

Driving Proteins Off DNA Using Applied Tension

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ABSTRACT Proteins that bind DNA so as to reduce its end-to-end length can be dissociated by application of force. The thermodynamics of this process are discussed, with special attention to the case of histones bound to DNA (i.e., a string of nucleosomes, or chromatin fiber). The histone octamer is predicted to be driven off chromatin fiber for tensions >2 piconewtons.

INTRODUCTION

Molecular biotechnology has made possible micromanipulation experiments where the responses of a single long DNA to tensions and torques are measured (Bensimon, 1996). This has allowed DNA bending (Smith et al., 1992; Bustamante et al., 1994), twisting (Strick et al., 1996), and stretching (Smith et al., 1996; Cluzel et al., 1996) to be studied in thermal equilibrium, and in unprecedented detail. These experiments typically anchor one end of a >10 μm -long DNA to a surface, and the other to a colloidal particle (e.g., a magnetic bead of 3 μm diameter). A force is applied to the particle is pulled by some means (e.g., a magnetic field gradient); the stretching of the molecule is then measured directly using optical microscopy.

Recent DNA-stretching experiments revealed that it could be "denatured" by force: the length of a double helix was seen to increase by a factor of ~ 1.6 when the tension was ~ 65 piconewtons (pN) (Smith et al., 1996; Cluzel et al., 1996). The only reasonable explanation for the observed sharp, reversible transition from normal to "overstretched" DNA is that the tension disrupts the hydrogen and hydrophobic bonds that stabilize the double helix: the DNA was extended like a spiral spring. And in fact, the work done per length of DNA during this extension is roughly the tension of 65 pN (note an energy per length is a force) (Marko and Siggia, 1995; Marko, 1997). Converting this to $k_B T$ units (1 $k_B T/\text{nm} = 4.1$ pN at 300 K) we find that the work done is ~ 15 $k_B T/\text{nm}$, enough to strongly perturb the DNA double helix, which is stabilized by a comparable cohesive energy per length (Breslauer et al., 1986).

The same methodology can be extended to proteins that bind to DNA. Imagine a protein bound to DNA such that an amount ℓ of DNA contour length is "stored" in a "loop." Examples are lac repressor, which can bind two distant DNA sites to form a DNA loop (Finzi and Gelles, 1995); and histone octamers, around which DNA wraps to form the

nucleosomes in eukaryote chromatin (van Holde, 1989; Wolffe, 1995). The protein sticks to the DNA because of some binding enthalpy ϵ . If we suppose the DNA to be under tension f , then if the protein comes off the DNA, an amount of work of roughly $f\ell$ can be done. In thermodynamic equilibrium, a tension $f \approx \epsilon/\ell$ is therefore sufficient to drive the protein off the DNA.

To correctly infer thermodynamic and elastic parameters from biophysical experiments that probe protein-DNA interactions with force requires a quantitative statistical mechanics calculation. The following section formulates such a theory and applies it to the liberation of histones from chromatin fiber. In the Discussion we go beyond a strictly thermodynamic description and outline some of the non-equilibrium effects to be expected experimentally in experiments on chromatin. Although we focus on DNA-protein interactions, our theory applies equally well to any case where tension in an elastic polymer causes structural change, e.g., the overextension of the protein titin from sarcomeres (Rief et al., 1997; Kellermayer et al., 1997; Tskhovrebova et al., 1997).

THEORY

One protein bound to DNA

As described above, consider a protein which when bound to DNA, stores a contour length ℓ in a loop. When the protein is off, the binding enthalpy ϵ is lost, but some translational entropy is gained (the liberated protein can move throughout the solution volume), and some work is done by the tension. Thus, the free energy difference between the protein "off" and "on" states is

$$\Delta G = k_B T \log \phi + \epsilon - f\ell \quad (1)$$

where ϕ is the concentration of the protein in solution. Below, we lump the ideal-gas free energy together with the enthalpy as $\mu \equiv k_B T \log \phi + \epsilon$. A low solution concentration reduces μ , favoring liberation of the protein from DNA (μ is reduced by $2.3k_B T$ for each 10-fold dilution of ϕ). The protein will be predominately in solution when Eq. 1 is negative, or when $f > f^* = \mu/\ell$; otherwise it is bound to the DNA, with contour length ℓ stored.

Summing over the two states, we obtain the partition function $Z = 1 + \exp(-\Delta G/k_B T)$. The equilibrium protein off probability is therefore just

$$p_{\text{off}} = \frac{1}{1 + \exp(\Delta G/k_B T)} \quad (2)$$

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where for now we do not consider the entropic elasticity of the looped DNA [this is a good approximation if μ/ℓ greatly exceeds the 0.1 pN necessary to more or less fully extend the DNA loop in the off state (Smith et al., 1992); the entropic elasticity of the loop can be taken into account if necessary, see below]. The average extension of the binding domain (distance between the ends of the loop) is just $\langle z \rangle = \ell p_{\text{off}}$.

The free energy difference, Eq. 1, can be reexpressed as a function of extension (or p_{off}) rather than force if we first invert Eq. 2 to find $f(\langle z \rangle)$, and then compute the free energy for the ensemble where p_{off} is fixed, by Legendre transformation:

$$F \equiv -k_B T \log Z + f \langle z \rangle \quad (3)$$

$$= p_{\text{off}} \mu + k_B T [p_{\text{off}} \log p_{\text{off}} + p_{\text{on}} \log p_{\text{on}}]$$

where the on state is populated with probability $p_{\text{on}} = 1 - p_{\text{off}}$.

What order of magnitude is f^* ? A typical DNA-protein interaction involves $\epsilon \approx 20k_B T$, corresponding to an association constant of $K_a = \exp[20] \text{ M}^{-1} \approx 10^9 \text{ M}^{-1}$ [the DNA binding affinities of many proteins are rather precisely characterized: the lac repressor-operator $K_a \approx 10^9 \text{ M}^{-1}$ (Hsieh et al., 1987); the free energy of DNA loop formation by lac repressors has also been measured (Hsieh et al., 1987; Finzi and Gelles, 1995); the cro repressor-operator $K_a \approx 10^{12} \text{ M}^{-1}$ (Kim et al., 1987); the trp repressor-operator $K_a \approx 10^{10} \text{ M}^{-1}$ (Hurlburt and Yanofsky, 1992); the transcription factor TFIIC2-B block $K_a \approx 10^{11} \text{ M}^{-1}$ (Boulanger et al., 1987); and the TBP-TATA box $K_a \approx 10^8 \text{ M}^{-1}$ (Perez-Howard et al., 1995)]. For a DNA loop of $\ell = 50 \text{ nm}$ (~ 150 base pairs, or a persistence length), stabilized by $\mu = 20k_B T$, we obtain $f^* = 1.6 \text{ pN}$. This is a low force compared to that needed to denature DNA (65 pN); f^* is further reduced for larger ℓ . Of course, this result refers to thermodynamical equilibrium; one must wait a time $\gg t_{\text{off}}$ for the protein in question. There may be large barriers to equilibration of the protein between the off and on states.

The width of this transition as a function of f can be expected to be sharp. For one protein, the transition width is due to thermal fluctuations of magnitude $k_B T$. Therefore a force below f^* by an amount $\sim k_B T/\ell$ will be sufficient to allow thermal fluctuations to partially populate the off state (see Eq. 2). The large difference between ϵ for strongly bound proteins and $k_B T$ leads to a sharp transition. This width for the example cited above is approximately $k_B T/50 \text{ nm} = 0.08 \text{ pN} \ll f^*$.

Physicochemical properties of chromatin fiber

Before discussing the stretching of chromatin fibers, we review their basic physical properties. At its lowest level of organization, the "10 nm fiber," chromatin consists of a string of "nucleosomes," each of which consists of 146 bp of DNA wrapped in ~ 1.75 turns around an octamer of histone proteins (van Holde, 1989; Wolffe, 1995). Thus about a 50-nm length of DNA is "stored" in each nucleosome. Successive nucleosomes are quite closely packed; here we suppose that there is one every 200 bp. So, a chromatin fiber can be thought of as a long series of the DNA-protein units described in the previous section.

Studies of the equilibrium between nucleosomes and naked DNA + octamers indicate that the binding enthalpy of each octamer is $\approx 20k_B T$ for "physiological" 0.15 M univalent salt solution (Cotton and Hamkalo, 1981; Ausio et al., 1984a, b). A similar estimate of the total binding enthalpy follows from recent measurements of the probability of exposure of nucleosome-wrapped DNA to a restriction enzyme (Polach and Widom, 1995). These experiments also make it clear that DNA fluctuates on and off the histones, and therefore that our statistical-mechanical approach is appropriate.

The interaction between histones and DNA is partially electrostatic, as evidenced by the salt dependence of the binding energy (Ausio et al., 1984a, b; Stacks and Schumaker, 1979). In fact, chromatin can be reconstituted from bare DNA and histones by dialysis from high ($\approx 1 \text{ M}$) salt, down to physiological values ($\approx 200 \text{ mM}$).

Native chromatin fiber, in addition to the histone octamer (2 each of H2A, H2B, H3, and H4 histones) and DNA, contains additional "linker" histones (H1 or H5). H1/H5 "caps" the nucleosome, sitting near where the wrapping of DNA begins and ends, on the 20- to 50-bp of "linker" DNA separating successive nucleosomes. The linker histones are an important determinant of higher order chromatin structure, the so-called 30 nm fiber, the in vivo structure of which remains controversial (Wolffe, 1995; Horowitz et al., 1994). One aim of chromatin-stretching experiments is to elucidate the differences between H1/H5-rich and H1/H5-depleted fiber.

Preliminary data (Castro, 1994) indicate that the 30-nm fiber, like DNA itself, can be modeled as a flexible polymer. Its persistence length is $A_1 \approx 30 \text{ nm}$ (versus 50 nm for bare DNA), and its contour length or segment size is roughly $1/10$ that of the 1–2 kb of DNA contained within it (estimates of this compaction factor range from $1/6$ to $1/40$). Given that there are 20 to 50 bp between successive octamers, this persistence length is consistent with chromatin flexibility being due to bending of the linker DNA. Both this persistence length and contour compaction are consistent with recent studies of the random-walk structure of interphase chromatin in HeLa cells (Sachs et al., 1995).

An important question about chromatin structure is whether or not the octamers can "slide" along the DNA. This is important to molecular genetics since nucleosome-wrapped DNA is relatively inaccessible to DNA-binding proteins; regulation of nucleosome position can conceivably control gene expression. Much effort has gone into the study of nucleosome positioning (FitzGerald and Simpson, 1985; Simpson, 1991), either by sequences that preferentially adhere to histones (Shrader and Crothers, 1989) or by DNA binding proteins that clamp onto a sequence, thereby blocking histone sliding (Kornberg, 1981; Fedor et al., 1988). In this paper sliding (or absence thereof) is important since it increases the entropy of bound octamers, and thus affects the free energy balance between bound and free histones. Below, we consider the two extreme cases where the histones freely slide (we consider them to form a one-dimensional "gas"), and where they have absolutely fixed positions and therefore no sliding entropy.

Chromatin fiber under tension

The fiber properties will be assumed to be a function of ψ , the fraction of the DNA length occupied by bound octamers. For the case of naked DNA, $\psi = 0$, the free energy versus extension is that of a "worm-like chain" (WLC) (Bustamante et al., 1994):

$$\frac{F_{\text{WLC}}}{k_B T} = \frac{L}{A} \left[\frac{(z/L)^2}{2} - \frac{z/L}{4} + \frac{1}{4(1-z/L)} - \frac{1}{4} \right] \quad (4)$$

where L is the total contour length, $A \rightarrow A_0 \approx 50 \text{ nm}$ is the persistence length, and z is the end-to-end extension. The force needed to separate the ends of the chain by a distance z is just $\partial F_{\text{WLC}}/\partial z$.

For closely packed nucleosomes arrayed as in closely packed 10 nm "beads-on-a-string," or as in 30-nm fiber, we have $\psi \approx 1$, and we assume that Eq. 4 continues to apply (Castro, 1994), with $L \rightarrow L_1 = L_0/10$ and $A \rightarrow A_1 = 30 \text{ nm}$. Here L_0 is the total length of DNA in the sample irrespective of whether nucleosomes are attached or not. Given these two limits for A and L , we interpolate for $0 < \psi < 1$ by retaining Eq. 4 with A and L given by:

$$L = \psi L_1 + (1 - \psi) L_0 \quad (5)$$

$$A = \psi A_1 + (1 - \psi) A_0$$

In addition to the conformational free energy (Eq. 4), we must include the binding free energy, and the nucleosome rearrangement entropy, both as a function of ψ . The rearrangement entropy depends on whether or not the nucleosomes are able to freely slide along the DNA. If there is no sliding, then the rearrangement free energy is just Eq. 3 with $p_{\text{on}} \rightarrow \psi$ (this is the free energy of a 1-d lattice gas where each nucleosome position plays the role of one lattice site). If in the close-packed state ($\psi = 1$), there is one

positioned nucleosome every contour length ℓ of DNA, we have the free energy:

$$F_{\text{positioned}}(z, \psi) = F_{\text{WLC}} + \frac{L_0}{\ell} [(1 - \psi)\mu + k_B T \psi \log \psi + k_B T (1 - \psi) \log(1 - \psi)] \quad (6a)$$

where we note that $L_0 \psi / \ell$ is just the number of octamers bound to the DNA. The same “chemical potential” $\mu = k_B T \log \phi + \epsilon$ is used here as in the previous section. When a numerical value is needed, we will assume $\ell = 68 \text{ nm}$ (200 bp).

If the nucleosomes can freely slide, their rearrangement entropy (the final two terms of Eq. 6a) is slightly modified. The sliding entropy may be taken to be that of hard cores of length ℓ , sliding along the one-dimensional DNA of length L_0 , which is known exactly (Tonks, 1936). The free energy in this case is:

$$F_{\text{sliding}}(z, \psi) = F_{\text{WLC}} + \frac{L_0}{\ell} \left[(1 - \psi)\mu + k_B T \psi \log \frac{\psi}{1 - \psi} \right] \quad (6b)$$

Note that with free sliding, as $\psi \rightarrow 1$, the rearrangement entropy diverges logarithmically; with positioned nucleosomes (Eq. 6a) the entropy stays finite in this limit.

In either case, ψ is determined by minimization of one of Eqs. 6. The force for given extension z is just

$$f = \frac{\partial F}{\partial z} = \frac{k_B T}{A} \left[\frac{z}{L} + \frac{1}{4(1 - z/L)^2} - 1/4 \right] \quad (7)$$

All dependence on ψ enters through A and L .

This model has the virtues of simplicity and straightforward application to experiment with a minimal number of parameters. There is only one (μ) with the dimensions of energy related to nucleosome binding; or in the case of 30 nm fiber, binding of octamer + linker histones to DNA, considered as a unit. The bending flexibility of the fiber is given by A_1 , and also can be expected to be different for 10 nm and 30 nm fibers. Equations 6 therefore ignore any processes intrinsic to the linker histones, either their elastic deformation or unbinding if uncoupled to that of the histone octamer. If the nucleosomes spontaneously clump together, their mutual binding energy is another neglected parameter. Distinctions between structures of the 30-nm fiber and the 10-nm “beads-on-a-string” are reflected only in the two length parameters ℓ and L_1 .

Fig. 1 shows the result of Eq. 6b and 7, when nucleosomes are free to slide, for a variety of μ . For strong binding ($\mu \gg k_B T$), the critical force f^* at which the nucleosomes are released, is nearly as sharply defined as in the case of a single protein. The parameter L_1/L_0 controls the extension just prior to nucleosome release. In the opposite limit of $\mu/k_B T$ near zero or negative, the extension curve is nearly that of naked DNA, since essentially no nucleosomes will be bound.

In Fig. 1 *b* at low force, the nucleosome binding fraction ψ is slightly below close-packing ($\psi = 1$): at nonzero temperature, the nucleosome sliding entropy diverges as $\psi \rightarrow 1$, implying that there will always be some space between nucleosomes (note that the on-off entropy diverges less strongly in this limit). For $f \rightarrow 0$ and large μ , $1 - \psi \approx k_B T / \mu$, thus one can expect a few percent of the DNA to be bare thanks to sliding entropy.

Fig. 2 shows extension versus force for the same μ values as in Fig. 1, for the case where the nucleosomes are positioned (using Eq. 6a). Overall, the behavior is similar to that of Fig. 1 *a*, but with a sharper onset of the transition ($z/L_0 < 0.5$). This occurs because the on-off entropy is less effective at keeping octamers off the DNA at low forces than is the sliding entropy: for $f \rightarrow 0$ and $\mu \gg k_B T$, the minimizing density is $1 - \psi \approx \exp(-\mu/k_B T)$. Therefore, much more closely packed nucleosomes are expected at low force, and the transition from the $\psi \approx 1$ state is expected to be more abrupt, in the case where there is no sliding (without sliding, the transition becomes essentially that of the simple on-off model of Eq. 2). On the side of the transition going to large extension with most nucleosomes

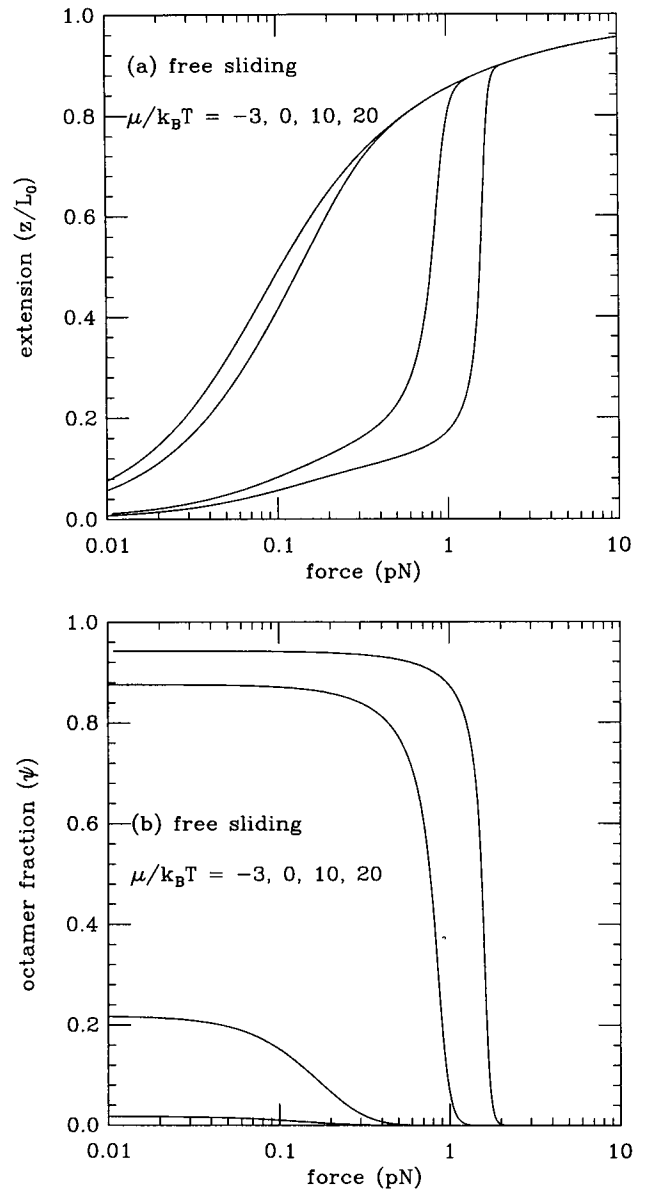


FIGURE 1 Extension (a) and octamer binding fraction (b) versus applied force for model of chromatin fiber under tension, with free nucleosome sliding (Eq. 6b). The curves from left to right are for $\mu/k_B T = -3, 0, 10$, and 20. Extension is as a fraction of total DNA length. For $\mu/k_B T \ll 1$, the binding fraction ψ is essentially zero and the force-distance behavior is that of naked DNA. For $\mu/k_B T \gg 1$, octamers are tightly bound at low forces, and are dissociated when the force exceeds a well-defined threshold. The transition width and $1 - \psi$ at zero force get smaller as μ is increased.

removed (for $z/L_0 > 0.5$), there is essentially no difference between the sliding and nonsliding cases since the rearrangement entropies in Eqs. 6a and 6b become essentially the same.

In the first experiments, we propose that just μ , A_1/A_0 , and L_1/L_0 be fit for H1/H5-depleted fibers: L_0 itself essentially can be read off from the high force limit (perhaps in a modified high salt buffer to be sure that the nucleosomes are released). From the thermodynamic data one expects $\mu \approx 20k_B T$ in the absence of linker histones. In the case of H1/H5-containing fibers, the simplest possibility is that the present model with different values of ℓ , A_1/A_0 , L_1/L_0 , and $\mu > 20k_B T$ fits experimental data. Only if

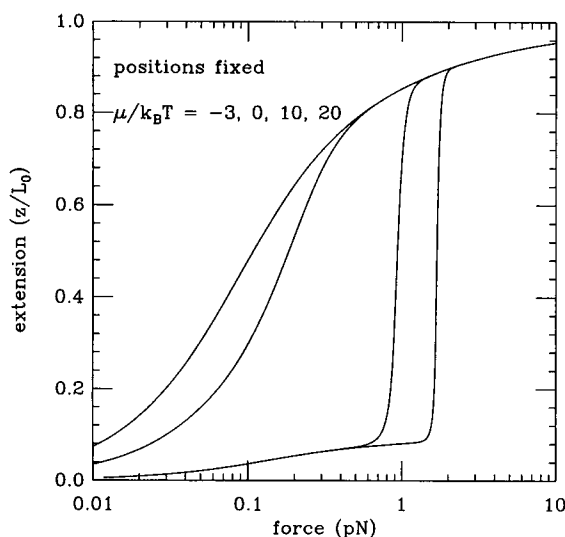


FIGURE 2 Extension versus force for chromatin fiber under tension, for the same parameters as in Fig. 1, but with fixed nucleosome positions (Eq. 6a). The curves correspond to $\mu/k_B T = -3, 0, 10$, and 20 from left to right. Below these transitions, $\psi = 1$; above, $\psi = 0$. The transitions occur at forces about the same as those of Fig. 1 *a*; they differ in that they have a sharper onset for $\mu/k_B T \gg 1$ (compare the regions where $z/L_0 < 0.5$).

some force-distance behavior qualitatively different from that discussed above is observed (e.g., an additional force "plateau" in the low force regime) should one contemplate trying to add additional parameters to describe the linker histones.

DISCUSSION

We have seen that a protein bound to DNA by a characteristic free energy μ , which stores a length ℓ in a loop or wrapping, will be liberated when a tension $f^* \approx \mu/\ell$ is applied. Thus force can be used as well as dilution to study the dissociation of proteins. In conjunction with dilution studies, force-distance studies can give direct structural information about the geometry of DNA binding. We have also developed a model for a string of such proteins bound to DNA, with application to chromatin fiber in mind.

Our chromatin stretching model has three regimes: for low forces, the fiber has some entropic elasticity; at the threshold force $\approx f^*$, the nucleosomes start to come off and the fiber greatly lengthens; finally, for high forces the elasticity of naked DNA is seen. The basic phenomenon of the "titration" away of the histones by the applied force should be very robust. However, one must keep in mind many effects outside the scope of this paper.

The most serious idealization in this chromatin stretching model is the assumption of thermodynamic equilibrium during both stretching and relaxation of the fiber. Rapidly ejecting a histone octamer from DNA into a physiological salt buffer will cause it to fall apart into its component histones, which may then nonspecifically adhere to the DNA (Kornberg et al., 1989) (note that the plausible range of forces required to remove the nucleosomes is much less than the ~ 20 pN required to denature a protein). To avoid

this, a histone chaperone is needed such as one uses in chromatin reconstitution protocols. Polyglutamic acid, or just short, single nucleosome segments of DNA will bind and stabilize the octamers when they are expelled from the host chromatin by tension, and allow their exchange back when the tension is relaxed. If the octamers do not cluster when reconstituting, then the equilibrium constant between the octamer concentration on the ~ 200 bp segments and that on the long fiber is a direct measure of the sliding entropy in Eq. 4.

Under favorable conditions, a force and distance measurement could be made quickly enough to see the renaturation kinetics, or some portion of it associated with wrapping the DNA around the histones. High enough kinetic barriers might exist for the loss of octamers, so as to slow this process down sufficiently that it too be studied mechanically. In an extreme case that reassociation kinetics exceed the time of the experiment, the force-extension curve will follow the rightmost curve in Fig. 1 *a* on extension, and the leftmost (naked DNA) curve on retraction.

Our chromatin model also assumes that the fiber is perfectly homogeneous. This is certainly not true: octamers may have preferred positions, defined either by DNA sequence or by the binding of other nonhistone proteins, that lead to inhomogeneous octamer binding (Shrader and Crothers, 1989). Furthermore, native chromatin will without doubt contain other nonhistone proteins that determine its higher-order structure, which are beyond our ability to even guess about. However, the qualitative results of our model should apply even with appreciable inhomogeneity. Note that the difference between the sliding and nonsliding force-extension curves is relatively small (compare Figs. 1 *a* and 2). Strong inhomogeneity should not destroy the transition from nucleosome-rich to nucleosome-poor DNA, and the transition force will still indicate the characteristic strength of the interactions holding the octamers to DNA.

A related question is whether there is tendency for the nucleosomes to "clump together" along a chromatin fiber, due to inter-nucleosome cohesion. Some preliminary evidence exists for this at high salt (Shrader and Crothers, 1989; Klevan and Crothers, 1977; Noll et al., 1980). If this occurs, one can expect a kind of "phase coexistence" of bare DNA and dense nucleosomes to occur as a chromatin fiber is extended. The model described above assumes no such clumping (either annealed or quenched random positions are supposed in the free-sliding and fixed-position cases considered) but one can easily include such effects using a two-phase model. Experimentally, one would expect to see a force plateau as the nucleosomes were removed with bare DNA elasticity above the transition, similar to Figs. 1 *a* and 2. Hence it will be hard to prove the existence of clumping using force data alone, and probably not useful to introduce the additional binding energy parameter necessary to describe such a phase unless its existence can be shown by other means.

It is possible to control the twist (more precisely, the double helix linkage number) of bare DNA while it is being

pulled (Strick et al., 1996): for $\approx \pm 5\%$ excess twist the force necessary to obtain a given extension is appreciably increased relative to untwisted DNA (a comparable but not identical effect is observed for either direction of twisting; untwisting is most physiologically relevant since the DNA in most cells is underwound by $\sim -5\%$; however, positive supercoiling is also of physiological importance, see below). For chromatin, it is known that positive supercoiling (overtwisting, or positive excess linkage) favors the release of histones 2A and 2B, while histones H3 and H4 are retained (Jackson et al., 1994): implications as to how nucleosomes respond to the passage of RNA polymerase have been drawn from that study (since DNA is overtwisted ahead of the polymerase, this effect will destabilize upstream nucleosomes). Given these two experimental results, it appears practical and of both physical and biological interest to ascertain the effect of supercoiling of chromatin fiber, specifically the force and rate at which the histones dissociate.

If the experiments suggested above prove possible, many directions for investigation will be opened up. As mentioned above, the effect of H1/H5 linker histones on chromatin could be quantitatively studied by force-distance measurements. We have retained only a single energy parameter in Fig. 1 *a* and 2, and have thus neglected the elasticity of the DNA-histone contacts (entry/exit angles) as modulated by H1/H5. Chromatin length compaction and flexibility, and kinetic properties, could all change with linker histones, necessitating additional parameters. Also, sliding/positioning might be qualitatively different in the two cases.

The bulk of this paper has focused on chromatin fiber, but it must be kept in mind that other DNA-binding proteins may be profitably studied in stretching experiments. A basic candidate is lac repressor, which can bind two distant sequences, forming a DNA loop (Finzi and Gelles, 1995). Since a large amount of length can be stored in this loop, the transition force could be very low. One can imagine fabricating a DNA with a series of lac-repressor binding sites, and then forming a series of loops which could then be broken by an applied force. A speculative biological question is whether this kind of force-sensitive DNA looping is ever used in gene regulation.

An even more intriguing possibility is the study of non-histone chromosome packaging proteins, such as the XCAP proteins that are essential for maintaining metaphase chromosome structure (Hirano and Mitchison, 1994; Hirano, 1995). Since the apparent function of chromosome packaging proteins is to decrease the length of chromatin, the kind of force-distance study described in this paper is clearly pertinent.

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