Linker Histone Interaction Shows Divalent Character with both Supercoiled and Linear DNA†

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ABSTRACT: The interaction of linker histone H1 with both linear and superhelical double-stranded DNA has been investigated at low ionic strengths. Gel mobility retardation experiments demonstrate strikingly different behavior for the two forms of DNA. First, the experiments strongly suggest that linker histone binds to superhelical DNA in a negatively cooperative mode. In contrast, binding of linker histone to linear DNA under the conditions employed here shows no cooperativity. Second, binding of linker histone to linear DNA results in aggregation of histone−DNA complexes, even at very low levels of input histone H1. Because H1 has been shown to interact as a monomer, this aggregation is evidence of the divalent character of the linker histone, for without H1's ability to bind to two duplex strands of DNA, aggregation could not occur. Although aggregation can be made to occur with superhelical DNA, it can do so only at near-saturation levels of input histone H1. Finally, in direct competition, linker histone binds to superhelical DNA to the complete exclusion of linear DNA, indicating that the linker histone's function is related to the crossover structures that differentiate superhelical DNA from linear DNA. We develop a model that explains the observed behavior of binding of linker histone to superhelical DNA that is consistent with both the divalent character of the linker histone and the negative cooperativity by which linker histone and superhelical DNA interact.

The binding of the "linker histones" (H1,[1] H5, and their cognates) to DNA has long been a subject of interest to molecular biologists. Aside from its importance as a model for nonspecific DNA−protein interactions, understanding the way in which these proteins bind DNA may help clarify the still-unresolved problem of how they bind to the nucleosomal structure in chromatin (*1*). This, in turn, bears on the very important question of the role linker histones may play in regulating the accessibility of DNA in a polynucleosomal structure. That the interaction of linker histones with DNA may be complex is suggested by structural studies which indicate the presence of two potential DNA binding sites on the globular domain of each linker histone, in addition to the possibility of binding by the positively charged N- and C-terminal tails (*2*−*4*).

A number of the earlier experimental studies have indicated that, at least at higher salt concentrations, binding of linker histones (or their isolated globular domains) to linear DNA is positively cooperative (*5*−*8*). Several of these authors have noted that the process becomes noncooperative at low salt concentrations (ca. 25 mM NaCl). The quantitative studies of Watanabe (*7*), which are the only ones to actually measure free histone concentrations and thus equilibrium constants, report that cooperativity decreases but does not vanish at low salt concentrations. Careful cross-linking studies indicate that this cooperativity results from a preference for protein−protein interactions when the histone is bound to DNA (*6*, *8*).

Linker histone−DNA interactions have also been studied as a function of DNA superhelicity. The early experiments gave somewhat confusing results with respect to whether superhelical DNA was preferred (*9*−*12*). However, more recent studies have argued for a clear preference of linker histone for superhelical DNA (*13*, *14*). In an attempt to clarify these sometimes conflicting reports, we have performed a further analysis of H1−DNA interactions, including experiments in which we directly compare the binding of histone H1 to a supercoiled plasmid and its linearized and relaxed circular forms. The results provide new insight into these DNA−histone interactions.

EXPERIMENTAL PROCEDURES

DNA Preparations. Plasmid pML2αG contains the entire coding region and 800 bp of the 5′- and 350 bp of the 3′-flanking regions of the mouse α-globin gene cloned into a pBR322 derivative (*15*). DNA purification was performed using standard procedures (*16*). The plasmid so prepared exhibited a high degree of superhelicity, as judged from the gel shift with respect to the relaxed circular form (see Figures 1−3). The exact value was not determined, but is not necessary for these studies.

Plasmid pML2αG was linearized by digestion with restriction endonuclease *Eco*RV (New England Biolabs). The reaction was stopped by the addition of EDTA to a final concentration of 12.5 mM, and the DNA samples were extracted with phenol and chloroform (1:1) and precipitated

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[1] Abbreviations: bp, base pairs of DNA; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; H1, linker histone H1; H5, avian erythrocyte linker histone H5.

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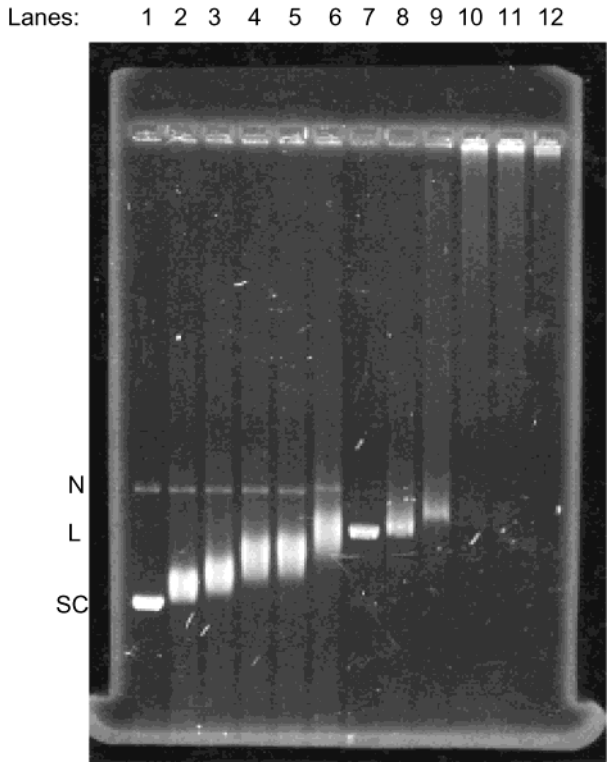

FIGURE 1: Gel mobility retardation assay of binding of H1 to plasmid pML2αG DNA. Titration of (lanes 1−6) the supercoiled form of plasmid DNA and (lanes 7−12) the linear form of plasmid DNA with increasing amounts of histone H1 on a 1.0% agarose gel. Letters to the left of the gel denote the positions of the three forms of DNA: N, nicked relaxed form of DNA; L, linear DNA; and SC, supercoiled DNA. The protein to DNA ratios (w/w) are 0, 0.05, 0.1, 0.15, 0.2, and 0.25 for lanes 1−6, and 7−12, respectively. The DNA concentration is 30 μg/mL in each case.

with ethanol. The pellets were dissolved in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA at a DNA concentration of 0.1 mg/mL. The concentration of DNA was determined spectrophotometrically by using an extinction coefficient of 20 mL cm^{-1} mg^{-1} at 260 nm.

Isolation of Histone H1. Chicken histone H1 was isolated from erythrocytes under nondenaturing conditions as described by Garcia-Ramirez et al. (*17*).

Gel Mobility Retardation Assays. Histone H1 was incubated with DNA in a total volume of 20 μL of a solution composed of appropriate volumes of the histone H1, DNA, distilled and deionized water, and a 4 μL aliquot of a stock incubation buffer composed of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% Triton X-100, and 20 mM NaCl for 1 h at room temperature. Thus, unless otherwise indicated, the salt concentration for incubation was 4 mM NaCl and the DNA concentration 30 μg/mL. The mixture was routinely electrophoresed through 1.0% agarose gels at 8−10 V/cm at room temperature in a Tris acetate/EDTA (TAE) buffer [40 mM Tris acetate (pH 7.5) and 1 mM EDTA]. Gels were stained with 0.1 μg of EtBr/mL for 20 min, briefly destained, and photographed with Polaroid 667 (Polaroid) film.

RESULTS

The Conformation of the DNA Molecules Plays a Major Role in Determining the Mechanism of Binding of H1 to DNA. As shown in Figure 1, a comparison of gel electrophoretic patterns for H1−DNA complexes in which the DNA is in either the supercoiled or the relaxed (linear) state shows very different behavior. In this experiment, precisely the same amounts of H1 have been added in each corresponding lane (i.e., lanes 1 and 7, 2 and 8, etc.) for supercoiled and linear DNA, yet the behavior is qualitatively different. As the amount of H1 added increases, the level of retardation of the DNA band increases in a regular manner in the lanes containing supercoiled DNA (lanes 1−6). This is definitely not observed for the comparable titration of H1 to linear DNA (Figure 1, lanes 7−12). In this case, some small, but noticeable, retardation is seen at low histone/DNA ratios. Then, there is an abrupt transition to complete aggregation of the DNA into the wells of the gel, which occurs at an H1/DNA (w/w) ratio of ~0.1.

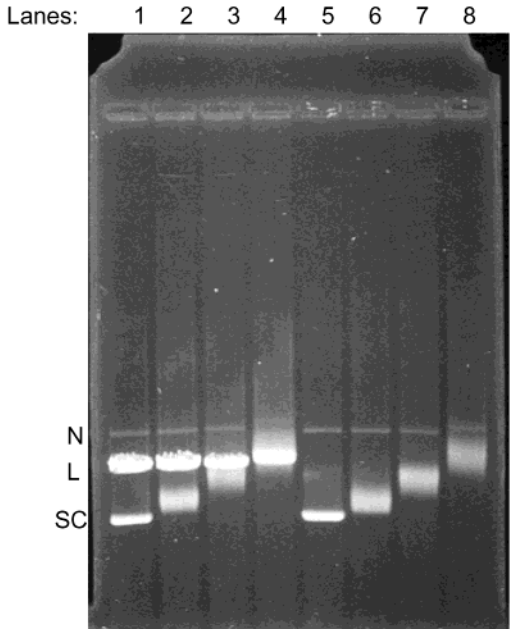

FIGURE 2: Gel mobility retardation assay of binding of H1 to supercoiled and linear DNA on a 1.0% agarose gel. Lanes 1−4 contained a mixture of supercoiled and linear DNA titrated with increasing amounts of histone H1, in direct competition. Letters to the left of the gel denote the positions of the three forms of DNA: N, nicked relaxed form of DNA; L, linear DNA; and SC, supercoiled DNA. Lanes 5−8 contained supercoiled DNA only, as a control. The histone H1 to DNA ratio (w/w) is 0 in lanes 1 and 5, 0.2 in lanes 2 and 6, 0.5 in lanes 3 and 7, and 0.8 in lanes 4 and 8. The total DNA concentration is 30 μg/mL in each case.

The data for the binding of supercoiled DNA by H1 shown in Figure 1 exhibit a peculiar feature. In each lane into which H1 is added (lanes 2−6), all of the DNA is migrating in a single, relatively sharp band, retarded relative to the DNA in the control lane (lane 1). The sharpness of the retarded band has, we feel, important significance to the mode of binding by supercoiled DNA; this will be discussed later.

Histone H1 Binds to Supercoiled DNA Preferentially over Linear DNA or Relaxed Circular DNA, in Direct Competition Experiments. Ivanchenko et al. (*14*) reported that H1 preferentially binds to superhelical pBR322 DNA over the linear or relaxed circular forms. We find exactly the same behavior for the plasmid used in this study (Figure 2). Histone H1, in the presence of both the supercoiled and linear DNA of the same sequence, will bind exclusively to the supercoiled DNA, leaving the relaxed conformer completely untouched. Note, too, that there is a small amount of residual relaxed circular DNA contaminating the samples of super-

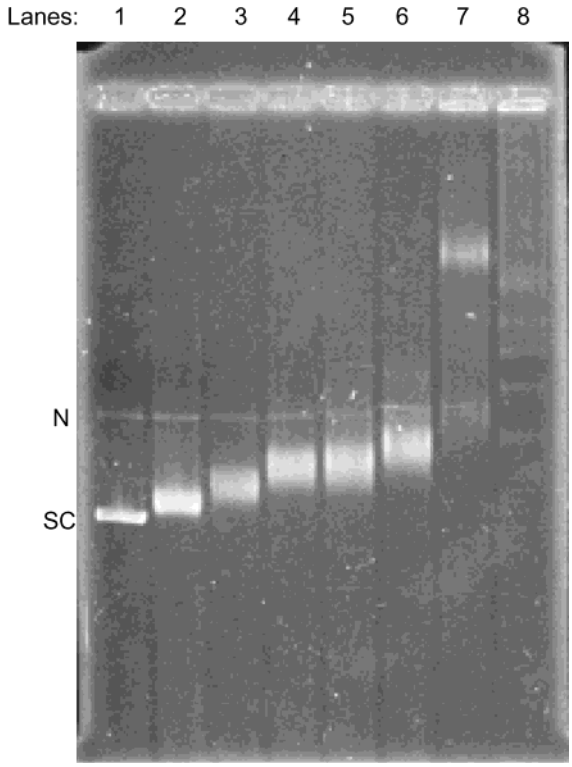

FIGURE 3: Gel mobility retardation assay of supercoiled DNA titrated with histone H1 on a 1.0% agarose gel. Letters to the left of the gel denote the positions of supercoiled (SC) and nicked circular (N) DNA. The ratios of histone H1 to DNA (w/w) are 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.5, and 0.7 for lanes 1−8, respectively. The DNA concentration is 30 μg/mL.

coiled DNA (Figure 1, lanes 1−6; Figure 2, all lanes), but this is also left completely untouched by H1. Thus, in the presence of all three conformations of DNA, supercoiled, linear, and relaxed circular, H1 binds exclusively to supercoiled DNA. Obviously, the affinity of H1 for highly supercoiled DNA must be orders of magnitude greater than that for relaxed DNA. Thus, there is some aspect of the supercoiling of DNA, not present in linear DNA, or relaxed circular DNA, that is the feature that the linker histone recognizes. Since the sequence of the DNA is exactly the same for all three forms in this experiment, it is some structural aspect which the H1 molecule preferentially selects. Previous work in this laboratory has suggested that crossovers of duplex strands in superhelical DNA provide a structural feature with which the H1 molecule preferentially interacts (*13*, *18*, *19*). Crossovers of DNA in this context signify the duplex strands of DNA that overlap upon one another as a result of the plectonemic writhing of the superhelical DNA.

Aggregation of the Histone−DNA Complex Occurs at High Levels of H1. Figure 1 (lanes 7−12) demonstrates that at the low salt concentrations used here even small amounts of H1 lead to an aggregated complex with linear DNA that is so large that is does not enter the gel. All of the DNA is involved in this aggregation, none being left free. The corresponding effect of the ratio of H1 molecules to superhelical DNA molecules was studied by titrating measured amounts of the DNA plasmid pML2αG with precise increasing amounts of chicken erythrocyte linker histone H1 and continuing that titration to high levels of H1 (Figure 3). As the amount of H1 relative to DNA increases, the migratory retardation of the electrophoresed samples increases, until, at a critical ratio of H1 to DNA, there is an abrupt transition at which the samples no longer penetrate the gel matrix, suggesting that superhelical molecules have aggregated into very large, polymeric complexes. The amount of H1 necessary to create the transition to aggregation differs greatly for the two different forms of DNA, i.e., linear and superhelical DNA. The significance of this difference will be discussed in the following section.

These data strongly support the notion (*2*, *4*) that the linker histone contains two DNA-binding sites which make it possible for each linker histone molecule to bind to two duplex strands of the linear DNA, for there is no explanation for the aggregation of the DNA in *any* form, as observed in Figures 1 and 3, other than one in which each linker histone molecule binds to two duplex DNA strands. This is a key observation, fundamental, we believe, to the role of the linker histone in the nuclear function of the nucleosome, as well as to our theoretical treatment of the experimental data on the linker histone's mechanism of interaction with superhelical DNA, discussed below.

DISCUSSION

Comparison of Binding of H1 to Superhelical and Linear DNA. Two differences in the binding of H1 to linear and superhelical DNA demand attention. First, even small amounts of H1 produce (under the low-salt conditions used here) complete incorporation of all of the linear DNA molecules into extremely large aggregates (Figure 1). On the other hand, although superhelical DNA will also aggregate, it will do so only at much higher concentrations of added H1 (Figure 3, lanes 7 and 8). This additional H1 is most certainly bound to the superhelical DNA, for there is not sufficient free H1 left to bind to the available competing relaxed, circular DNA (Figure 3, lanes 2−6), except at the very highest H1 loading [H1/DNA ratio (w/w) of ∼0.7], at which point aggregation of the superhelical DNA has also begun (Figure 3, lanes 7 and 8). Indeed, this level of bound histone corresponds to a histone molecule per 45 bp of DNA. This is the site size deduced by Clark and Thomas (*6*), although somewhat smaller than the value of 65 bp obtained by Watanabe (*7*). Given either value, it is clear that the superhelical DNA must be nearly saturated with H1 when its aggregation begins.

This argues for a strong preference, on the part of superhelical DNA, for *intramolecular* binding of H1. Furthermore, this binding is evidently *much* stronger than the *intermolecular* binding characteristic of the linear DNA. This is abundantly evident from Figure 2, where the two forms (as well as the third, relaxed circular form of DNA) are in direct competition for the ligand (see also ref *14*). The absence of significant binding to H1 molecules by relaxed DNA indicates that there can be very little free H1 in solution.

As mentioned in the introductory section, earlier studies were ambiguous with regard to the preference of linker histones for superhelical DNA. However, earlier studies from our laboratory (*14*) as well as the work presented here demonstrate unequivocally that highly supercoiled DNA is very much preferred by these histones.

It seems reasonable that the explanation for this preference is to be found in the plectonemic structure of supercoiled

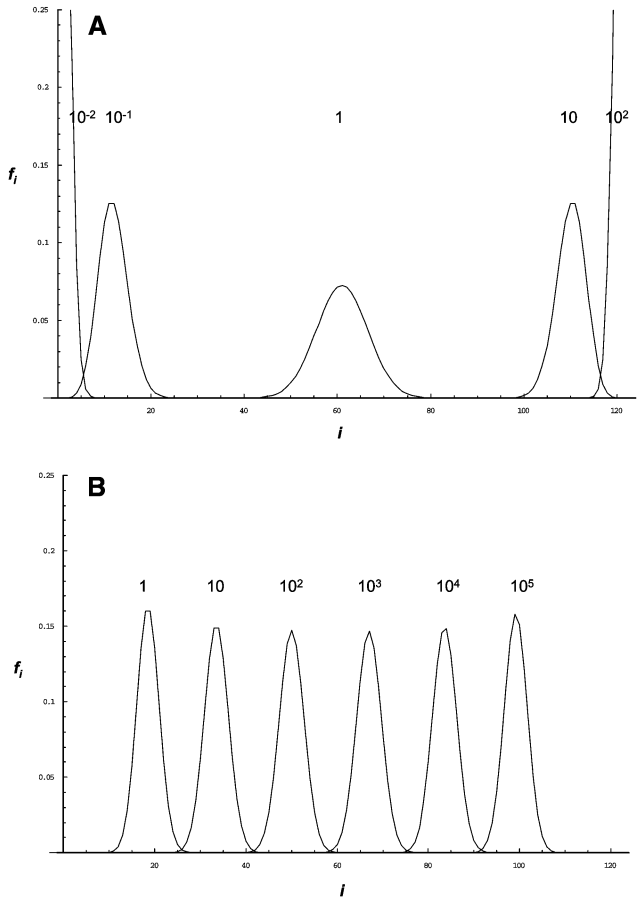

FIGURE 4: Effects of global cooperativity on ligand occupancy distributions. Distributions of occupancy (f_i) were calculated for models involving 120 binding sites, as estimated for the plasmid pML2αG DNA. The number of sites was estimated on the basis of the size of the plasmid and the site size given by Clark and Thomas (*6*). (A) Distribution for noncooperative binding ($\alpha = 1$) at five values of k_1[A]. (B) Distribution for negatively cooperative binding ($\alpha = 0.95$) for six values of k_1[A]. Values of k_1[A] are given above each curve.

DNA. In such a conformation, the DNA duplex repeatedly crosses itself, providing multiple opportunities for binding to two opposed sites on the histone molecule (*4*).

It should be noted that none of our experiments demonstrate the positive cooperativity in binding of H1 to DNA reported by other authors (*6*, *7*). This is undoubtedly a consequence of the low salt concentrations used in our experiments.

Possible Negative Cooperativity in Binding of H1 to Superhelical DNA. Close inspection of the gel electrophoretic patterns for superhelical DNA shown in Figures 1−3 reveals a curious phenomenon. As more histone is titrated onto the DNA, the electrophoretic band is retarded, *but remains quite sharp*, and does not appear to have the broad distribution that one would expect from noncooperative binding (the binomial distribution; see Figure 4A). The DNA molecules are behaving as if some factor inhibits binding of histone to DNA molecules that already have H1 bound to them, while favoring, instead, the binding of H1 to less-occupied DNA molecules. This is a characteristic of negative cooperativity.

Would we expect negative cooperativity in the binding of H1 to superhelical DNA? The answer is yes. We have shown above and previously that supercoiled DNA binds H1 much more strongly than does linear or relaxed circular DNA. Further, it has been established by two kinds of studies that binding of linker histones *unwinds* supercoiled DNA (*20*, *21*). If unwinding to a more weakly binding conformation is a consequence of binding, then that binding must be negatively cooperative. The kind of negative cooperativity suggested here is different from previous models of cooperativity in binding to a DNA lattice [that of McGhee and von Hippel (*22*), for example]. In earlier models, ligand−ligand interaction was invoked to explain the effect. This is a kind of "local" cooperativity. What we propose is a "global" cooperativity, in which the binding of each ligand changes the affinity of *all* of the remaining substrate sites for additional ligand. The model is developed in the Appendix, and some sample calculations are carried out to demonstrate that the kind of effects we see are in fact predicted (see Figure 4B).

The basic postulate of the model is that the microscopic binding constant (k_i) for binding to a molecule with i sites already occupied is given by

$$k_i = \alpha k_{i-1} \tag{1}$$

where α is a constant parameter that will be lower than unity for negative cooperativity and greater than unity for positive cooperativity. If $\alpha = 1$, the usual case of noncooperative binding to identical sites is obtained.

It is easy to show (Appendix) that the distribution of site occupancy is given by

$$f_i = \left[\frac{n!}{(n-i)!i!}\alpha^{P_i}(k_1[\mathrm{A}])^i\right] \Big/ \sum_{i=0}^{n}\frac{n!}{(n-i)!i!}\alpha^{P_i}(k_1[\mathrm{A}])^i \tag{2}$$

where f_i equals the fraction of molecules with i sites occupied out of a total of n. The microscopic binding constant for the first site is k_1, and the ligand concentration is [A]. The number P_i is equal to $i(i-1)/2$. Some simple calculations are illustrated in Figure 4, which contrasts the negatively cooperative binding with noncooperative binding. Note that with the global negative cooperativity model there is a clustering of liganded species, as observed. Because of the very strong power dependence of α, even an α value slightly lower than unity will have a very marked effect when the number of sites is large. It is not possible from our data to estimate the value of α, but it probably lies between 0.9 and 1.0. Finally, it should be emphasized that these experiments do not *prove* negative cooperativity; it simply seems to be an expected and reasonable explanation for the distribution.

Interaction of H1 with Linear DNA. At the low ionic strengths used in this study, we do not observe the positive cooperativity reported by previous workers for the interaction of H1 with linear DNA. In our experiments, the DNA was mixed with histone at a defined salt concentration (usually 4 mM) and then electrophoresed, in most cases, in gel buffer without additional salt. The onset of positively cooperative behavior has been reported to occur only at salt concentrations in the range between 25 and 50 mM (see, for example, refs *5*, *6*, and *23*), although Watanabe (*7*) notes some weak cooperativity at a concentration as low as 20 mM NaCl.

In any event, our experiments do not reveal the highly cooperative bimodal distribution between naked DNA and highly complexed DNA observed at higher salt concentrations. Instead, we find that above a critical H1/DNA ratio,

all of the DNA is incorporated into aggregates that are too large to enter the gel (Figure 1). The transition is very abrupt, and it occurs at an H1/DNA ratio much below that at which complexes between H1 and supercoiled DNA begin to aggregate.

This process closely resembles the formation of infinite networks from random association of multivalent polymers, and there exists a theory that should describe it. In 1952, Goldberg (*24*) developed a model to describe the precipitation of a multivalent antigen by divalent antibodies. The theory predicts that an abrupt transition from soluble complexes to an infinite network will occur at a critical degree of reaction. In our context, the divalent antibody corresponds to H1, the multivalent antigen with f binding sites to the DNA plasmid used in these experiments. Using the value of 45 bp from ref *6* as an estimate of the H1 site size, we expect ≅120 sites (f) on the plasmid DNA. Equation 25 of Goldberg when written in terms of our parameters gives the critical fraction of DNA sites reacted, P_c, as

$$P_c = \left[\frac{2}{f(f-1)}\frac{[\mathrm{H1}]}{[\mathrm{DNA}]}\right]^{1/2} \tag{3}$$

where [H1]/[DNA] is the input ratio of H1 to DNA molecules.

Because $f \gg 1$ in this case, eq 3 reduces to

$$P_c = \frac{1}{f}\left(2\frac{[\mathrm{H1}]}{[\mathrm{DNA}]}\right)^{1/2} \tag{4}$$

We observe that the critical point for aggregate formation occurs at a histone/DNA ratio (w/w) of ∼0.1 (Figure 1). This corresponds to an [H1]/[DNA] ratio of ≅17, expressed as a molar ratio. Equation 4 then predicts $P_c = 0.048$. That is, 4.8% of the DNA sites (or 5.7 sites/molecule) are occupied at the precipitation point.

If the H1/DNA input ratio at the critical point is 17 (see above), then only approximately one-third of the H1 molecules are bound to DNA; two-thirds are free. Is this consistent with binding data? The only quantitative data (*7*) give a value for the nucleation binding constant (the appropriate value in this low-H1 domain at low salt concentrations) of $\sim 5 \times 10^5\ \mathrm{M}^{-1}\ \mathrm{L}^{-1}$. We write

$$K = \frac{[\mathrm{H{\cdot}site}]}{[\mathrm{H}][\mathrm{site}]} \tag{5}$$

where [site] corresponds to the concentration of unoccupied DNA sites and [H] to that of free histone. Expressing this in terms of the extent of reaction defined by Goldberg (the fraction of sites occupied), we find

$$K = \frac{P}{(1-P)[\mathrm{H}]} \tag{6}$$

or

$$[\mathrm{H}] = \frac{P}{(1-P)K} \tag{7}$$

When $P = 0.048$ and $K = 5 \times 10^5$, we find $[\mathrm{H}] \cong 1 \times 10^{-7}$ M = 2.1 μg/mL. Since the total histone concentration at the precipitation point was 3 μg/mL, the calculation indicates that two-thirds should be free, as predicted above. Thus, the results are internally consistent. We do not deny the reported positive cooperativity at higher salt concentrations. However, at these very low salt concentrations, where histone−histone interactions appear to be minimized, the major role of the linker histone with this divalent character appears to be to allow network formation between DNA molecules. It should be emphasized that if linker histones each possessed one DNA-binding site, network formation would be impossible, according to the Goldberg model.

In summary, the behavior of both superhelical DNA and linear DNA in response to H1 binding at low salt concentrations has been given rational explanations, consistent with a model for linker histone that predicates two binding sites on each histone molecule. It seems likely that the existence of two strong DNA-binding sites on each linker histone H1 molecule, as observed in these experiments, and treated in our theoretical explanations, is important for the nuclear function of H1, in binding DNA strands exiting and entering the nucleosome.

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APPENDIX

Global Cooperativity in Binding to Nucleic Acids. The model assumes that the addition of each ligand molecule to a superhelical nucleic acid molecule with many potential binding sites produces an overall change in the nucleic acid molecule that perturbs equally the binding affinities of all remaining unoccupied sites in that nucleic acid molecule. The model does not include either ligand−ligand interactions or site-overlap effects [cf. McGhee and von Hippel (*22*)]. Neglect of the latter will lead to errors at high occupancy, but we see no way to include this complication.

We write the sequential addition of ligands to nucleic acid molecules as

$$\mathrm{N} + \mathrm{A} \overset{k_1}{\rightleftharpoons} \mathrm{NA} \quad k_1 = [\mathrm{NA}]/([\mathrm{N}][\mathrm{A}]) \tag{A1}$$

$$\mathrm{NA} + \mathrm{A} \overset{k_2}{\rightleftharpoons} \mathrm{NA}_2 \quad k_2 = \alpha k_1$$

$$\mathrm{NA}_2 + \mathrm{A} \overset{k_3}{\rightleftharpoons} \mathrm{NA}_3 \quad k_3 = \alpha k_2 = \alpha^2 k_1$$

$$\mathrm{NA}_3 + \mathrm{A} \overset{k_4}{\rightleftharpoons} \mathrm{NA}_4 \quad k_4 = \alpha k_3 = \alpha^3 k_1$$

In general

$$\mathrm{NA}_{i-1} + \mathrm{A} \rightleftharpoons \mathrm{NA}_i \quad k_i = \alpha^{i-1} k_1 \tag{A2}$$

Writing the equilibrium relationships for individual steps in the reaction

$$[NA] = k_1[N][A]$$

$$[NA_2] = k_2[NA][A] = k_2k_1[N][A]^2 = \alpha k_1{}^2[N][A]^2$$

$$[NA_3] = k_3[NA_2][A] = k_3k_2k_1[N][A]^3 = \alpha^3 k_1{}^3[N][A]^3$$

In general

$$[NA_i] = \alpha^{P_i} k_1{}^i[N][A]^i \tag{A3}$$

where the P_i values are given by the equation $P_i = i(i - 1)/2$. To obtain the overall concentration of species with i ligands, we must multiply by the statistical factor

$$\overline{[NA_i]} = \frac{n!}{(n-i)!i!}\alpha^{P_i}(k_1[A])^i[N] \tag{A4}$$

Thus, the fraction of molecules that will have i ligands bound is $\overline{[NA_i]}/\sum_{i=0}^{n}\overline{[NA_i]}$ or

$$f_i = \frac{\dfrac{n!}{(n-i)!i!}\alpha^{P_i}(k_1[A])^i}{\displaystyle\sum_{i=0}^{n}\frac{n!}{(n-i)!i!}\alpha^{P_i}(k_1[A])^i} \tag{A5}$$

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