA by using [γ-^{32}P]ATP. The 20–50-μL incubation was done in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM β-mercaptoethanol, and at least 1 μM ATP containing 1–2 μg of DNA. The reaction mixture was incubated at 37 °C with 2 units of T4 kinase for 1 h and then terminated with excess EDTA and cooling on ice. In some cases, the incubation mixture was diluted with an excess of cold sample and then immediately digested with pancreatic DNase I as above.

End labeling of the isolated DNA fractions with ^{32}P was done as above but with 50 mM Tris-HCl instead of the 10 mM Tris-HCl. When this DNA was to be reconstituted with histones, the end-labeled sample was diluted with an excess of cold DNA and reconstituted immediately as above. The unreacted [^{32}P]ATP and $^{32}PO_4$ were removed during the reconstitution dialysis.

[1] It has come to our attention that this method for analysis resembles in certain respects a method proposed by Baldwin (1959).

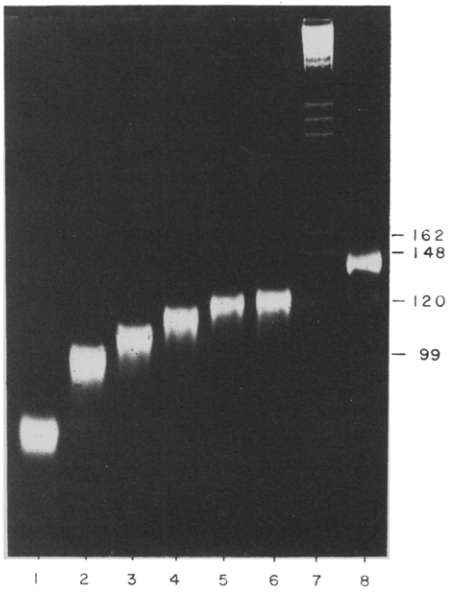

FIGURE 1: One of several sets of DNA fragments resolved by preparative gel electrophoresis. Selected fragments were electrophoresed on a 6% polyacrylamide slab gel. Channels from left to right contain (1) 69 bp DNA; (2) 94 bp; (3) 105 bp; (4) 114 bp; (5) 120 bp; (6) 123 bp DNA; (7) *Hae*III restriction fragments of PM2 DNA (sizes indicated to the right); (8) 144-bp DNA from core particles.

Table I: Reconstitutions Using 1 mol of DNA/mol of Histone Octamer

DNA size (bp)[a]	protein/DNA (w/w)		$s_{20,w}$ at		
	obsd[b]	predicted[c]	2.5×10^{-4} M[d]	0.01 M[e]	0.15 M[f]
144 ± 5	1.2 ± 0.1	1.15	10.0, 10.0	10.9 ± 0.1	11.1
125 ± 9	1.40, 1.41	1.38	9.0	10.4 ± 0.3	11.3 ± 0.8
111 ± 6	1.38, 1.42	1.51	8.7	10.0	14.0
102 ± 14	1.6 ± 0.1	1.66	9.0, 9.5	16.5	>20

[a] ± indicates half-width of DNA at peak half-height. [b] ± indicates standard deviation of ⩾3 samples. This also applies to $s_{20,w}$ values. [c] Assuming 108 000 and 650/bp for the molecular weight of the histone octamer and DNA, respectively. [d] 2.5×10^{-4} M EDTA, pH 7.2. [e] 0.01 M Tris–cacodylate, 7×10^{-4} M EDTA, pH 7.2. [f] 0.15 M NaCl, 0.01 M Tris–cacodylate, 7×10^{-4} M EDTA, pH 7.2.

Results

The DNA molecules used in these reconstitution studies were obtained by electrophoretic separation of DNA fragments produced by extended digestion of chicken erythrocyte nuclei with micrococcal nuclease. Figure 1 shows the fractionation profile of a representative electrophoresis. While not completely homogeneous in length, DNA fractions were obtained in large enough quantities to allow physical characterization of reconstituted products. The exact sizes of the individual DNA fractions were determined by coelectrophoresis with *Hae*III restriction fragments of bacteriophage PM2 which have been calibrated against SV40 restriction fragments. Fragments of sizes 177 bp, 161 bp, 144 bp, 125 bp, 111 bp, 105 bp, 102 bp, 94 bp, 90 bp, and 65 bp were reconstituted with histone cores and then used for the sedimentation and DNAse I digestion studies. Fractions containing 123 bp and 144 bp of DNA were reconstituted for the thermal denaturation experiments described below. The half-width of all fractions at

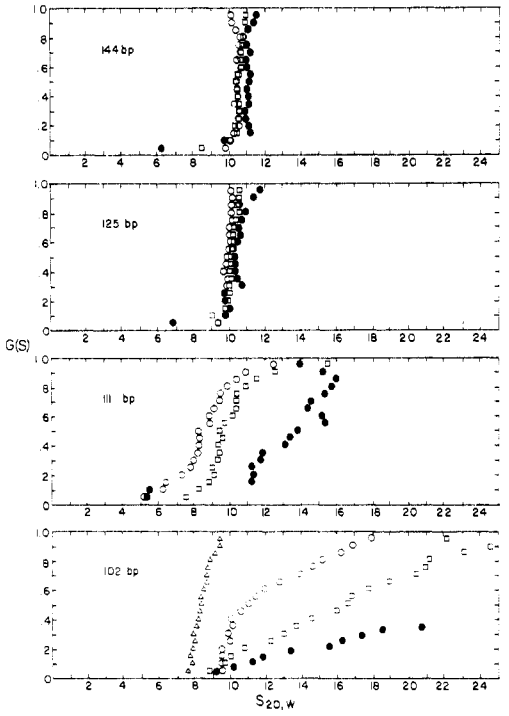

FIGURE 2: Distribution of sedimentation coefficients ($G(S)$) for particles reconstituted with one DNA molecule each of the lengths indicated. The buffer solutions employed are as follows: (○) 5 mM Tris–cacodylate, 0.35 mM EDTA; (□) 10 mM Tris–cacodylate, 0.7 mM EDTA; (●) 0.15 M NaCl, 10 mM Tris–cacodylate, 0.7 mM EDTA; (△) 0.25 mM EDTA. All are at pH 7.2.

peak half-height was estimated to vary between 5 bp and 14 bp (see Table I). This is surely an overestimate of the heterogeneity for it does not take into account diffusion, the finite initial band width, nor any effects of sequence heterogeneity on migration rate. In all cases, reconstitution was accomplished by simply mixing the appropriate DNA with salt-extracted core histones in 2 M NaCl and slowly lowering the ionic strength by gradient dialysis. The results of these reconstitutions are discussed below.

Reconstitution with Single DNA Fragments Shorter Than 144 Base Pairs Forms Stable Complexes Very Similar to Core Particles. In the first set of experiments,histones and DNA were mixed at a molar ratio of one histone octamer (two each of the four core histones H2A, H2B, H3, and H4) per DNA fragment. As Table I indicates, each DNA size down to 94 bp could combine with core histones to form particles with a 1:1, mole histone octamer:mole DNA ratio. In the case of 90-bp and 65-bp DNA, complete precipitation of the complex occurred and no attempt was made to characterize the product. Nevertheless, soluble, well-defined particles could be obtained with 90-bp and 65-bp DNA if the DNA:histone ratio was increased (see below). In all of the reconstitution products tested, the histone stoichiometry appeared to be identical with that of the native core particles in that the four core histones were present in equal amounts.

Particles reconstituted from DNA about 125 bp in length resemble very closely the 144 bp reconstitutes and native core particles. The measured protein/DNA ratio indicates each has one molecule of DNA associated with one histone octamer (see Table I). These particles sediment with very homogeneous boundaries at 10–11 S (Figure 2, top) at all ionic strengths studied. A thermal denaturation experiment was performed with a fraction containing 123 bp. As Figure 3 shows, the main phase of the thermal denaturation is identical with that in core particle, attesting further to the fidelity of reconstitution. A point of interest is the smaller premelt found for the 123-bp reconstitute. We have estimated the premelt for this particle to correspond to 26 bp of DNA, leaving 97 bp in the main transition. This is consistent with our suggestion that the main transition in the core particle involves about 100 bp of central DNA (Weischet et al., 1978). In fact, we have found that thermal denaturation of nucleosomes containing DNA as long as 184 bp leads to the same result; always an amount of about 100 bp is found in the main transition (K. G. Tatchell, unpublished experiments). Very similar results, using nucleosomes with longer DNA molecules, have been obtained by M. Cowman and G. Fasman (private communication).

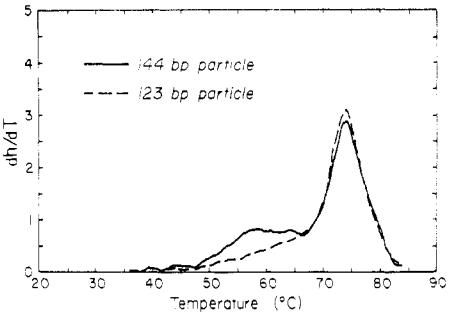

FIGURE 3: Differential profile of thermal denaturation of reconstituted particles containing 144-bp and 123-bp DNA. The hyperchromism h is defined as $h = 100(A_{260} - A°_{260})/A°_{260}$, where A_{260} is the absorbance at T (corrected for thermal expansion) and $A°_{260}$ is the absorbance at 20 °C. (See Weischet et al. (1978) for additional details.)

Particles with Less Than 120 bp of DNA Exhibit Aggregation at High Ionic Strength. The sedimentation velocity studies of particles containing 1 histone octamer and 1 DNA molecule are described in Table I, and representative sedimentation coefficient distributions are shown in Figure 2. We interpret these results in the following ways.

(1) At ionic strengths of 0.01 and higher, particles containing short DNA fragments exhibit aggregation. This can be detected in 0.15 M salt at 111 bp; particles of roughly dimer size (14–16 S) are observed. Particles containing even shorter DNA associate to very large aggregates ($s_{20,w} > 20$) in this medium.

(2) In 0.01 M Tris, aggregation can be detected when DNA fragments of 105 bp or smaller are employed in the reconstitution. As has been noted above, attempts to reconstitute with 1 DNA molecule 90 or 65 bp in length lead to immediate precipitation. This we interpret as extreme cases of the above-mentioned aggregation.

(3) In very low ionic strength (2.5×10^{-4} M EDTA), this tendency to aggregation is much suppressed. Particles containing 102-bp DNA yield a nearly homogeneous boundary at 8–10 S. The sedimentation coefficients observed at this low ionic strength are consistently lower than the values observed for the monomer in 0.01 M Tris; this is almost certainly in part an electrostatic effect on the sedimentation; it also could involve in part the conformational change detected by Gordon et al. (1978). The change in s with decreasing DNA size in this buffer is complex, for as DNA size decreases the molecular weight decreases somewhat, but the net negative charge also decreases. The net charge on the particle will reach zero when the DNA size is decreased to between 90 and 65 bp, the exact point depending on the (unknown) extent of counterion binding. Particles containing 94-bp DNA do show a certain degree of heterogeneity in the sedimenting boundary, indicating that aggregation can occur even at this low ionic strength if the DNA length is short enough.

Table II: Reconstitutions Using 2 mol of DNA/mol of Histone Octamer

DNA size (bp)[a]	protein/DNA (w/w) obsd[b]	protein/DNA (w/w) predicted[c]	$s_{20,w}$ at 2.5 × 10^{-4} M[d]	$s_{20,w}$ at 0.01 M[e]	$s_{20,w}$ at 0.15 M[f]
105 ± 7	1.15, 1.20	0.81	7.5	9.0, 9.0	11.0, 11.4
94 ± 6	0.83, 1.00	0.90	6.3	10.0, 10.5	10.5, 11.3
65 ± 5	1.20, 1.40	1.30	7.5 ± 0.8	10.3, 10.6	11.0

[a-f] See corresponding footnotes in Table I.

The Histone Core Will Accept More Than One Short DNA Molecule. We observed that only one 144-bp DNA molecule would reconstitute with the core histones, even in the presence of excess DNA (Tatchell & Van Holde, 1977). With significantly shorter DNA molecules, on the other hand, we find that the histone octamer can bind more than one DNA fragment.

In these experiments, core histones and DNA were mixed together at a molar ratio of 1:2 (histone octamer:DNA fragment). Unlike the result with 144-bp DNA, where half the DNA in such reconstitutes would remain free in solution, sedimenting in the analytical ultracentrifuge with a sedimentation coefficient identical with that of free DNA, less than half of the shorter DNA would sediment as free DNA in these experiments. In the case of 65-bp DNA, no free DNA boundary existed; each histone core was binding exactly two DNA molecules.

The particles made at these high DNA:protein input ratios were fractionated from any free DNA by centrifugation through sucrose gradients and studied in the analytical ultracentrifuge. A summary of their properties is presented in Table II. Note that, whereas the 65-bp and 94-bp fragments reconstitute to give a stoichiometry close to 1:2 (histone octamer:DNA fragment), the particles made with 105-bp DNA contain, on the average, less DNA than would be expected for a 1:2 stoichiometry, but more than predicted for a 1:1 stoichiometry. Thus, this sample must be heterogeneous, containing both kinds of particles. Apparently 100 bp is the approximate limit at which two DNA molecules will still be accepted.

The sedimentation behavior observed when two short DNA molecules are added to one histone core is quite different than that found for a 1:1 stoichiometry. With two DNA molecules, we find no evidence for aggregation, even at higher ionic strength. This behavior is not surprising, for these particles will carry quite large negative charges, and most will have protruding DNA ends.

In very low ionic strength, on the other hand, the particles containing two DNA molecules behave quite differently. Whereas those containing a single DNA molecule exhibited slightly decreased sedimentation coefficient values because of charge effects in 2.5 × 10^{-5} M EDTA, the "two-DNA" particles exhibit broad, heterogeneous sedimentation coefficient distributions extending down to 5–6 S. The results with the 65-bp particle are of particular interest, for this particle contains nearly the same amount of DNA as the core particle itself, but in two pieces instead of one. It cosediments with the core particle in 0.15 M Tris, but behaves very differently at 0.25 mM EDTA (Figure 4). Such a difference is consistent with either a partial dissociation or drastic conformational change (see Discussion).

The DNA Does Not Bind Randomly on the Histone Core. Assuming the 144 bp of DNA in the core particle wraps symmetrically around the histone octamer, the placement of

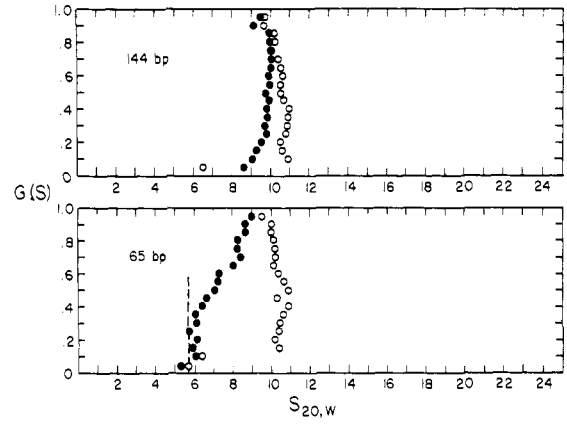

FIGURE 4: Sedimentation coefficient distributions ($G(S)$) for particles reconstituted with one 144-bp DNA molecule (top) or two 65-bp DNA molecules (bottom). Solution conditions are (○) 10 mM Tris-cacodylate, 0.7 mM EDTA; (●) 0.25 mM EDTA. The broken line indicates the sedimentation coefficient predicted for a hemisome (see Discussion).

the smaller DNA molecules can be ascertained by using the method of Simpson & Whitlock (1976). The DNA is labeled on the 5′ end with ^{32}P, reconstituted with histones, and then digested with pancreatic DNase I, and the resulting DNA fragments are run on denaturing polyacrylamide gels and autoradiographed. Simpson & Whitlock (1976) showed that certain sites on the nucleosome were very resistant to DNase I digestion while other sites were easily cleaved. Using this same method of analysis on reconstituted particles, we can accurately define the position of the DNA with respect to these protected and exposed sites.

We are concerned about the actual sizes of the single-strand DNA fragments produced by DNase I. They have been generally considered to be multiples of ten bases. But the DNA in the core particle is not 140 bp in length. We now obtain a value around 145 bp, as do others (Mirzabekov et al., 1978). The core DNA size—determined on nondenaturing gels—is 144 bp, while the core size on denaturing gels is somewhat larger (146–148 bases) (D. Lohr, private communication). We suspect that this apparent difference may arise from the 2-base overlaps of 5′ ends produced by micrococcal nuclease cleavage (Sollner-Webb et al., 1978). If the DNase I bands extend at a constant multiple, from zero bases to the full length of the core particle, the interval between bands is 10.3–10.4 bases rather than 10.0 bases. Recent experiments (Lutter, 1979) confirm a 10.3–10.4 base average spacing. The DNase I cutting sites are not all evenly spaced. We will refer to each DNase I DNA band by its number (from 1 to 14) and not by its absolute size.

If the DNA of reconstituted particles goes on the histone core randomly, the resulting autoradiogram of the DNase I digestion should be a smear. If, on the other hand, the placement of DNA is not random but phased with respect to the internal protection sites, a series of bands should appear on the autoradiogram. The stringency of this test should be emphasized. Suppose, for example, there was an equal probability of placing either 5′ end of the DNA anywhere ± 5 bp from the required frame. This would obliterate the ^{32}P-banding pattern.

The results from such experiments, presented in Figure 5, indicate that the placement of the DNA is not random. Reconstitution with 144-bp DNA yields particles which give a clear banding pattern in the autoradiogram similar to the pattern from native particles (Simpson & Whitlock, 1976; Lutter, 1978). The near absence of bands 3, 6, 8, and 11 attest

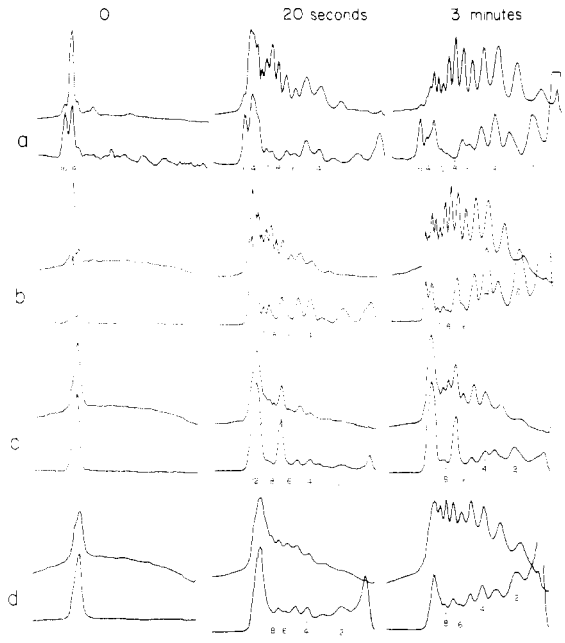

FIGURE 5: Gel electrophoresis of single-strand DNA fragments from DNase I digestions of native and reconstituted particles. In each frame, the upper curve is a scan of the gel stained with ethidium bromide; the lower is an autoradiograph of fragments labeled at the 5′ end with ^{32}P. Times of digestion are indicated at the top. (a) Native core particles; (b) particles reconstituted with 144-bp DNA; (c) particles reconstituted with 125-bp DNA; (d) particles reconstituted with 111-bp DNA.

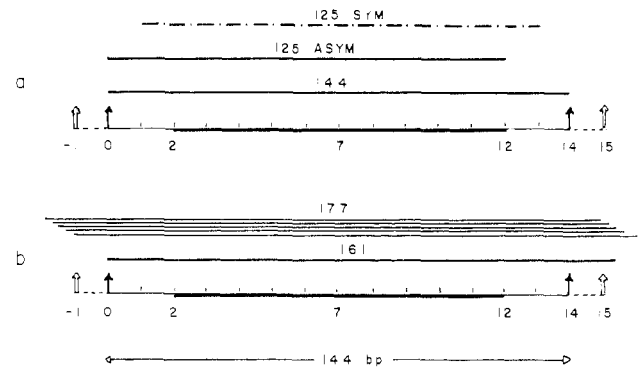

FIGURE 6: Schematic representations of the binding of DNA molecules to histone cores. The marks 0–14 represent the DNase I cutting sites, located about 10.4-bp apart (on the average), and spanning a total of 144 bp. The heavy line represents the inner, most stable region of approximately 100 bp. The broken lines extending to −1 and 15 represent the extension to a "full" DNA length of about 166 bp. The solid arrows show the strong sites at 0 and 14, the open arrows the weak sites at −1 and 15. (a) Putative positions of the 144-bp DNA and the symmetrically and asymmetrically located position for 125-bp DNA. (b) Putative position for the 161-bp DNA and the range of position available to 177-bp DNA. See Discussion.

to the fidelity of reconstitution. However, minor differences are found between the patterns obtained from native and reconstituted core particles. In particular, band 7 is consistently stronger in digests of the reconstituted core particles. We do not know the significance of this result, but it could possibly reflect small differences in the structure of the reconstituted core particle. Also of interest is the fact that our native core particle preparation is not uniformly end labeled. The core particle preparations used for these experiments contained a small quantity of 170-bp particles which were preferentially labeled in the kinase reaction. Presumably, the DNA ends in the 170-bp particles are more accessible to the kinase enzyme. Sollner-Webb et al. (1978) have observed the same phenomenon. While the 170-bp DNA made up only a few percent of the DNA in the core particle preparation, the differential labeling causes the 170-bp DNA to make up nearly 50% of the end-labeled DNA (compare bands 16 and 14 at $t = 0$ in Figure 5a). This preferential labeling is not observed when kinasing free DNA. Interestingly, the 170-bp particles appear to be more resistant to DNase I digestion. After 3 min of DNase I digestion, the 170-base band is of significantly greater intensity than the 144-bp band (see Figure 5a). Simpson (1978) has observed that chromatosomes (carrying DNA longer than core DNA, plus lysine-rich histones) are quite resistant to DNase I. We cannot exclude the possibility that the small fraction of 170-bp particles in our preparation also contains lysine-rich histones.

Another peculiar feature of all of the digestion experiments with end-labeled DNA is illustrated in Figure 5. The bands below 4 in the autoradiogram do not coincide with the corresponding bands stained by ethidium bromide. In particular, the autoradiogram bands are consistently larger than the corresponding ethidium bromide bands. The ethidium bromide bands represent averages of many different (predominantly internal) fragments, while the autoradiogram scans represent a specific fragment from the 5′ end of the DNA. The extra length of the 5′-end fragment may be explained in part by the 2-base "overlap" of the 5′ end demonstrated by Sollner-Webb et al. (1978), but the additional 2- or 3-base difference is more difficult to explain, particularly because it does not seem to be present in the larger bands (>4).

The 125-bp and 111-bp reconstitutes, when end labeled and then digested with DNase I, also show a clearly discernible series of bands on the autoradiograph (see Figure 5). The existence of such well-defined bands in these digests, remarkably sharp considering the broad size distribution of the DNA fragments used in reconstitution, attests to a striking fidelity in DNA alignment with respect to the histone core. We have used the autoradiogram profile to determine where the 125-bp and 111-bp DNA molecules are placed on the histone core. In the case of the 125-bp particle, for example, we may ask whether the DNA is placed symmetrically or asymmetrically with respect to the 144-bp DNA placement? The symmetric mode would result if the 125-bp DNA were displaced about 10 bp in on each end, whereas the asymmetric mode would result from placement of one DNA end at the site of the 144-bp end and the other end recessed by about 20 bp. These two different modes are illustrated in Figure 6a. Either of these arrangements should give sharp DNase I patterns with end-labeled DNA. However, the two different modes should give clearly distinguishable banding patterns upon digestion with DNase I, assuming the bands are defined by histone binding.

The banding pattern for the symmetric mode should be identical with the pattern with 144-bp DNA but with each ^{32}P-labeled band shortened by about 10 bases. The highly protected sites at band 8 and band 6 from the core should now be found at band 7 and band 5, respectively, in the symmetric particle. The banding pattern predicted for the asymmetric mode is more complicated because the ends of the DNA are not in identical histone environments. Cutting from one 5′ end would result in a 144-base pattern down-shifted by about 20 bases while cutting from the other 5′ end would be identical with the 144-bp pattern. Model cutting patterns for these two modes are represented in Figure 7 for the 125-bp particle along with a densitometer tracing of the autoradiogram. While the actual autoradiogram does not correspond exactly to either of the two simple models presented, it very strongly supports the asymmetric mode of DNA placement. Strong peaks in

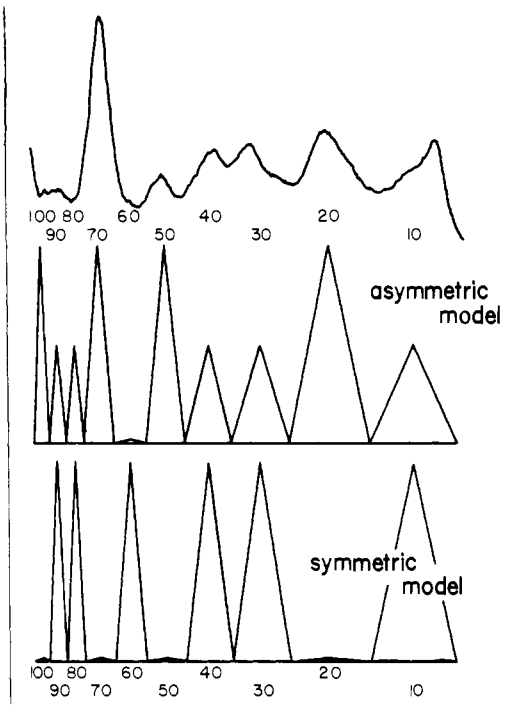

FIGURE 7: A comparison between the observed pattern of end-labeled DNA fragments obtained after 3-min digestion of particles reconstituted with 125-bp DNA and "theoretical" patterns predicted for the asymmetric and symmetric models in DNA placement (Figure 6). The model patterns are calculated by assuming that cuts cannot occur at sites roughly 30, 60, 80, and 110 bases from the 5′ end (bands 3, 6, 8, 11) but can occur with equal probability at other sites. The peak heights represent the predicted number average cutting frequencies. The calculation, although semiquantitative, makes the point that cutting frequencies should be low for bands 2, 5, and 7 if the placement were symmetric, contrary to observations.

the autoradiogram at bands 5 and 7 and weak ones at band 6 and 8 are predicted by the asymmetric model but virtually rule out the symmetric model. As mentioned above, band 7 is consistently stronger than would be predicted in all of our reconstitutes. In fact, in the 125-bp reconstitute it becomes the most prominent band. A part of its strength can derive from the fact that the cutting site 70 bases from the *recessed* end (right end, Figure 6a) coincides with the location of the 90-base cut in core particles. However, this is not a strongly cut position (Lutter, 1978) so that this explanation in itself is not sufficient.

The placement of 111-bp DNA cannot be easily modeled as either symmetric or asymmetric since the DNA is approximately three DNase I bands (31 bp) shorter than the core particles. Nevertheless, the similarity in the autoradiogram of the 111-bp pattern and the core particle pattern again suggests an asymmetric model. The reasons for this similarity will be discussed below.

Reconstitution with Longer DNA Reveals the Limits of Defined Placement. The experiments described above indicate a surprising degree of fidelity in placement of the DNA. Since these are random sequences, we cannot attribute this to specific sequence phasing. This led to the question: how long can the DNA be, and still be placed specifically with respect to whatever marker sites are aligning the shorter DNA fragments in reconstitution? Therefore, we have carried out reconstitution experiments using DNA fragments 161 bp and 177 bp in length.

When reconstituted particles containing these DNA lengths and 1 mol of histone octameter were digested with DNase I, and the single-strand fragments separated on denaturing gels

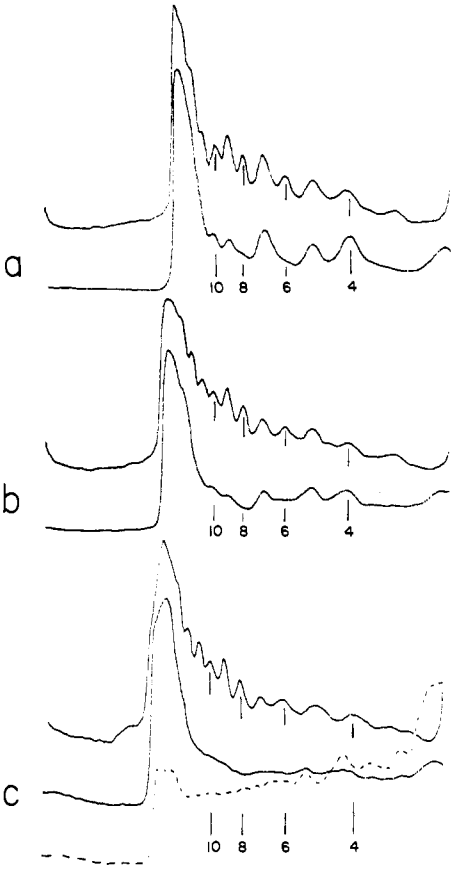

FIGURE 8: Comparison of DNase I digestion patterns of particles formed by reconstitution with (a) 142-bp, (b) 161-bp, and (c) 177-bp DNA. In each case, the DNA fragments were run on denaturing gels, and first stained with ethidium bromide (upper scans) and then autoradiographed to reveal the pattern of 5′-end-labeled fragments (lower scans). Each corresponds to 20-s digestion time, except for the broken line in c, which shows the autoradiograph of the fragments obtained from the 177-bp particles after 3-min digestion.

were stained with ethidium bromide, the result shown in Figure 8 was obtained. In all cases, DNA bands up to number 14 were resolved, and (at least at early digestion times) one or two more faint bands can be seen for the larger DNA samples. On the other hand, the autoradiographs of the end-labeled fragments (Figure 8) are strikingly different. The results found with particles made with 161-bp DNA are virtually identical with those with 144-bp DNA. On the other hand, almost *no* end-labeled bands at all can be seen when 177-bp DNA is used. The former result (with 161-bp DNA) is in apparent contradiction with Simpson's (1978) recent result with the 160-bp chromatosome (but see below). The second can only be interpreted as showing that 177 bp is too long to be placed on a histone core with the 5′ end in a "phasing" position. The observation of very weak bands can be accounted for by the presence of a small amount of shorter DNA molecules in the preparation.

Discussion

It is now clear that stable nucleosomal particles can exist with a single piece of DNA smaller than the "core" size of 144 bp. The particle reconstituted with 125-bp DNA contains 20 bp less DNA than the core particle, but is still very stable to thermal denaturation and has a sedimentation coefficient nearly identical with that of the core particle. As the length of the DNA is further decreased, particles can still form with the usual histone:DNA molar stoichiometry, but these "short-DNA" particles tend to associate at higher ionic

strengths. If the DNA length is appreciably less than 100 bp, aggregation is so pronounced that the entire preparation precipitates. This aggregation phenomenon was observed earlier by Noll & Kornberg (1977) and Bakayev et al. (1977) who found that chromatin digested to DNA fragments shorter than 120 bp tends to precipitate during a micrococcal nuclease digestion. The actual mechanism for this self-association is still obscure; the simplest hypothesis is that histone cores will tend to spontaneously aggregate in salt unless the electrostatic repulsion from the net negative charge contributed by the DNA is sufficient.

The fidelity of reconstitution as demonstrated with ^{32}P-end-labeled DNA is surprising. The DNA size classes used were far from homogeneous, with a half-width at half-height of 5–14 bp; yet particles made from these fragments gave a sharp banding pattern upon digestion with DNase I. We have calculated that a variability of only ±5 bp in the distance between the labeled DNA ends and the DNase I cutting sites should obliterate the banding pattern. These results seem highly improbable unless one end of the DNA is always phased with respect to the DNase I cutting sites. The results indicating an asymmetric mode for reconstitution of the smaller DNA fragments support this idea. During the reconstitution with DNA molecules shorter than the usual 144 bp, one end of the DNA seems always to bind at sites 0 or 14 in Figure 6, while the other end is recessed. This will assure that one ^{32}P-labeled DNA end will always be phased with respect to the DNase I cutting sites, no matter how heterogeneous the DNA population was, and give a definite banding pattern upon digestion with DNase I. It is even conceivable that the 5′-phosphate which has been added plays a role in locating the end; the extra negative charge could strengthen the interaction at this point. Since the labeling is relatively low, many molecules may contain only one labeled end. This would help explain the clarity of the pattern.

An alternative hypothesis derives from an idea advanced by Trifonov & Bettecken (1979). They have suggested that the location of DNase I cutting sites on the DNA is dictated not by the locations of binding sites on the histone core but by a "beat" effect between the nonintegral number of base pairs per turn (~10.4) and the narrow angular range in which phosphodiester bonds are assumed to be accessible for cutting. If such were the explanation, the DNA could lie anywhere along the prescribed path on the protein core, and still give the same DNase I cutting pattern, so long as the *ends* of the DNA were always constrained to the same angular orientation with respect to the core. While it is hard to exclude this model as a partial explanation, it is not easy to see why DNA ends, placed at different positions along the binding path, would be constrained to the *same* angular orientation everywhere. In fact, the opposite would be expected.

Nevertheless, our data do not allow us to distinguish unambiguously between different mechanisms for generating the cutting pattern. Certainly they are consistent with a variant of the Trifonov–Bettecken model in which the DNA end prefers a particular site (0 or 14) and is rotationally oriented at that site, thus dictating the subsequent cutting sites.

On the other hand, it is possible to make strong arguments for the "binding site" model for DNase I protection, based on energetic considerations. For example, the asymmetric mode of DNA placement seems reasonable from an energetic standpoint. If specific sites on the histone core bind DNA more strongly than others, the most stable state for any DNA is that in which a maximum possible number of strong binding interactions are filled. The fact that the 144-bp DNA is placed regularly with respect to the core indicates that at least the end sites (0 and 14, Figure 6) must be binding sites. Indeed, Mirzabekov et al. (1978) have shown that histone H3 binds near the 3′ end of the DNA. Since H3 (together with H4) appears to play a primary role in nucleosome assembly (Camerini-Otero et al., 1976), strong bonding at the DNA end is to be expected. In the case of reconstitutions with 144-bp DNA, the most favorable state is identical with the native particle, with both sites (0 and 14) complexed with DNA (see Figure 6a). With shorter DNA, however, it is not possible to fulfill all the binding requirements. One alternative, of course, is to bind more than one fragment of DNA to this histone core. This does seem to occur to a small extent if excess DNA is present during the reconstitution. If no excess DNA is present, the DNA molecule will fit onto the histone core in such a way as to satisfy as many strong protein–DNA interactions as possible. If we accept the assignments of histone binding sites made by Mirzabekov et al., we will expect that the 0- and 144-bp locations represent strong binding sites. This is the minimal assumption to explain our results. The proposed symmetric model for the binding of 125-bp DNA, with each DNA end set in about 10 bp with respect to the ends, would leave *two* strong binding sites unfilled, at positions 0 and 14. The asymmetric model, on the other hand, would leave only one of these strong binding sites unfilled (See Figure 6a). Hence, from such an argument the asymmetric binding mode would be predicted for the 125-bp particle.

If the regions on the protein core beyond the ends of the core DNA (that is, from positions 0 to −1 and 14 to 15, Figure 6) bind DNA only weakly, the alignment of one end of 125 bp at 0 or 14 is also understandable. The DNA so aligned will cover a strong end site (0 or 14) *and* all of that 100-bp long region which is most resistant to thermal denaturation. In the two-chain binding model of Mirzabekov et al. (1978), it is this ~100-bp central region in which *both* chains are histone bound. To displace the DNA in *either* direction would thus be energetically unfavorable.

Similar considerations can explain the behavior of 161-bp and 177-bp reconstitutes, if we postulate weak binding sites at locations −1 and 15 (Figure 6). The 161-bp DNA is not long enough to cover both of these sites; we estimate that 165–170 bp would be needed to do so. Hence, it will maximize interaction by binding one end at the 0 or 14 site, the other near the 15 or −1 site (see Figure 6b). We have strong evidence that under certain circumstances the histone octamer can protect approximately 168 bp of DNA from micrococcal nuclease, even in the *absence* of lysine-rich histones (Weischet et al., 1979).

The 177-bp DNA, on the other hand, can cover all sites, *and can do so in a variety of ways*, with ends overlapping to varying degrees (Figure 6b). Thus, there is no prescribed location for its 5′ ends, and one should not see a strongly defined pattern in the autoradiograph. This is the result observed.

If the strong interactions determine the placement of small DNA molecules on the histone core, the placement of two 65-bp DNA molecules should be quite specific. One end of each DNA will bind at the sites (0 and 14), while the other end will sit near the proposed dyad axis of the core particle. This particle should be very similar to the 144-bp particle except for having a cleaved DNA; indeed the 65-bp reconstitute does act similarly to the 144-bp particle, as judged by analytical sedimentation, under most conditions. Differences are seen, however, between the 144-bp and the 65-bp particles at extremely low ionic strength (Figure 4). This is the same

ionic strength at which Gordon et al. (1978) have observed a small reproducible change in the sedimentation coefficient of core particles. Oudet et al. (1978) and Poon & Seligy (1978) have both observed a partial unfolding of nucleosomes at low ionic strength. Oudet et al. (1978) find that the 20 or so nucleosomes on the SV-40 chromosome unfold into about 40 slightly smaller particles. Poon & Seligy (1978) have found a similar phenomenon in isolated mono- and dinucleosomes.

If the nucleosome does open up into half-nucleosomes or "hemisomes" at low ionic strength, the 65-bp reconstitute should be a good candidate for observing this conformational change. The continuous strand of DNA across the two halves of the core particle is broken in the 65-bp particle, which should allow dissociation instead of simple unfolding. We have predicted the sedimentation coefficient for the proposed hemisome, assuming both the core particle and hemisome to be oblate ellipsoids. The ellipsoid dimensions used for the core particle (55 Å × 110 Å) and hemisome (27.5 Å × 110 Å) are taken from electron diffraction (Finch et al., 1977) and neutron scattering (Pardon et al., 1977) data. The calculation is not very sensitive to the precise dimensions. By assuming no change in partial specific volume, the expected ratio of S(hemisome)/S(core particle) is 0.57. Since at low ionic strength the sedimentation coefficient for the core particle is approximately 10 S, the predicted sedimentation coefficient for the hemisome is 5.7 S. This value is close to the lower limit for the sedimentation coefficient distribution observed with the 65–70-bp reconstitutes at low ionic strengths (see the broken line in Figure 4). It is significantly larger than the sedimentation coefficient expected for free 65-bp DNA under these conditions ($s_{20,w} < 4$ S). The lower value for the sedimentation coefficient of the core particle (10.0 S vs. 10.9 S) is here assumed to be due entirely to electrostatic effects of the extremely low ionic strength buffer. However, if this decrease is in part due to a conformational change (Gordon et al., 1977), the value we obtain for the hemisome may be too low. If we use a value of 10.5 S for the sedimentation coefficient of the core particle, neglecting half of the ionic strength decrease as due to possible conformational changes, we obtain 6.0 S for the hemisome, still a reasonable result. We do not know at the moment why the $G(S)$ profile for the 70 bp reconstitution is so very broad. Two possible explanations can be considered. (1) Perhaps not all of these particles *can* cleave; in some cases a DNA fragment may cross the dyad and bind the halves together. Considering what has been seen above, in terms of fidelity of chain placement, we think this unlikely. (2) The broad $G(S)$ curve may represent a reaction boundary; the dissociation reaction may be in rapid equilibrium with reassociation. Such a rapid monomer–dimer reaction would yield a single boundary. Certainly, more study of these particles is called for.

In summary, while the evidence is strong that these "two-DNA" particles dissociate at low ionic strength, we do not yet know much about the process. For example, we have no evidence that the 65-base particle dissociates into homologous pieces; the possibility still exists that the dissociation may produce heterologous particles, i.e., one particle containing only H3 and H4 and one particle containing only H2A and H2B.

Reconstitutions with DNA molecules shorter than 144 bp help illustrate the nonrandom nature of the protein–DNA interactions within the nucleosome. The placement of these short DNA molecules seems to be directed by these interactions in ways that we still do not fully understand. At least three kinds of "binding" domains within the core particle are indicated by the melting experiments and reconstitution experiments.

(1) An "inner region" of about 100 bp seems, from the thermal denaturation studies, to be most strongly bound to the histone core.

(2) The points at 0 and 14 (Figure 6), spanning about 144 bp, seem to be of special significance, for they act both as strong limits to micrococcal nuclease digestion and as "ordering points" in reconstitution.

(3) Weak interactions at points about 10 bp beyond the core limits at 0 and 14 are suggested both by the quasistability of a particle with about 160–170 bp of DNA during digestion and by the properties of reconstitution utilizing longer DNAs.

We feel it is important that these results can, in general, be explained by one simple principle: in reconstitution, the DNA, whatever its size, is attached so as to maximize bonding with the histone core.

Added in Proof

A very recent paper on nucleosome reconstitution (Chao et al., 1978) has come to our attention. Using defined-sequence DNA fragments, these authors find evidence for preferred alignments of such DNA on the nucleosome core. On the other hand, they do not find clear end-labeled DNase I patterns with "random" sequence DNA (145 ± 10 bp), in contrast to our results. We do not know whether this apparent discrepancy results from differences in the reconstitution procedures or from some other cause.

Acknowledgments

We wish to thank Dr. R. Simpson for providing us with a preprint of a paper prior to publication and Drs. M. Cowman and G. Fasman for discussion of their thermal denaturation experiments. We thank Dr. Dennis Lohr for information on sizes of DNase I digestion products and many helpful discussions and Dr. R. T. Kovacic for information on sizes of *Hae*III fragments of PM2 DNA. The expert technical assistance of Georgia Riedel is gratefully acknowledged. We thank Dr. C. Tahourdin for the gift of polynucleotide kinase and Dr. R. McParland for the gift of [γ-^{32}P]ATP. Finally, we wish to thank Dr. W. Weischet for convincing us, against stubborn resistance, that particles containing more than 145 bp of DNA are significant.

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Quenching of Fluorescence of Bleomycins by Ferrous Ion and Its Correlation with DNA-Breakage Activity†

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ABSTRACT: The quenching of fluorescence of various bleomycin-related compounds by Fe(II) and Cu(II) was demonstrated and used to study DNA–metal ion interactions. The quenching by both Fe(II) and Cu(II) was stoichiometric, sensitive to (ethylenedinitrilo)tetraacetic acid but insensitive to NaCl or urea. Fe(III) and Mg(II) failed to induce quenching. The Fe(II)-induced fluorescence quenching was extensive for drugs shown to be active in DNA breakage: 70–80% for bleomycin A_2, deamidobleomycin A_2, and bleomycin B_1 and 50–60% for tallysomycin A and phleomycin D_1. Quenching kinetics of these drugs showed a rapid phase followed by a slow phase. Subsequent addition of Cu(II) caused no further change if maximal quenching by Fe(II) had been obtained. β-Mercaptoethanol accelerated the slow phase with little change in the extent of maximal quenching. The Fe(II)-induced quenching was less significant for drugs with less activity in DNA breakage: 20% for the C-terminal peptide fragments Wa and Wb and 30% for three bleomycin A_2 derivatives with substitutions at α-NH_2 of the β-aminoalanine moiety. Subsequently added Cu(II) readily displaced Fe(II) from the less active drugs, indicating a weak Fe(II)–drug interaction. The Cu(II)-induced quenching varied only slightly with all the tested compounds (30–50%). Subsequent addition of Fe(II) to the Cu(II)-quenched drugs caused little change. β-Mercaptoethanol added before, but not after, the addition of Cu(II) markedly reduced the quenching by Cu(II). These results indicate a correlation of quenching by Fe(II), but not by Cu(II), with DNA-breakage activity of the drugs, probably relating to the integrity of α-NH_2 at the β-aminoalanine moiety. It is suggested that a proper complex formation of Fe(II) with active drugs, especially the ligation at the α-NH_2 site, may induce specific conformational changes or other chemical events, which can be facilitated by β-mercaptoethanol, and thus favor the subsequent oxygen ligation and DNA breakage.

The bleomycins are a family of glycopeptide antibiotics which show various antitumor activities and have been clinically used in chemotherapy of certain human tumors (Blum et al., 1972; Umezawa, 1973–1976; Crooke & Bradner, 1976; Müller & Zahn, 1977; Goldberg et al., 1977). The antitumor activity may be related to the ability of bleomycins to cause single- and double-strand breaks of DNA (Suzuki et al., 1969; Haidle, 1971; Müller et al., 1972; Takeshita et al., 1974).

The mechanism of DNA breakage by bleomycins is not yet clearly defined. A variety of effectors have been suggested to be involved in or to affect the DNA-breakage activity of bleomycins. These effectors, among others, include metal ions such as Fe(II) and Cu(II), reducing agents such as β-mercaptoethanol, and chelators such as EDTA[1] (Suzuki et al.,

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0006-2960/79/0418-2880$01.00/0