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Solution studies of the interactions between the histone core proteins and DNA using fluorescence spectroscopy

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Abstract

The equilibrium interactions between histone H2A-H2B and H3/H4 subunits with 200 base pair chicken erythrocyte DNA have been studied by monitoring the fluorescence polarization of a long-lived fluorescence probe covalently bound to the histone subunits. These studies have brought to light the formation of highly asymmetric complexes exhibiting very high histone/DNA stoichiometries as well as very high apparent affinities. The stoichiometries observed for these non-nucleosome complexes depended both upon the concentration of the histones and the concentration of the DNA 200mer. The observed stoichiometries varied approximately between 4 and 16 histone octamers/DNA 200mer and the affinities were in the nanomolar range. These results are discussed in terms of their *in vitro* as well as their possible *in vivo* significance.

Keywords: Fluorescence spectroscopy; Histone core proteins; Histone–DNA complexes

1. Introduction

The free energies of interaction between histone core proteins and DNA undoubtedly play a major role in determining the state of chromatin throughout the eukaryotic cell cycle. For a general review of chromatin structure and function see the text by Van Holde [1]. It has long been known, however, that nucleosomes do not form with high yield spontaneously upon mixing the core proteins with DNA. Generally, a salt dialysis method in the presence or absence of urea is employed for reconstitution of nucleosomes, (e.g [2–8]). Alternately, a variety of polyanionic macromolecules have been used to induce slow reassociation into nucleosomes of core octamer and DNA (e.g. [9–16]). In addition, care must be taken to work at appropriate protein to DNA ratios and to keep the absolute concentration of the protein relatively low [13]. All of these procedures and precautions must be taken apparently because of reactions which compete with the nucleosome formation reaction. The appearance of non-native nucleosomes or nucleo-protein particles carrying more than the complement of one

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Abbreviations: DNS—5-(dimethylamino)-naphthalene-1-sulfonyl; bp—base pair; DNA 200mer—200 base pair double stranded DNA isolated from nuclease digestion of chicken erythrocyte chromatin.

octamer per 145 base pair oligonucleotide has been observed [13,17]. In general, however, the nature of these competing reactions and the affinities involved have remained relatively obscure. Rather than look upon these interactions as a nuisance to be either eliminated or ignored, we have chosen to examine the solution interactions of the histones with DNA under a variety of conditions with the underlying hypothesis that the competition between the multiple binding modes of histones to DNA may intervene in the changes in chromatin structure during different stages of the cell cycle. Protein associations between histone octamers may assist in keeping chromatin relatively condensed during replication. Since nucleosome assembly is thought to involve initial binding of H3/H4 molecules followed by the H2A-H2B dimers to form native nucleosomes [18–20] we also investigated the differential effects between the DNA binding to core histones and its binding to H3/H4 alone.

Recently we reported results concerning the interactions between the protein subunits of the histone core octamer obtained from fluorescence polarization measurements of histone core proteins labeled with the fluorescent dye, DNS (5-(dimethylamino)-naphthalene-1-sulfonyl) [21,22]. The fluorophore polarization, p, is related to its rotational correlation time, τ_c, of the fluorophore through the Perrin equation [23]:

$$1/p - 1/3 = (1/p_0 - 1/3)(1 + \tau/\tau_c) \qquad (1)$$

where τ is the fluorescence lifetime and p_0 is the limiting polarization. Since the correlation time (τ_c) is proportional to the frictional rotational coefficient, one can use fluorescence polarization measurements to observe changes in the size or shape of macromoleculer complexes, since smaller, spherical complexes have lower frictional coefficients than do larger, or asymmetric particles. In those studies we found that H3/H4 tetramers aggregate to form octamers and perhaps higher order aggregates, and that this aggregation was enhanced by increasing salt concentration. Addition of H2A-H2B dimers to H3/H4 in stoichiometric amounts resulted in fluorescence polarization changes consistent with the formation of core octamer. In this present work, we use changes in the fluorescence polarization of the labeled core proteins to study their binding to 200 base pair DNA oligonucleotides obtained from nuclease digestion of chicken erythrocyte chromatin.

2. Materials and methods

2.1 Purification of histones

Histones were purified from erythrocytes isolated from whole chicken blood as previously described [21,24]. Briefly, chromatin was isolated by centrifugation, treated with micrococcal nuclease and applied to a hydroxylapatite column. A salt gradient was then used to elute either pure core histones or to obtain H3/H4 and H2A-H2B purified core subunits. The histones were then dialyzed into 100 m*M* Tris, 100 m*M* KCl, pH 7.1 and stored at 0°C. Purity was confirmed by electrophoresis in a 15% SDS polyacrylamide gel at 25 mA for 5 hours. Proteins were visualized with Coomasie Blue. Tris UltraPure was obtained from ICN Biochemicals (Costa Mesa, CA). All other salts and buffers used in fluorescence measurements were checked and found to be free of fluorescence impurities.

2.2 Purification of 200 bp DNA

DNA bound to the hydroxylapatite column used to purify the histones (see above) was eluted with 0.5 *M* KH_2PO_4, pH 6.7. The DNA was ethanol precipitated, washed with 70% ethanol, dried, and resuspended in a small volume of 100 m*M* Tris, 100 m*M* KCl, pH 7.1. The DNA was then electrophoresed on a 2% agarose gel at 50 V for 5 hours. The band corresponding to 200 bp was excised and the DNA liberated from the gel using the Gene Clean kit (BIO 101, La Jolla, CA). Purity of the 200 bp DNA was confirmed by electrophoresis on a 2% agarose gel.

2.3 DNS labeling of histones

The histones to be labeled were first dialyzed against a 100-fold excess volume of 0.24 *M* potas-

sium phosphate, 5% dextrose, pH 8.0 for 3 hours. The dialyzed histones were then incubated with a ten-fold molar excess of 0.02 *M* dansyl chloride from a stock solution in dimethylformamide for ten minutes at room temperature. The protein–dye solution was then loaded onto a Sephadex G-25 column (1.5 cm i.d. × 15 cm) equilibrated with 100 m*M* Tris, 100 m*M* KCl, pH 7.1 and eluted at 1.0 ml/min. The labeled protein, detected with a handheld UV lamp, eluted in the void volume while the free dansyl chloride was retained. The labeled protein was centrifuged in a microfuge for ten minutes to remove any precipitate. The labeling ratio was determined the method of Jameson [25] where 4500 cm^{-1} M^{-1} was used a.s the molar extinction coefficient of the dye at 340 nm and 8000 cm^{-1} M^{-1} and 20000 cm^{-1} M^{-1} were used as the molar extinction coefficient of the dimer and tetramer respectively at 275 nm. The above labeling conditions resulted in labeling ratios of approximately 1 DNS per H2A-H2B dimer and 1 DNS per H3/H4 tetramer.

2.4 Fluorescence polarization measurements

Fluorescence polarization values of the DNS protein conjugates were measured on a Greg PC (ISS, Inc., Champaign, IL). All measurements were performed at room temperature (21°C). DNA titration experiments were carried out by pipeting the appropriate amount of stock DNA (96 μM) into the cuvette containing the histone solution. This solution was then incubated at room temperature for 5 min before measurements were made. Salt titrations were similarly performed. Excitation was 340 nm with a 16 nm bandpass in excitation. Polarization of the emitted light was measured in the L-format through a cuton filter (L42 from Hoya Optics, Inc., Freemont, CA) followed by an emission monochromator with a 16 or 8 nm bandpass set at 500 nm in order to exclude any scattered exciting light.

2.5 Time-resolved fluorescence measurements

Lifetime and differential phase measurements of the DNS–protein conjugates were carried out using a multifrequency phase fluorometer similar to that described by Gratton and Limkeman [26]. The excitation source, the 325 nm line from a continuous wave helium cadmium laser (Liconix, Sunnyvale, CA), which was modulated using the double acousto-optic modulation setup described by Piston and co-workers [27]. Emission was observed through an L-42 cuton filter. Multifrequency phase and modulation data were measured relative to 1,4-bis(5-phenyl-2-oxazoyl)benzene, POPOP, (Eastman Kodak, Syracuse, NY) with a lifetime of 1.32 ns [28]. The standard phase and modulation errors were $\pm 0.2°$ and ± 0.004, respectively. The lifetime measurements were carried out using magic-angle excitation. These data were analyzed in single curve mode using the program, Globals Unlimited (Laboratory for Fluorescence Dynamics, University of Illinois, Urbana, IL). A limiting polarization of 0.4 was used for the DNS label [29].

2.6 Gel electrophoresis of H3 / H4–DNA complexes

Solutions of 5 μM H3/H4 with 0.5, 5, or 50 μM 200 bp DNA were electrophoresed in a 12% native acrylamide gel with a Tris/Borate/EDTA running buffer at 65 V for 12 hours. The complexes were visualized by staining with either ethidium bromide, which exclusively stains the DNA, or silver stain, which will visualize both DNA and protein.

2.7 Nuclease protection assay

Solutions of 5 μM H3/H4 with 0.5, 5 or 50 μM 200 bp DNA in 100 m*M* Tris, 100 m*M* KCl, 1 m*M* $CaCl_2$, pH 7.1 were digested with micrococcal nuclease (104 U/μmol DNA) for 4 minutes at 37°C. The reaction was stopped by the addition of EDTA (ten-fold excess to the Ca^{2+} in the buffer). Histones were removed from the DNA by first increasing the salt concentration to 2 *M* then performing a phenol–chloroform extraction. The DNA was then ethanol precipitated, dried and resuspended in 100 m*M* TRIS 100 m*M* KCl, pH7.1. Next, 2 μg of DNA from each sample was electrophoresed in a 2% agarose gel at 50 V for 5 hours and visualized by staining

with ethidium bromide. Solutions of 5 μM in histone octamer of partially purified chromatin was similarly treated as a control for the protection assay.

3. Results

3.1 Titrations of labeled core octamer with DNA 200mer

Titrations H2A-H2B-DNS mixed with unlabeled H3/H4 in 2 to 1 molar ratios with 200mer DNA were carried out and the steady-state fluorescence polarization was monitored. These studies, shown in Fig. 1(a), were carried out in 0.2 M NaCl and at pH 7.0 because in our previous fluorescence studies we demonstrated that at protein concentrations of 1 μM and below, core octamer is in equilibrium with free dimers and tetramers, H3/H4 octamers and H2A and H2B monomers [21,22]. The existence of oligomerization equilibria is borne out by the increase in the H2A-H2B-DNS polarization value observed in Fig. 1(a) for increasing core octamer concentrations in absence of any DNA. As can be seen in Fig. 1(a), the polarization titration profiles are quite complex. At protein concentrations between 0.1 and 0.4 μM in total core octamer, the polarization increases immediately upon addition of DNA, reaches a peak value and then decreases to a plateau value. The peak polarization value of each curve increases with increasing protein concentration. In addition, the apparent binding affinity is also highly protein concentration dependent.

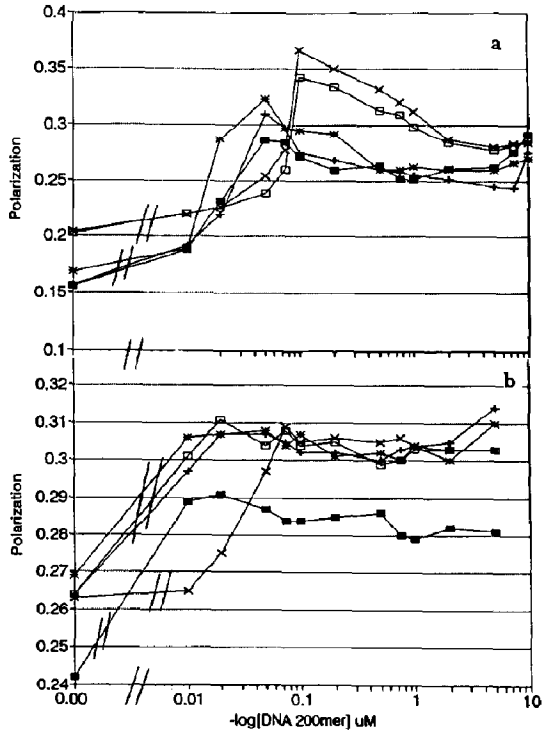

Fig. 1. (a) Titration of core histones containing H2A-H2B-DNS with 200 base pair chicken erythrocyte DNA. DNS polarization vs. [DNA 200mer] was measured for five protein concentrations: (■) 0.1 μM core, (+) 0.2 μM core, (*) 0.4 μM core, (□) 0.6 μM core, and (×) 1.0 μM core. (b) Titration of histones containing H3/H4-DNS with 200 base pair chicken erythrocyte DNA. DNS polarization vs. [DNA 200mer] was measured for five protein concentrations: (■) 0.05 μM H3/H4 octamer, (+) 0.1 μM H3/H4 octamer, (*) 0.2 μM H3/H4 octamer, (□) 0.3 μM H3/H4 octamer, and (×) 0.5 μM H3/H4 octamer.

We have estimated the complex stoichiometries assuming that at the peak value all of the protein is bound to DNA. This assumption is supported by three observations. First and most importantly, the actual values of the polarization measured at the peaks is quite close to the limiting value for the probe in a glass (0.4). These unusually high polarization values are certainly also indicative of a highly asymmetric complex as one would expect from the observed stoichiometries. The presence of even a small amount of free protein would cause the polarization values to be much lower. In fact, in the absence of any significant changes in the lifetimes (which we demonstrate below) and assuming no local mobility of the probe, the maximum fraction of free protein calculated from the observed and limiting polarization values is 7%. Second, the curve shifts abruptly upon increasing the protein concentration from 0.4 to 1.0 μM in core. Such a protein concentration dependent shift indicates that all of the available DNA is bound by protein and that beyond a certain protein concentration (or below a certain DNA concentration), the histones

bind as a higher order oligomer. Third, the fact that these curves reach a peak and then decrease indicates that as the DNA concentration increases all the protein molecules are bound up and as more DNA 200mer becomes available (beyond the peak) the proteins partition themselves among the excess DNA molecules, causing a decrease in polarization beyond the peak value. It is important to note that at the peak polarization value, the octamer-to-DNA ratio is different for each titration curve, indicating that larger complexes are formed in presence of increasing amounts of protein. However, the concentration of DNA at which the peak is observed is approximately constant for the first three protein concentrations and then the last two. In interpreting the forms of these titration profiles, we must bear in mind that the observed polarization profiles are the result of an increase in polarization as protein binds to the added DNA, followed by (and overlapping) a decrease in polarization as enough DNA becomes available for competition against the higher order complexes. The peak value is the point at which the decrease in polarization due to the formation of smaller complexes becomes the most important factor. Over the small range of protein concentrations in the first three curves, the polarization at the peak is higher for the higher protein concentrations, but the competition from the added DNA appears to occur at the same DNA concentration, because in all cases, it is the DNA and not the protein which is the limiting factor for formation of the smaller complexes. The curves for the last two protein concentrations are shifted abruptly to higher DNA concentrations. This means that more DNA is necessary to bind the protein. The only reasonable explanantion for this would be that the protein at these concentrations exists as a higher order oligomeric species and that the DNA must compete with protein for binding. (Note the increase in the polarization for the protein alone at these concentrations.) In addition, since the observable is polarization, at the lower DNA concentrations, there may be a small shift in species from oligomeric protein to protein bound to DNA and this without any large change in polarization. Clearly, these titrations demonstrate that there is quite a complex interplay between protein and protein–DNA species which is a very sharp function of the concentrations of the components.

3.2 Titration of labeled H3 / H4 with 200mer DNA

Since the binding of H3/H4 to DNA is thought to be the first step in nucleosome formation [18,19], the same titration experiment was carried out using the labeled histone tetramers, referred to as H3/H4-DNS. We had shown previously the tendency of these tetramers to form higher order aggregates in solution (most likely predominantly octamers). This aggregation is both protein and salt concentration dependent. Under the conditions of these titrations, (200 mM NaCl, pH 7.0) the solution at all H3/H4 concentrations should be mostly tetrameric, although in equilibrium with both dimers and higher order aggregates [21,22]. The H3/H4-DNS concentrations will be expressed here in terms of octamer to facilitate comparison with the core octamer results.

As can be seen in Fig. 1(b), the addition of 200mer DNA results in an increase in the fluorescence polarization of the labeled H3/H4. Again, a peak value is reached at relatively low 200mer concentrations, followed by a small decrease. This peak effect is not as dramatic as in the case of the core octamer with labeled H2A-H2B, but it is nonetheless observed. This indicates that the free DNA is not as able to compete away the H3/H4 complexes as the entire core complexes to form protein–DNA species of lower stoichiometries. Thus, the relative affinity of the H3/H4 higher order complexes is higher than that of formed with all four core proteins. Alternately, the affinity of the lower stoichiometric species in the case of the full core complement may be greater than for the H3/H4 proteins alone. The peak polarization value is also a function of the protein concentration. At 0.05 μM H3/H4-DNS octamer, the peak as well as the plateau polarization value, is much lower than at the higher protein concentrations. As in the case of the core octamer, the curve at 0.5 μM H3/H4 octamer (0.6 μM for core) is shifted to an approximately five-fold higher 200mer concentration. The apparent H3/H4 octamer to DNA ra-

tios at the peak also vary from 4 to 10 octamers bound per 200mer.

When carrying out binding studies on systems in which both the approximate affinities as well as the degree of cooperativity are unknown, it is important to explore the widest ranges of concentration possible in order to determine whether the observed binding phenomena is occurring under equilibrium or stoichiometric conditions. We therefore extended the titrations below 10 nM in 200mer using 0.05 μM H3/H4-DNS This titration, along with a control titration at 0.5 μM in H3/H4-DNS octamer, can be found in Fig. 2. It can be seen that the entire curve for the 0.05 μM H3/H4 octamer solution is shifted to lower concentration by approximately 1.3 log units. A shift of one log unit is generally considered to be indicative of binding in the stoichiometric, rather than equilibrium limit, although these titration curves are certainly much more complex than those which give rise to simple stoichiometric binding profiles. Referring to the plots in Fig. 2, it can be seen that the midpoint of the titration curve for the 0.5 μM H3/H4-DNS octamer occurs at 0.03 μM 200mer. This means that 0.03 μM DNA apparently binds 50% of a 0.5 μM solution of H3/H4-DNS octamer, or 0.25 μM octamer. Thus there are apparently on average, approximately 10 octamers of H3/H4-DNS bound per DNA 200mer. However, at the peak polarization value for this curve, the concentration of 200mer is 0.1 μM and the apparent octamer to 200mer ratio is only 5. We can therefore conclude that as in the case of the core proteins, the apparent stoichiometry of the H3/H4-DNS/DNA complexes changes along the titration curve.

3.3 Titration of the end complex with NaCl

Salt titrations of 6 concentrations of H3/H4-DNS octamer and 10 μM DNA 200mer were performed to verify that dissociation does indeed occur. As can be seen from the plots in Fig. 3, a decrease in polarization is observed upon increasing the concentration of NaCl, indicating that the protein–DNA complex is destabilized by high salt, as is the case also for native nucleosomes. The values plateau at relatively high polarizations, however, because the proteins themselves form large aggregates with very high affinity as the salt concentration is increased [21,22]. A decrease in the polarization for the two lowest protein concentrations with decreasing salt concentration to 0.05 M NaCl is indicative of a destabilization of the complex most likely due to repulsion between the positively charged protein subunits. We point out here that there is no effect of chloride ions on the lifetime of the DNS residue *per se*. However, there is a small increase in the lifetimes of the DNS labeled proteins as a function of salt. This is due to the aggregation of the proteins as a function of salt [21].

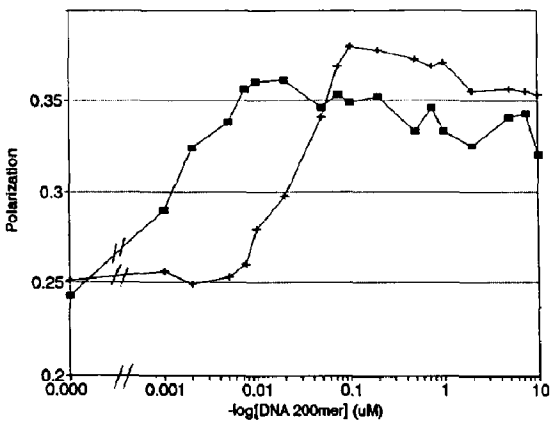

Fig. 2. DNS polarization H3/H4-DNS binding to 200mer DNA extended to 1 nM 200mer DNA for (■) 0.05 and (+) 0.5 μM in H3/H4–DNS octamer.

3.4 Time-resolved anisotropy of the labeled histone complexes with DNA 200mer

Lifetime and differential phase and modulation measurements were carried out on the solutions of 1.0 μM core protein solution containing H2A-H2B-DNS and unlabeled H3/H4 in molar ratios of 2 H2A-H2B dimers to one H3/H4 tetramer in presence of 0, 0.1 μM and 10 μM 200mer DNA. These time-resolved anisotropy experiments serve to differentiate the local motions of the fluorophore from the overall tumbling of the complex, both of which contribute to the steady-state polarization. The frequency response of the intensity and differential polarization can

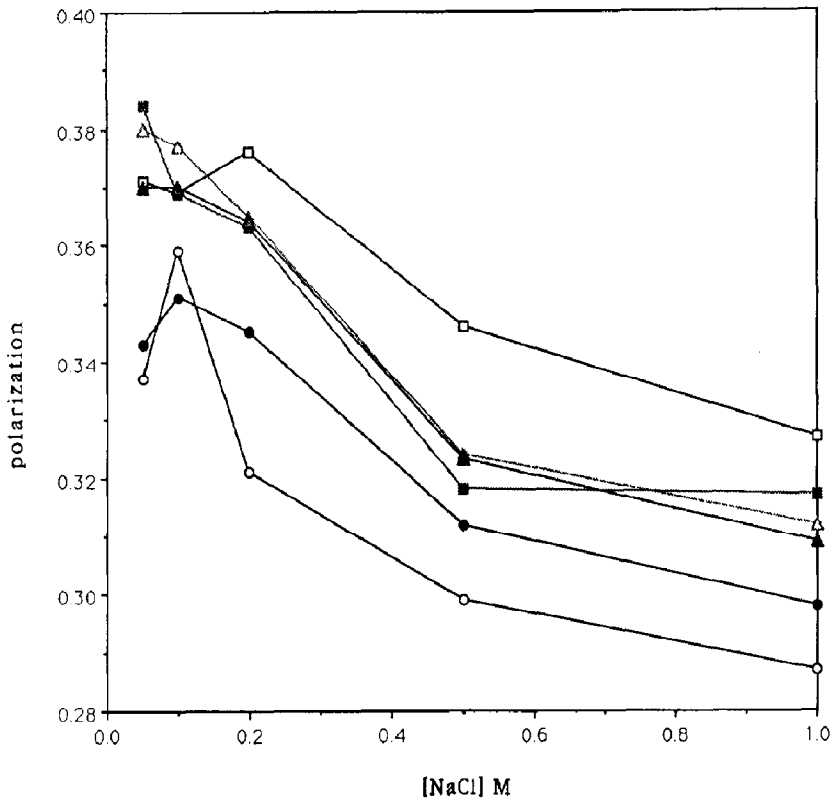

Fig. 3. Titration with NaCl of the complexes obtained in presence of varying H3/H4–DNS concentrations and 10 μM 200mer DNA. (○) 0.05 μM H3/H4 octamer, (●) 0.1 μM H3/H4 octamer, (□) 0.2 μM H3/H4 octamer, (■) 0.3 μM H3/H4 octamer, (▲) 0.4 μM H3/H4 octamer, (△) 0.5 μM H3/H4 octamer.

be found in Figs. 4(a)–(f). All samples exhibited similar lifetimes with average values of 10.3, 11.6 and 10.5 for the solutions with 0.0, 0.1 and 1.0 μM DNA 200mer, respectively. The absence of any significant change in the lifetime confirms that the observed polarization differences do not result from any changes in lifetime. We analyzed the DNS multi-frequency data terms of a triple exponential decay with lifetime values of approximately 14 ns, 2–4 ns and a short component whose value depended upon the solution. These lifetime values are quite consistent with those obtained for DNS labeled histones in our previous studies [21].

Using the recovered lifetime values as fixed quantities,the differential polarization data were fit, with best fits obtained for non-associative models with three rotational rates. In a non-associative model, we assume that there is no particular association of the decays with the rotational rates, and that all lifetime components are linked to all rotational rates. For both the core and H3/H4 interactions with the 200mer DNA, the complexity of the equilibria is such that absolute assignments of correlation times to molecular species is obviously not feasible. The fast rotational component has been observed for every conjugate of DNS with protein and is taken to correspond to the fast local motions of the DNS. The long one is seen to be indicative of large complex formation. The middle correlation time is the most difficult to interpret. As has been shown for the lac repressor intrinsic decay and rotational rates by site-directed mutagenesis [30],

the recovered values correspond to some linear weighted combination of the real values. Thus for a system as complex as the present one, this middle correlation time likely arises from some combination of free protein dimers and monomers and the grouping the rotations of the larger complexes into a single correlation time.

The values recovered from the fits can be found in Table 1. In absence of any DNA, the long correlation time was 71 ns. This is slightly too high for an octamer of 120,000 Da molecular weight. One would expect a correlation time for the octamer ranging from 47 to 61 ns depending upon whether one assumes the degree of hydra-

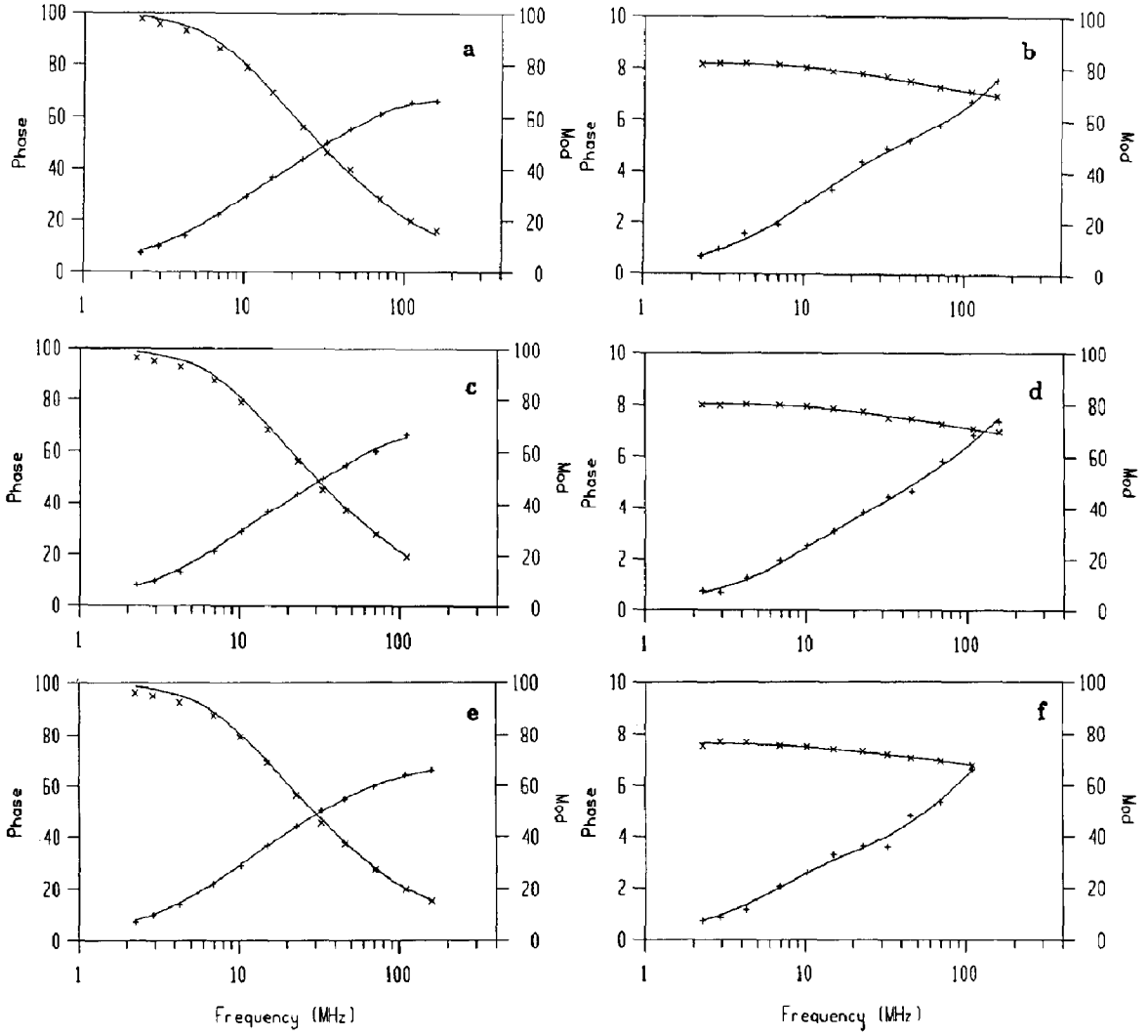

Fig. 4. Time-resolved fluorescence of H2A-H2B-DNS and H3/H4 in a 2:1 molar ratio, (a) and (b) the frequency response of the fluorescence intensity and differential polarization respectively for (a) and (b), of the protein solution alone at a 1 μM total core protein concentration; (c) and (d) the frequency response of the fluorescence intensity and differential polarization respectively for (c) and (d), of the protein solution at a 1 μM total core protein concentration and in presence of 0.1 μM 200 base pair DNA; (e) and (f) the frequency response of the fluorescence intensity and differential polarization respectively for (d) and (e), of the protein solution at a 1 μM total core protein concentration and in presence of 10.0 μM 200 base pair DNA.

Table 1

Time resolved fluorescence results of H2A-H2B-DNS + H3/H4 ± 0.1 or 10 μM DNA 200mer

Solution	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{c1} (f_1) [a] (ns)	τ_{c2} (f_2) [a] (ns)	τ_{c3} (f_3) [a] (ns)	Chi^2
1.0 μM core	13.5	3.7	0.2	71 (0.13)	5.2 (0.23)	0.14 (0.64)	0.5
1.0 μM core + 0.1 μM 200mer	13.5	3.7	0.2	66 (0.19)	3.9 (0.18)	0.14 (0.63)	0.7
1.0 μM core + 10 μM 200mer	13.5	3.9	0.6	45 (0.32)	3.7 (0.07)	0.3 (0.61)	0.9

[a] f_i values correspond to fractional depolarization weighting factors.

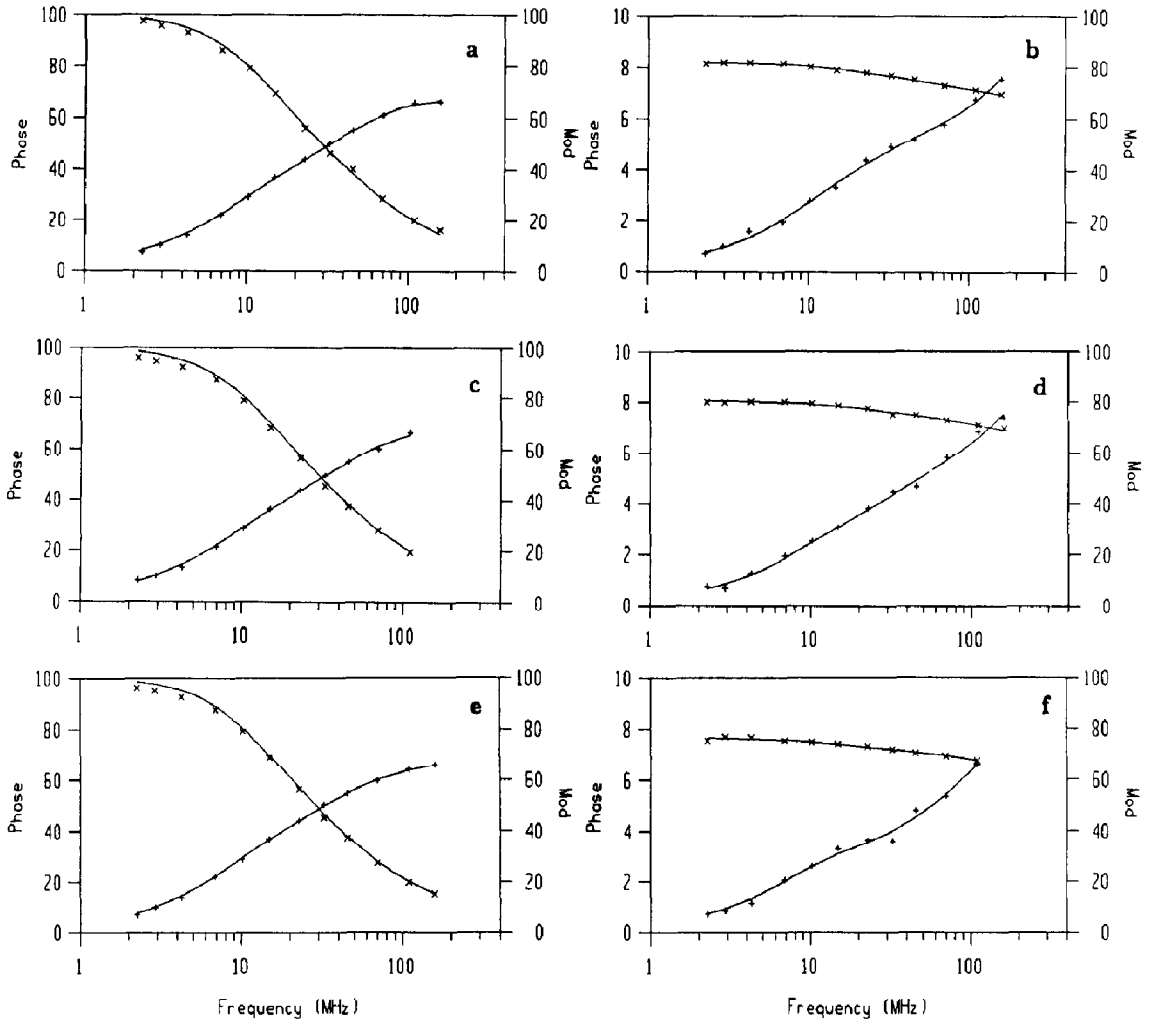

Fig. 5. Time-resolved fluorescence of H3/H4-DNS (a), and (b) the frequency response of the fluorescence intensity and differential polarization respectively for (a) and (b), of the protein solution alone at a 0.5 uM total H3/H4-DNS octamer concentration; (c) and (d) the frequency response of the fluorescence intensity and differential polarization respectively for (c) and (d), of the protein solution at a 0.5 μM total H3/H4-DNS octamer concentration and in presence of 0.1 μM 200 base pair DNA; (e) and (f) the frequency response of the fluorescence intensity and differential polarization respectively for (e) and (f), of the protein solution at a 0.5 μM total H3/H4-DNS octamer concentration and in presence of 10.0 μM 200 base pair DNA.

tion to be 0.3 or 0.6 g H_2O per g protein. This higher value for the long correlation time indicates either a certain degree of asymmetry and perhaps some higher order aggregation of the histones themselves. This is not inconsistent with our previous studies on the histone aggregation characteristics. The long correlation time, however, only accounts for 13% of the total depolarization, the rest being due to local DNS motions and the tumbling of smaller aggregates. In presence of 0.1 μM DNA, according to the plot in Fig. 1(a), the peak polarization value has been reached. The value of the long rotational correlation time recovered from the analysis of the data in Fig. 4(c) remains essentially unchanged near 66 ns, although its fractional contribution to the total depolarization increased to approximately 19%, causing an increase in the steady-state polarization. In presence of 10 μM DNA, the long correlation time recovered from the data in Fig. 4(e) decreases to 46 ns, whereas the fractional contribution to the total depolarization increases to 32%. This decrease in the rotational correlation time is consistent with the decrease observed for the steady-state fluorescence polarization and is indicative of the partitioning of the histone molecules onto the excess DNA.

The time-resolved intensity and anisotropy was also examined for the H3/H4-DNS solutions in absence and in presence of 200mer DNA and can be found in Figs. 5(a)–(f) for the three solutions (0.5 μM H3/H4–DNS octamer alone, in presence of 0.1 μM 200mer DNA and in presence of 10 μM 200mer DNA). Again the changes in the lifetimes were small and the values recovered from the best fit to a triple exponential decay can be found in Table 2. The consistently longer value for the longest and middle lifetime components for the H3/H4–DNS compared to the H2A-H2B–DNS was also observed in our earlier studies of histone protein interactions [21] and presumably represents a more solvent shielded DNS environment in the samples. The differential phase/modulation plots showed large changes upon addition of DNA. These data were analyzed in terms of three rotational rates which can be found in Table 2. Again the long rotational correlation time in absence of any DNA (75 ns) was too long for an H3/H4 octamer and is indicative of asymmetry and/or an equilibrium including higher order aggregates. This is consistent with our previous studies of histone aggregation [21,22]. In the H3/H4-DNS solutions, a much larger fractional depolarization stems from this long correlation time, 43%, compared to 13% for the core with H2A-H2B–DNS and is consistent with the higher steady-state polarization values observed for the solutions of H3/H4–DNS alone. In presence of 0.1 μM DNA, the data are much more difficult to fit and there appears an infinitely long correlation time. The fraction of the depolarization due to this component increases to 50%, again at the expense of the middle component and results in the observed large increase in steady-state polarization. Such an infinitely long correlation time (relative to the scale of the fluorescence lifetime) indicates the existence of very large (and probably elongated) particles. Upon

Table 2

Time-resolved fluorescence results of H3/H4–DNS ± 0.1 or 10 μM DNA 200 mer. Comparison between experimentally found and calculated time constants

Solution	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{c1} (f_1) [a] (ns)	τ_{c2} (f_2) [a] (ns)	τ_{c3} (f_3) [a] (ns)	Chi^2
1.0 μM octamer	21	9.5	2.1	75 (0.43)	3.7 (0.18)	0.15 (0.39)	0.7
1.0 μM octamer + 0.1 μM 200mer	21	9.5	2.3	∞ (0.5)	5.4 (0.12)	0.18 (0.38)	4.4
1.0 μM octamer + 10 μM 200mer	22	8.4	1.3	190 (0.53)	11 (0.1)	0.22 (0.37)	0.6

[a] f_i values correspond to fractional depolarization weighting factors.

further addition of up to 10 μM 200mer, the long correlation time drops slightly to 190 ns, consistent with the slight decrease in steady-state polarization and partitioning of the molecules onto the excess DNA yielding smaller particles. Under these conditions, 52% of the total depolarization is due to the long correlation time and the fast rotational correlation time remains relatively unperturbed by DNA binding.

3.5 Gel electrophoresis characterization of the complexes

Since fluorescence polarization experiments indicated that large elongated complexes were formed between histone H3/H4 proteins and 200 base pair DNA, we subjected the complexes to non-denaturing polyacrylamide gel electrophoresis in order to gain further information concerning their size and shape. Solutions of 0.5 μM H3/H4 octamer and 0.1, 1 and 10 μM 200mer were electrophoresed in a 12% non-denaturing polyacrylamide gel and stained with ethidium bromide. A photograph of the gel is shown in Fig. 6. By comparison with the standards in lane 1, it can be seen that the free DNA which is present at a ten-fold molar excess of DNA to protein (lane 4), migrates in accordance with its molecular weight, however, no additional band is de-

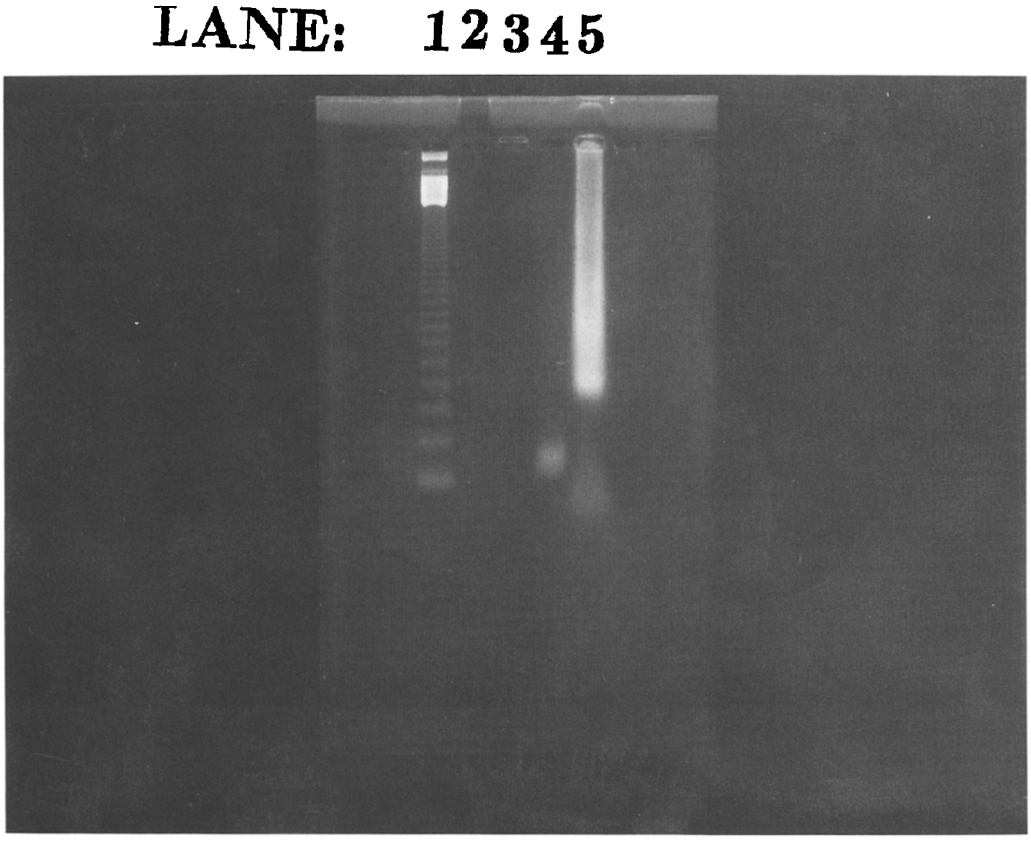

Fig. 6. Gel electrophoresis of the complexes formed under varying conditions between histones H3/H4 and 200 base pair DNA. Lane 1 shows the migration of standard DNA 123 base pair ladder. Lane 5 represents the migration of native mono and multi-nucleosomes isolated from partial micrococcal nuclease digestion of chicken erythrocyte chromatin. The reconstituted complexes of H3/H4 (at 5 μM in tetramer) with 0.1, 1.0 and 10.0 μM DNA 200mer were loaded in lanes 2–4, respectively. The gel was 2% in agarose.

tected. Moreover, when the protein is present at 10:1 or 1:1 molar ratios of protein to DNA, there is no band observed in the lane. Silver staining of these gels did not result in the detection of protein in the lanes, and only a small amount was observed in the wells. This is in accordance with our conclusion that there is no free DNA in these solutions. Apparently the complex does not enter the gel, either because it is too large and asymmetric or because the large number of positively charged histones bound to the DNA actually reverses the sign of the overall charge of the complex. It can be seen that in this gel system, nucleosomes isolated from partial nuclease digestion of chicken erythrocyte chromatin do enter the gel. The bright spot at the bottom of the smear is taken to be a mononucleosome, whereas the smear itself is ascribed to the existence of di-, tri- and higher order nucleosomes in the digestion mixture. Some free DNA is also observed in this lane.

3.6 Micrococcal nuclease protection assays

The free DNA, native core nucleosomes, and the complexes of 0.5 μM H3/H4 with 0.1, 1 and

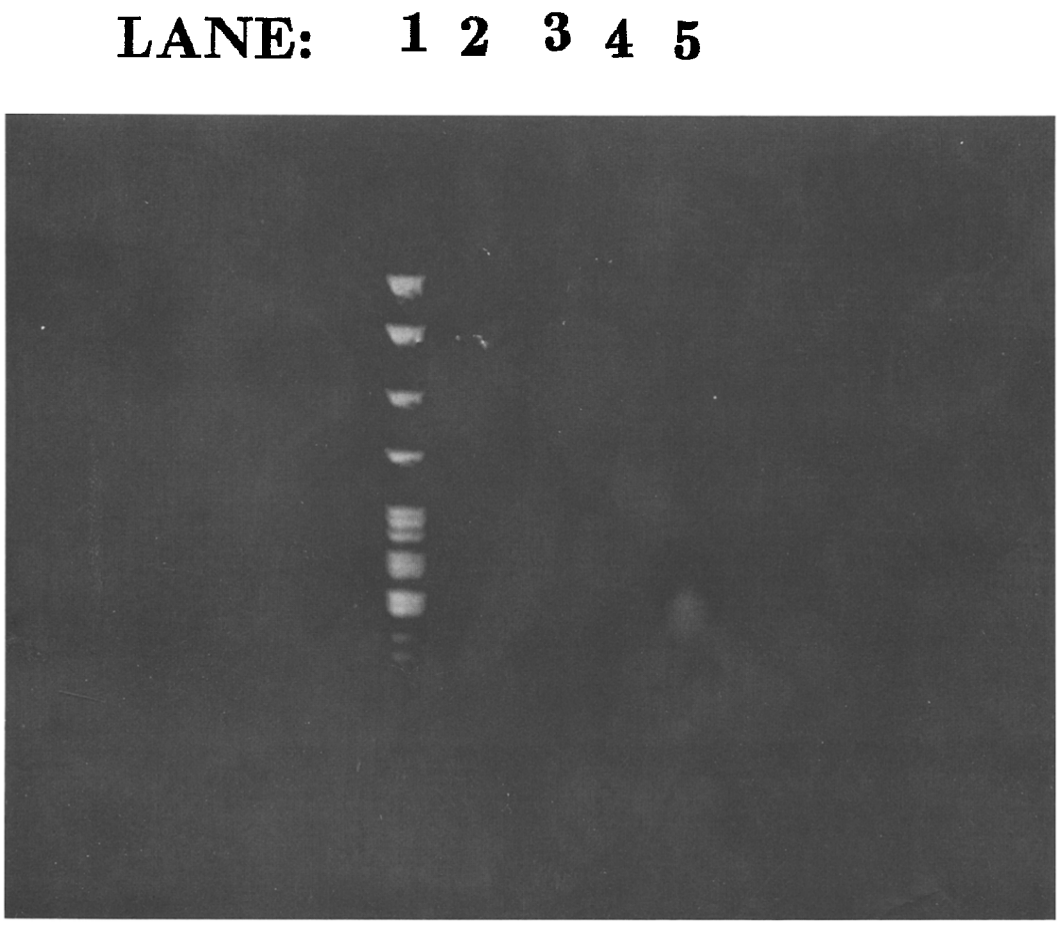

Fig. 7. Gel Electrophoresis of the DNA recovered from micrococcal nuclease digestion of the complexes of the histones with the 200mer DNA. Lane 1—Msp I standards, lane 2—2.5 μM H3/H4 octamer in presence of 0.5 μM DNA 200mer, lane 3—2.5 μM H3/H4 octamer in presence of 5 μM DNA 200mer, lane 4—2.5 μM H3/H4 octamer in presence of 50 μM DNA 200mer, and lane 5—nucleosomal DNA migrating at ~145 bp.

10 μM DNA were subjected to digestion by micrococcal nuclease. The DNA was purified from the protein in the reaction mixtures by phenol extraction and the products were run on an agarose gel and stained with ethidium bromide. A photograph of the gel can be seen in Fig. 7. In this gel a distinct band was observed for native nucleosomes due to the protection of the DNA in the nucleosome structure by the histone proteins (lane 5). The untreated DNA in absence of any protein also appears at the appropriate molecular weight (lane 2). However, no protected band was observed for any of the reconstituted complexes of H3/H4 with DNA.

4. Discussion

Fluorescence polarization studies have brought to light the complexity of the binding of histone core proteins to DNA. The strong protein concentration dependence of the DNA binding profiles demonstrates that there are multiple modes of interaction of the histone core proteins with DNA. Although we cannot determine using this methodology the stoichiometries of the various species in solution under any given conditions, it is clear that their relative populations are highly dependent upon both the protein and the DNA concentrations. The most striking result presented here is the high apparent affinity of these proteins for the DNA to form species which clearly do not correspond to the core particle. Despite the complexities of the stoichiometries of binding, the simple fact that association between the core proteins and the DNA is observed at 0.05 μM H3/H4 in an apparently stoichiometric, rather than equilibrium mode attests to the fact that apparently, the affinity is higher than $2\ 10^7$ M^{-1}. The equilibria between protein subunits and protein and DNA are obviously highly coupled and quite complex. The species formed under these equilibrium conditions, at least in the case of the H3/H4 complexes, quite clearly do not resemble nucleosomes. Interestingly, these high affinity complexes show no protection of the DNA from nuclease, leading to the conclusion that while the affinity of the complexes is high, nuclease can compete effectively for binding. We have also made the observation that the stoichiometry of the complex changes along the titration curves, whether one moves along the DNA concentration or the histone concentration axis. We interpret this observation as follows. At low concentrations of DNA, as many histones as possible bind to the piece of DNA, giving rise to a very high stoichiometry, elongated complex, consistent with the long rotational correlation times observed under these conditions. As more DNA is added, the proteins partition themselves onto the excess DNA forming complexes of lower stoichiometry. Both the steady-state polarization and the rotational correlation times of these particles decrease compared to the values found at low DNA concentrations. For both the total core proteins and the H3/H4 proteins, the stoichiometries were also highly dependent upon the concentration of the protein. This is due to the fact that the proteins themselves form aggregates of differing sizes and composition [21,22,33], and that these interactions are coupled to DNA binding.

Above 0.5 μM core or H3/H4 octamer concentrations it takes a ten-fold greater DNA concentration to obtain binding and the complexes formed are apparently larger in size, indicating that there is an upper limit to the number of protein molecules which can be accommodated by the 200mer and also that different oligomeric forms of the protein are binding to the DNA at different protein concentrations.

DNA fibers smoothly coated by the histones have been observed using electron microscopy [31]. The binding of more than one complement of histone octamer to chromatin core particles was observed by Voodrow and Eisenberg [17]. Oohara and Wada [32] reported the non-cooperative saturation of DNA by H2A-H2B with stoichiometries corresponding to 7 dimers per 200 mer. Daban and Cantor [20] demonstrated using excimer formation that when H2A-H2B subunits were added immediately after H3/H4 molecules mixed with DNA, core particles were formed, whereas a dead-end non-nucleosome complex was formed when the H3/H4 molecules alone were present or when the H2A-H2B was added at long

times after the initial mixing. Sperling and Wachtel [33] have described polymers or fibers of histones which are helical in structure. Such a helical fiber could conceivably wrap around a DNA double helix. These authors reported 12 dimers of histones (or 3 octamers) per 330 Å repeat. Since a 200 bp oligonucleotide is approximately 720 Å length, this would allow 26 dimers or 4 octamers to bind. Such a stoichiometry is similar to the apparent stoichiometries which we report for the peak polarization values. However, some of the stoichiometries which we have observed at earlier points on the titration curves are even higher than this,indicating that in these cases, the complex must include additional protein–protein interactions.

Given these rather interesting observations on the equilibrium interactions of histones with DNA in solution, one is led to ask what eventual *in vivo* significance these interactions may hold. Probably the most obvious is that the free energies involved in these complexes are high in absolute value. This is why the reconstitution of nucleosomes *in vitro* requires high salt dialysis or polyanions to be successful. Certainly, these significant driving forces are present *in vivo* as well, and imply that the cell has found its own mechanism for competing against them. Furthermore, one may ask whether these high affinity competing binding interactions constitute for the cell, as for the investigator, a nuisance which requires a large amount of energy to overcome, or whether they actually serve some purpose. Recently a number of conflicting reports have appeared in the literature pertaining to the fate of nucleosomes during transcription. Losa and Brown [35] demonstrated that histones remained bound to transcribed the 5S RNA gene. Lorch and coworkers [34,36] using nucleosomes reconstituted onto either bacterial or adenoviral sequences and ligated to an SP6 promoter showed by gel electrophoresis that histones were displaced during transcription, while the histones on the 5S RNA gene were not. They concluded that the 5S RNA gene was an exception and that the general mechanism of transcription involves the displacement of histones. An interesting set of observations using two crosslinking reagents, one for lysines and one for histidines, was reported by a group of Soviet investigators [38,39]. Using a crosslinking reagent which chemically crosslinks the histidines found in the central core of the histone core octamer to DNA, Karpov and coworkers [38] found that crosslinking was significantly reduced in transcriptionally active genes. This led them to first conclude that histones were displaced during transcription. However, further studies [39] using the lysine reactive crosslinking reagent demonstrated that although the central core of the octamer does not remain bound to the DNA during transcription, efficient crosslinking through the lysine residues in the flexible C- and N-terminal domains of the proteins was observed for transcriptionally active chromatin. They suggested that a modified histone–DNA complex may form during transcription which allows the passage of the transcription machinery via a tails-mediated transient release of the DNA. Bonne-Andrea and coworkers [37] report that using a T4 *in vitro* replication system, nucleosomes remain bound to the artificial template during replication, and concluded that nucleosomes open up transiently to allow the passage of the replication fork.

Our results on the equilibrium interactions of histone core proteins with DNA demonstrate that there are multiple modes of binding other than that found in the nucleosome and that these are both energetically significant as well as coupled to the state of aggregation of the histones. Histone protein–protein interactions may aid in preserving the correct subunit ratios, local concentrations and relatively condensed chromatin state during replication and transcription. They could also play a role in the efficient binding of newly synthesized histones onto DNA, and in the formation of higher order chromatin structures. A number of factors including the salt concentration,the protein concentration, the relative ratio of H2A-H2B subunits to the H3/H4 subunits, the pH and the presence of other proteins and effector molecules can all work to influence these coupled protein–protein and protein–DNA equilibria. The fact that the complexes which we have observed *in vitro*, while relatively high in affinity, allow the passage of micrococcal nuclease sug-

gests that similar interactions could also allow the passage of the replication and transcription machinery *in vivo*. The regulation of the competition between the multiple binding modes between the histones and DNA and the histones themselves may in fact be the basis for changes in chromatin structure during different phases of the cell cycle.

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References

1 K.E. van Holde, Chromatin (Springer-Verlag, New York, NY, 1986).
2 P. Oudet, M. Gross-Bellaed and P. Chambon, Cell 4 (1975) 281–300.
3 J.E. Germond, B. Hirt, P. Oudet, M. Gross-Bellard and P. Chambon, Proc. Natl. Acad. Sci., USA 22 (1975) 1843–1847.
4 R.D. Camerini-Otero, B. Sonner-Webb and G. Felsenfeld, Cell 82 (1976) 333–347.
5 K. Tatchell and K.E. van Holde, Biochemistry 16 (1977) 5295–5303.
6 N. Ramsay, G. Felsenfeld, B. Rushton and J.D. McChee, EMBO J. 3 (1984) 2605–2611.
7 H.R. Drew and A.A. Travers, J. Mol. Biol. 186 (1985) 773–790.
8 W. Linxweiler and W. Horz, Nucl. Acids Res. 12 (1984) 9395–9413.
9 R.A. Laskey, B.M. Honda, A.D. Mills and J.T. Finch, Nature 275 (1978) 416–420.
10 A. Stein, J.P. Whitlock and M. Binou, Proc. Natl. Acad. Sci., USA 76 (1979) 5000–5004.
11 R.T. Simpson and R.W. Stafford, Proc. Natl. Acad. Sci., USA 80 (1983) 51–55.
12 W.C. Earnshaw, B.M. Honda and R.A. Laskey, Cell 21 (1980) 373–383.
13 S. Pennings, S. Muyldermans and L. Wyns, Biochemistry 25 (1986) 5043–5051.
14 J.D. Retif, B.T. Sewel, H.J. Greyling, S. Schwager and C. van Holt, FEBS Lett. 167 (1984) 170–175.
15 I. Oohara, A. Suyawa and A. Wada, Biochim. Biophys. Acta 741 (1983) 322–332.
16 J. Germond, J. Roviere-Yaniv, M. Yaniv and D. Brutlag, Proc. Natl. Acad. Sci., USA 76 (1979) 3779–3783.
17 G. Voodrow and H. Eisenberg, Nature 273 (1978) 446–448.
18 A. Worcel, S. Han and M.L. Wong, Cell 15 (1978) 969–977.
19 V. Jackson and R. Chafldey, Cell 23 (1981) 121–134.
20 J.-R. Daban and C.R. Cantor, J. Mol. Biol. 156 (1982) 771–789.
21 C.A. Royer, T. Ropp and S.F. Scarlata, Biochemistry 28 (1989) 6631–6637.
22 S.F. Scarlata, R. Rusch and C.A. Royer, Biochemistry 28 (1989) 6637–6641.
23 F. Perrin, J. Phys. Radium 1 (1926) 390–401.
24 R.H. Simon and G. Felsenfeld, Nucl. Acids Res. 6 (1979) 689–696.
25 D.M. Jameson, Ph.D. Dissertation, University of Illinois, Urbana, IL, 1978.
26 E. Gratton and M. Limkeman, Biophys. J. 44 (1983) 315–323.
27 D.W. Piston, G. Marriott, T. Radiveyovitch, R.M. Clegg, T.M. Jovin and E. Gratton, Rev. Sci. Instrum. 60 (1989) 2596–2600.
28 E. Gratton, M. Limkeman, J. Lakowicz, B.P. Maliwal, H. Cherek and G. Laczko, Biophys. J. 46 (1984) 479–486.
29 G. Weber, Biochem. J. 259 (1951) 155–164.
30 C.A. Royer, J.A. Gardner, J.M. Beechem, J.-C. Brochon and K.S Matthews, Biophys. J. 58 (1990) 363–378.
31 G. Moyne, F. Harper, S. Saragosti, S. and M. Yaniv, Cell 30 (1982) 123–130.
32 I. Oohara and A. Wada, J. Mol. Biol. 196 (1987) 389–397.
33 R. Sperling and E.J. Wachtel, Adv. Prot. Chem. 34 (1981) 1–61.
34 Y. Lorch, J.W. LaPointe and R.D. Komberg, Cell 42 (1987) 203–210.
35 R. Losa and D.D. Brown, Cell 50 (1987) 801–808.
36 Y. Lorch, J.W. LaPointe and R.D. Komberg, Cell 55 (1988) 743–744.
37 C. Bonne-Andrea, M.L. Wong and B.M. Alberts, Nature 343 (1990) 719–726.
38 V.L. Karpov, O.V. Preobrazhenskaya and A.D. Mirzabekov, Cell 36 (1984) 423–431.
39 G.A. Nacheva, D.Y. Guschin, O.V. Preobrazhens, V.L. Karpov, K.-K. Ebralidse and A.D. Mirzabekov, Cell 58 (1989) 27–36.