Analysis of the Competition between Nucleosome Formation and Transcription Factor Binding*

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We have studied the competitive binding of histones and the Rous sarcoma virus internal enhancer binding factor (IBF) factor (which recent studies indicate is almost certainly cEBPβ). We find that histones and IBF are incapable of forming a ternary complex with a 159-base pair (bp) fragment of DNA containing a single IBF binding site and that histones and factor are mutually exclusive in binding. We have analyzed the various physical parameters of binding, in an attempt to understand how the factor might establish an exclusive binding site in the cell. The stability of the nucleosome and the factor-DNA complex have been determined, and in addition a minimum value for the affinity of the histone octamer has been computed. We found that in simple competition the IBF can successfully compete, only if the substrate DNA is shorter than 140 bp. The relevance of these results is discussed in terms of a kinetic model for successful factor competition during the replication of the factor binding site in the cell.

Many experiments have been done to show that nucleosomes are able to block formation of transcription complexes. Using either RNA polymerase II or III transcription systems, full assembly of nucleosomes on the transcriptional template invariably results in the loss of transcriptional competence of that template, presumably because nucleosomes block access of transcription initiation factors to the promoter (1–7). However, the mechanism of chromatin assembly in vivo is almost certainly different from that used in the model systems described above.

There is, both in vivo and in vitro, evidence that both nucleosomes and trans-acting factors are at least transiently displaced by the passage of a replication fork during DNA replication (8–12). Although it is not known if histones and factors are totally displaced from the DNA, replication significantly randomizes nucleosome structure and also can allow for significant remodeling of the factors binding to DNA. Therefore, after passage of a replication fork, both histones and factors must be available to establish their contacts with newly replicated DNA. In general, newly replicated DNA is efficiently packaged into nucleosomes. However, the mechanism of chromatin assembly must permit an exclusive association of promoters and enhancers with the factors that bind them. This mechanism must be all the more proficient when one considers that several cis-acting elements for each of thousands of essential genes must be occupied by factor (with no failures) in order to maintain cell viability. The desired result could potentially be achieved if trans-acting factors were able to displace pre-established nucleosomes from their cognate sites. Although this strategy is used in a small subset of inducible genes (13–15), and indeed a mechanism to this end can be envisaged (16), the extent to which the cell uses the displacement of nucleosomes is unknown, and it may not be a general property of trans-acting factors. Furthermore, nucleosomes are presumed to bind DNA with fairly high affinity; therefore, it is likely that the cell would employ a chromatin assembly mechanism that would avoid having to remove nucleosomes to expose all the factor binding sites.

To address these issues, we have attempted to reconstitute this competition between histones and trans-acting factors in a simple model system using internal enhancer binding factor (IBF). Although there are several cloned DNA-binding proteins that could have been used for these studies, there is a concern that proteins produced in *Escherichia coli* lack certain post-translational modifications that might affect binding properties. Therefore, we chose to use a DNA-binding protein which was originally purified from baby hamster kidney (BHK) cells. IBF is a cEBPβ-related protein that binds a site within an enhancer that lies internal to the *gag* gene of the Rous sarcoma virus. The enhancer activates transcription in vivo when linked to a reporter gene. Furthermore, purified IBF stimulates in vitro transcription of promoters that contain the enhancer sequence (17, 18).

Based on the characteristics of well studied trans-acting factors, it is apparent that they differ significantly in their thermodynamic stability and in their ability to bind a nucleosomal site (19, 20). Therefore, we have characterized thermodynamic and kinetic properties of both histone and IBF complexes with DNA. Most transcriptional regulatory proteins characterized thus far bind their cognate sites with an association constant in the range of 10^9 to 10^12 M^-1 (21–31). This would seem to account for their ability to bind their cognate sites even though they are present at relatively low concentrations. However, eukaryotic trans-acting factors must also compete with nucleosomes for binding to specific sites. Therefore, it is relevant to ask whether simple competition for a binding domain is sufficient to allow for a high success rate in excluding histones. There have been surprisingly few attempts to quantitate the affinity of histones to DNA at physiological ionic strength. Although affinities of individual purified histone subtypes for DNA have been determined (32), no absolute affinity of the histone H3/H4 tetramer or the complete histone octamer is reported in the literature.

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1 The abbreviations used are: IBF, internal enhancer binding factor; BHK, baby hamster kidney; bp, base pairs; FMSF, phenylmethylsulfonyl fluoride; β-ME, β-mercaptoethanol.
(33). Therefore, we have also attempted to assess the strength of binding of histones to DNA and to compare the equilibrium and kinetic constants with those of the enhancer-binding protein.

Despite the results of the in vitro transcription experiments mentioned above, certain DNA-binding proteins (glucocorticoid receptor and GAL4; see Refs. 19, 20, and 34) appear to be able to bind their cognate sites even if that site is already occupied by a nucleosome. Apparently, these proteins are able to establish their specific contacts with a DNA sequence as it is displayed on the surface of a nucleosome. In contrast, our results with IBF indicate that prebinding of histones to a 159-bp DNA fragment containing a factor binding site will exclude the factor, and that prebinding the factor will exclude the histones. Binding histones and factor at the same time generates a mixture of both possible complexes. However, if the length of DNA is shortened, the ability of histones to bind is severely compromised, allowing trans-acting factor binding to predominate.

MATERIALS AND METHODS

Radio labeling of DNA Fragments—The Hind III end of a 159-bp HindIII/Real fragment of pBS-PO1 containing a single binding site for IBF was radiolabeled on ice with [α-32P]dATP using DNA polymerase I in the restriction enzyme buffer. The plasmid consists of a single copy of the IBF site inserted into the EcoR I site of the Bluescript (17) plasmid from Stratagene (17). A 152-bp EcoRI-Real fragment containing the Xenopus borealis somatic 5 S RNA gene (35) was obtained from plasmid pXBS201, which was kindly supplied by William Taylor.

Protein Purification—Hyperacetylated histones were isolated as described (19, 36), except that the hydroxyapatite column was poured using a ratio of 1 mg of chromatin to 1 ml of bed volume. In addition, 0.2 mM PMSF and 2 mM benzamidine were added to all the buffers. Upon isolation of chromatin by EDTA lysis of nuclei, the chromatin was washed twice with the column buffer in order to wash out residual Nonidet P-40, 0.2 M NaCl, and 2.0 M NaC1. Pure histones H3/H4 was eluted in the 2.0 M wash.

Partially purified IBF was obtained by phenyl-Sepharose and hydroxyapatite chromatography of BHK extracts. The peak fractions of the hydroxyapatite column were heated as described (17), and the supernatant was utilized for the binding studies. Nucleoplasmin and N1/N2 were purified as described (37).

Nucleosome Reconstitution—When using poly(dl-dC)(+dl-dC), either nucleoplasmin or N1/N2 were prebound to purified histones (amounts indicated in figure legends) at room temperature for 10 min prior to mixing with the DNA. The buffer in this case was 15 mM Hepes, pH 8.0, 150 mM NaCl, 50 mM PEG, 0.5 µg/ml bovine serum albumin, 0.01% Nonidet P-40, 0.2 mM PMSF, and 7 mM β-ME.

DNase I Digestion of Reconstituted Nucleosomes—Nucleosomes were reconstituted (in the absence of poly(dl-dC)(+dl-dC)) using nucleoplasmin to limit precipitation. One ng of the radiolabeled fragment was mixed with 0.5 ml of histone (1/100 volume of 1% SDS, 0.6 M sodium acetate, and 25 µg/ml yeast tRNA. The complexes were digested for 1 and 2 min with 0.5 unit/ml DNase I (Worthington) at room temperature. Free DNA was digested for 15 and 30 s with 0.1 units/ml DNase I. Digestion was terminated by addition of an equal volume of 1% SDS, 0.6 M sodium acetate, and 25 µg/ml yeast tRNA. The DNA was then precipitated and run on a 5% sequencing gel.

Isolation and Radiolabeling of Native Mononucleosomes—Five confluent 75-cm flasks of 293 cells (a human embryonic kidney cell line transformed by adenovirus type 5) (44) were harvested and washed free of serum. The cells were then washed two more times in buffer J (lacking Triton) and resuspended in 2 ml of buffer J with 1 mM CaCl2 and 50 mM NaCl. 200 units of micrococcal nuclease (Worthington) was added, and the sample was placed at 37°C for 15 min. Digestion was terminated by adding sample to 1 ml EDTA and 2 mM EGTA and placing on ice. Samples were spun at 16,000 × g for 10 min at 4°C. Supernatant containing released mononucleosomes was diluted to 10 ml with 70 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM EGTA, and 10 mM dithiothreitol. [γ-32P]ATP (150 µC; 6000 Ci/mmole) and 5 units of T4 polynucleotide kinase were added for 15 min at 37°C.

Determination of Kinetic and Affinity Constants—Histone binding at low dilution (without carrier DNA; Fig. 4) was performed by mixing pure histones and the radiolabeled 159-bp fragment in 10 mM Tris, pH 7.5, 125 mM NaCl, 6% glycerol, 250 µg/ml bovine serum albumin, 7 mM β-ME, 1 mM MgCl2, 5 mM CaCl2, 1 mM benzamidine, 0.25 mM EDTA, and 0.2 mM PMSF. Approximately 5 ng of pure histone H3 and H4 was bound to a range of concentrations of the 159-bp fragment for 45 min at room temperature. This incubation time was empirically determined to be adequate for full binding under these conditions. These samples were then analyzed on a 4% acrylamide gel in TGE (Tris, glycine, EDTA) buffer (17); the gel was dried and then exposed to an x-ray film. The bound and free bands were cut from the dried gel, and the amount of radioactivity in each was determined. Alternatively, the gel was scanned to determine the bound and free portions of the total DNA concentration, and then Scatchard analysis of these data was used to obtain an affinity constant.

RESULTS

Reconstitution of Nucleosomes on a 159-bp DNA Fragment—In order to compare the stability of a single nucleosome with that of a factor-DNA complex, we attempted to reconstitute a nucleosome that could be analyzed on a mobility shift gel. In order to make this comparison, a sufficiently large DNA fragment has to be used because a nucleosome contacts approximately 140–150 bp of DNA (38). A fragment slightly larger than this range was selected to ensure that normal histone-DNA contacts could take place. A single copy of the binding site for the IBF was cloned into the middle of the Bluescript plasmid polylinker (17). From the resulting plasmid, a 159-bp restriction fragment containing this binding site in the middle was then prepared for subsequent experiments.

To reconstitute nucleosomes, histones were complexed with nucleoplasmin in order to prevent aggregation and to facilitate formation of nucleosomal structure. (Identical results were obtained with another assembly factor: N1/N2.) When nucleoplasmin and histones were combined with a radiolabeled 159-bp fragment and poly(dI-dC)(+dI-dC) (average length = 8000 bp), a discrete retarded complex was observed on a mobility shift gel (Fig. 1; lane 2). The mobility of this complex was compared with mononucleosomes prepared in several different ways. First, mononucleosomes obtained by micrococcal nuclease digestion of 293 cell chromatin were radiolabeled and electrophoresed on a mobility shift gel (lanes 3 and 4). When the native nucleosomes were denatured with SDS, the free DNA appeared to be approximately 150–160 bp (data not shown). The mobility of the native mononucleosomes was identical to that of the reconstituted histone complex. Second, another widely used assembly method is to mix DNA fragments with chromatin (mono- or oligonucleosomes), adjust to high salt (2 M NaCl) and then slowly dialyze (or dilute) to physiological ionic strength (39, 40). Again, this method resulted in generation of a complex with the same mobility as the reconstituted mononucleosomes (lanes 8–10). When a surplus of histones was added, some slower complexes were occasionally observed (lane 8). However, in contrast to the complex that ran as a mononucleosome, these extra complexes were not stable to the addition of more poly(dI-dC)(+dI-dC) (lanes 9 and 10; see below). In conclusion, the complex formed by mixing pure histones and an assembly factor with the 159-bp DNA fragment appears to be a bona fide nucleosome.

Another characteristic of nucleosomes is a periodic 10-bp spacing of DNase I cleavage sites that result from the DNA sequence being displayed on the surface of the histone octamer (35, 41). The 159-bp fragment was reconstituted as above with enough histones to completely bind the fragment. This complex was digested with DNase I, and the digestion products were then denatured and run on a sequencing gel. It should be kept
in mind that a 10-bp pattern is only observed by this method if the nucleosome is precisely phased relative to the DNA sequence. A 4–5-bp variation in nucleosome positioning will obscure any periodicity in the DNase I cleavage pattern. As can be seen in Fig. 2, DNase I digestion of the reconstituted nucleosome gave rise to a pattern substantially different than that of naked DNA. With some exceptions, protected DNase I hypersensitive sites (open circles) were spaced at approximately 10-bp intervals along the sequence. The pattern of DNase I-hypersensitive sites in free DNA that are protected in the reconstituted nucleosome relative to free DNA. Open circles denote DNase I sites in free DNA that are protected in the reconstituted nucleosome.

Comparison of the Stabilities of Nucleosomes with Factor Complexes—In vivo, nucleosomes remain bound to the DNA on which they are deposited until they are perturbed by transcription or replication (8, 9). In vitro, histones do not dissociate from isolated chromatin unless the ionic strength is raised substantially above physiological levels (42). As shown in Fig. 3 (upper panel), the reconstituted histone complexes exhibit the same sort of stability. If we add twice the normal amount of poly(dI-dC)·(dI-dC) as competitor DNA to the reaction prior to the addition of histones, the amount of radiolabeled complex is reduced substantially (lane C). On the other hand, if the additional portion of competitor DNA is added after the initial complex is formed, the amount of complex is not reduced significantly over the time course of the experiment (75 min). This indicates that the off-rate of the nucleosome formed in our assembly assay is very low.

To compare the binding characteristics with those of an enhancer binding factor, we performed the same experiment with the internal enhancer binding factor and the same DNA fragment. A single binding site for this factor lies in the middle of the 159-bp fragment used above. The half-life of the factor-DNA complex was determined by adding a plasmid containing the IBF binding site at specific time points (Fig. 3, lower panel). In this case, the stability of the factor-DNA complex was much lower than the nucleosomal complex with a half-life of 20 min. We repeated this experiment with a 20-fold range of competitor

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**Fig. 1.** Reconstitution of nucleosomal complexes. 0.5 ng of the labeled 159-bp fragment from pBSPO1 (lane 1) was combined with 100 ng of poly(dI·dC)·(dI·dC), 150 ng of hyperacetylated core histones, and 200 ng of egg nucleoplasmin (lane 2). Lanes 8–10 show similar assemblies with 50 ng of poly(dI·dC)·(dI·dC). In lanes 9 and 10, an additional 50 ng of poly(dI·dC)·(dI·dC) was added prior to loading the gel. Mono- 

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**Fig. 2.** DNase I digestion pattern of nucleosomal complexes. The radiolabeled 159-bp fragment was reconstituted with amounts of histone which completely bind the DNA (data not shown). The DNase I digestion pattern of this complex is shown compared to the DNase I pattern of naked DNA (N). DNA polymerase I was used to fill in the 5' overhang created by HinfI so that one strand was uniquely labeled. A Maxam-Gilbert G cleavage reaction of the fragment (G) was used to derive base numbers. Filled circles show DNase I sites that are hyper-sensitive in the reconstituted nucleosome relative to free DNA. Open circles denote DNase I sites in free DNA that are protected in the reconstituted nucleosome.
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Competitive Factor Binding

Concentration and found the off-rates to be essentially the same (data not shown). Therefore, the dissociation of the factor complex is not accelerated by an intrastrand transfer mechanism and is presumed to be unimolecular.

Comparison of Equilibrium Constants of Nucleosomes with Factor Complexes—Since the off-rates indicate that a nucleosome, once formed, is more stable than the IBF complex, we decided to compare the equilibrium binding constants of these complexes. The affinities of several enhancer binding factors for their cognate sites have been determined using Scatchard analysis, and the association constants typically range from $10^9$ to $10^{12}$ M$^{-1}$ (21-31). The affinity of IBF for its site has been determined to be $1.1 \times 10^8$ M$^{-1}$ (43). Although one can determine an affinity constant for the binding of core histones to DNA, it cannot be directly compared to the affinity of the enhancer binding factor to its site because it is not a bimolecular reaction; free histones at physiological ionic strength exist as H2A/H2B dimers and H3/H4 tetramers. Consequently, the binding reaction can be represented by Reaction 1.

$$2 \text{H2A/H2B dimers} + 1 \text{H3/H4 tetramer} \rightarrow 159\text{-bp DNA fragment} \leftrightarrow \text{nucleosome}$$

**Reaction 1**

Furthermore, nucleosome assembly at physiological ionic strength is generally performed in the presence of an assembly agent such as polyglutamate or nucleoplasm. This further complicates a thermodynamic analysis of histone binding to DNA.

Nonetheless, it is possible to obtain an affinity constant for the binding of the H3/H4 tetramer to DNA. H3/H4 tetramers form complexes with DNA that are very similar to nucleosomes with a full complement of histones (35). H3/H4 alone can supercoil DNA, as well as form a complex that is resistant to micrococcal nuclease (44-48). In addition, the H3/H4 tetramer is able to recognize phase signals in a DNA sequence and position itself in the same way as a complete histone octamer (35). There is evidence that the H3/H4 tetramers are deposited on newly replicated DNA and that H2A/H2B dimers then associate with this complex to complete the nucleosome (10, 44, 49). Thus, the affinity of H3/H4 tetramers for DNA may be legitimately regarded as a minimum estimate of the affinity of the histone octamer for DNA and almost certainly represents the stability of the initial step of chromatin assembly. We have found that addition of small amounts (1-10 ng) of purified H3/H4 (without any assembly agent) to the 159-bp fragment results in the formation of a complex which migrates slightly faster than the complex formed with all the core histones. Others have observed that the mobilities of the H3/H4 and nucleosomal complexes are quite similar (47, 48). Little aggregation is observed if the amount of complexed DNA is kept below 50% of the total. So a constant amount of purified histone H3/H4 was bound to a 20-fold range of DNA concentrations. As can be seen in a Scatchard analysis of the resulting data (Fig. 4), the affinity of the H3/H4 tetramer for this 159-bp fragment is approximately $1 \times 10^9$ M$^{-1}$.

Since the 159-bp fragment contains sequences that do not normally exist in nucleosomes, it is possible that this DNA sequence may not be representative. Therefore, we repeated the affinity determination using a fragment from the X. borealis 5 S RNA gene, which has been extensively used for nucleosome positioning. Using the same analysis, we obtained an affinity constant of $3.7 \pm 2.0 \times 10^9$ M$^{-1}$. Octamers of the histones H3 and H4 have been observed when reconstitution occurs at higher concentrations (and at higher histone to DNA ratios; Refs. 35 and 50-52). At our much lower concentrations, it is
more difficult to determine the histone composition and stoichiometry of the complex. In this case, however, where the free DNA concentration exceeds that of the histone complex, the histone concentration would seem to be limiting; consequently, only tetramers should be associated with the DNA (35).

Comparison between Histones and Factor for Binding to the Same Site—Since the H3/H4 tetramer and IBF are binding with comparable affinity to the same DNA fragment, it seems unlikely that simple competition would account for the exclusive association of IBF with its site in vivo. Nonetheless, we decided to test this directly in a competitive binding assay. The experiments described below utilize purified histones and nucleoplasmin but the factor itself is only partially purified. Consequently, poly(dI·dC)·(dI·dC) is used as a nonspecific competitor to bind contaminating proteins. The much higher concentrations of histone required to saturate the poly(dI·dC)·(dI·dC) would normally lead to precipitation of the DNA in the binding reaction. Therefore, nucleoplasmin is used to assemble nucleosomes at the same ionic strength at which factor binding occurs. This complex binding mixture can be described as shown in Reaction 2,

\[
n_p - \text{histones} + \text{DNA}_n + \text{factor} + \text{DNA}_n^* = c_{n_p} + c_{n_p} + nuc_n + nuc_n + n_p
\]

where \(c_{n_p} = \text{factor complexed to 159-bp fragment}, c_{n_p} = \text{factor complexed to poly(dI·dC)·(dI·dC)}, nuc_n = \text{histones complexed to 159-bp fragment}, nuc_n = \text{histones complexed to poly(dI·dC)·(dI·dC)}, \) and \(n_p = \text{nucleoplasmin}.

With the use of poly(dI·dC)·(dI·dC), the mixture more closely approximates the situation in vivo where the histone concentration greatly exceeds that of factor. Histones bind both poly(dI·dC)·(dI·dC) and the radiolabeled 159-bp fragment with roughly equal affinity, whereas most of the factor becomes associated with the fragment. Although it is not known if histones are complexed with nucleoplasmin in vivo, it is likely that they are transported and deposited while associated with one or more chaperone proteins. Therefore, the use of nucleoplasmin is also expected to mimic at least part of the nucleosome assembly process.

Under these conditions, we first established the kinetics of binding for both complexes (Fig. 5). Both binding events occur quite rapidly with most of the binding occurring within 1 min. This result indicates that the time course of the experiment (10 min of binding) allows complete binding of either species. Additionally, the data of Fig. 5 indicate that the on-rates of factor and histone binding in this experiment are roughly equivalent. The fast on-rate of histone binding is somewhat surprising for two reasons. First, the use of nucleoplasmin might have been expected to retard the rate at which histones bind DNA, but this appears not to be the case. Second, this result appears to contradict the previous observation that although the histone H3/H4 tetramer has a comparable affinity to that of IBF, a complete histone octamer has a much slower off-rate than IBF; an equivalent affinity requires that the rate constants for histone binding should be much smaller than that of factor because: \(K_{on} = k_{off}/k_{on}.\)

One possible explanation is that a full nucleosome is significantly more stable than the H3/H4 tetramer, which was used for the affinity determination. Although this may be the case, the source of the apparent contradiction is principally due to the presence of nonspecific competitor in the binding reaction. The concentrations of histones and histone binding sites are approximately 200-fold greater than factor and factor binding sites, which dramatically increases the rate of reaction. As a result, the rate of histone binding has become fortuitously equivalent to the rate of the factor binding. In absolute terms, though, the rate constant for histone binding (without nucleoplasmin mediation) is smaller than that of the factor. In other experiments, we have observed that the rate of H3/H4 tetramer binding (at the low dilutions used in Fig. 4) is indeed substantially slower than that of IBF (data not shown). It is expected that the excess of histones and histone binding sites more accurately reflect the situation in vivo than if histones and factor were used in equimolar concentrations.

The next experiment was designed to establish whether factor binding and histone binding are mutually exclusive. In particular, we were interested in determining whether the factor could bind to a nucleosome or even somehow dislodge an already established nucleosome. The mobility of the nucleosomal complex is distinct from that of factor associated to the same DNA fragment (containing a single specific binding factor). Therefore, it is possible to differentiate binding of either histones or factor even when both are present. In Fig. 6, IBF was allowed to establish binding before the addition of histones.
Fig. 7. A, the IBF complex is supershifted by anti-LAP (cEBPβ) antibodies. Labeled DNA containing the IBF binding domain was incubated for 20 min with a nuclear extract from BHK cells and the IBF shift visualized by a gel shift analysis. All lanes contained BHK nuclear extract to which the following additions were made: lane 1, no addition; lane 2, preimmune serum; lane 3, antibody against purified LAP (cEPBβ); lane 4, antibody against cEBPα. Lane 5 contained DNA alone. B, nucleosomes exclude factor binding in presence of a large excess of factor. For this experiment we utilized bacterially expressed LAP which is the major component of IBF (unpublished data). Labeled DNA (the 159-bp fragment used in Fig. 7A) was mixed with a 200-fold excess of chromatin (from H4 cells) and the ionic strength raised to 2.0 M, and then lowered to 0.25 M to assemble histones onto the labeled fragment in nucleosomal form was separated from chromosomal DNA by centrifugation (20,000 rpm for 20 min). Increasing amounts of LAP were added to the nucleosome over a 10,000-fold concentration range, including sufficient LAP to drive the labeled fragment into the fully complexed form, as well as an excess of LAP to cause the fragment to precipitate and be retained at the top of the gel.

(lanes 3 and 4), and then the order of addition was reversed (lanes 5 and 6). It appears that either type of binding (histone or factor) is able to form a complex that is stable to addition of the other. When both proteins are added simultaneously (lanes 9–12), both complexes are observed in almost equal proportions. A more slowly moving band that might indicate concomitant binding of both histone and factor does not appear. Since a histone octamer has twice the molecular weight and much more positive charge than IBF, charge and molecular weight are not the principal determinants of the magnitude of the mobility shift. Rather, the shape of the complex seems to be most important. IBF is known to exist as an asymmetric dimer and therefore the binding of this asymmetric molecule on the surface of a nucleosome most likely cause the ternary complex to migrate more slowly than either a nucleosome or IBF-DNA complex (43). Therefore, the factor by itself is able to form a complex that is resistant to histone binding. In addition, factor is not able to bind its site when it is complexed to histones. However, it is possible that a specific nucleosome phasing relative to the IBF binding site may be able to obscure critical IBF contact points while another phasing would allow IBF binding.

Considering the faster off-rate of IBF as compared to histones, one might expect that extended incubation of the radiolabeled DNA fragment with both histones and IBF would eventually result in most of the DNA fragment becoming nucleosomal. However, this does not occur because the concentration of free histones is rapidly reduced upon binding to poly(dI·dC·dI·dC) and then is not sufficiently high to form nucleosomes on free DNA resulting from IBF dissociation.

In the experiment shown in Fig. 6, there is a significant amount of free DNA left after binding of histones and factor. Therefore, it might be possible that IBF could still bind its site on the surface of a nucleosome, albeit with a reduced affinity. We have performed this same experiment with higher concentrations of partially purified IBF and histones and have seen
Fig. 8. Length dependence of histone binding. A 28-bp oligonucleotide (derived from the intergenic sequence of the copy II H3/H4 locus of Saccharomyces cerevisiae) was self-ligated and labeled to create different multimers of the same sequence. These multimers were individually purified and equimolar amounts were mixed with 5, 10, and 20 ng of purified core histones. The resulting complexes were resolved on a mobility shift gel (top panel). The lane on the far right shows the mixture of all six multimers employed in this experiment. The bottom panel shows a similar experiment to Fig. 6 using the 159-bp fragment described above and a truncated version (86 bp) of the same fragment. The IBF binding site lies approximately in the middle of the 86-bp fragment as well. Each set of 10 lanes corresponds to lanes 1–8, 11, and 12 of Fig. 6. Equivalent amounts of histone were used for both DNA fragments.

smeared complexes that run more slowly than either the IBF or histone complexes (data not shown). However, these smeared complexes are also observed when higher amounts of either histones or IBF alone are incubated with the radiolabeled DNA fragment. Furthermore, the smeared complexes are not stable if poly(dI-dC)-(dI-dC) is added just prior to loading the gel. Since both IBF and histone complexes are stable to the addition of nonspecific DNA, we conclude that these complexes at higher concentrations of IBF and histones do not represent stable ternary complexes (histones-IBF-DNA) but rather are due to nonspecific factor binding and/or association of histones with DNA in a non-nucleosomal fashion.

In order to address how severely the presence of a nucleosome reduces the factor’s affinity for its site, we decided to fully reconstitute mononucleosomes with the same fragment and then challenge these complexes with an excess of factor. In order to permit us to utilize a large excess, we have employed bacterially synthesized LAP (cEBPβ), because much of the IBF binding is due to this agent, as is seen in Fig. 7A in which we document the ability of anti-LAP antibody to supershift the majority of the IBF gel shifted complex. Accordingly, we reconstituted mononucleosomes by combining the radiolabeled fragment and H4 cell chromatin in 2.0 M NaCl and then slowly dialyzed the mixture to physiological ionic strength. The high molecular weight chromatin was then removed by centrifugation, and the fully reconstituted, labeled fragment remained in the supernatant. Upon the addition of LAP/IBF/cEBPβ to the nucleosomal complex (Fig. 7B), no ternary complex formation was observed even with a 10–100 fold excess above the level required to saturate naked DNA. Therefore, we conclude that the presence of a nucleosome efficiently excludes IBF (LAP) binding.

Although both factor and histones bind equally to the 159-bp fragment in our experiments, we decided to determine if the length of the DNA fragment could affect the results of this competition. During DNA replication of a cis-acting element, the element exists transiently within a length of free DNA that is shorter than 150 bp. On this basis, a model that explains the preferential association of trans-acting factors with their cognate sites has been proposed (53). An important aspect of this model is the decreased affinity of histone to DNA fragments as their length is reduced (39, 54). Former experiments have shown this relationship using mixed sequence DNA fragments produced by nuclease digestion, and therefore their results may have been due to a preferential affinity of histones for certain sequences. To further verify this observation, a 28-bp oligonucleotide was self-ligated to generate oligomers of various sizes. These oligomers were labeled, purified, and used for binding purified histones at low dilution as described above. As can be seen in Fig. 8 (top panel), the affinity of core histones for shorter pieces of DNA is indeed reduced. There appears to be a minimum length of DNA (85–113 bp) that is required for any histone binding in this assay. Above 113 bp, affinity increases with length (as seen by the amount of free DNA at the highest histone concentration).

Because of the reduced affinity of histones for smaller fragments, one would predict that the outcome of the competition experiment would be changed if the DNA fragment size is reduced. The mutual exclusion experiment was repeated with a smaller piece of DNA (80 bp) to see if factor binding would now prevail. Even though an 80-bp fragment cannot properly form a nucleosome, it remained formally possible that some sort of histone complex with the shorter fragment could form and inhibit factor binding. However, Fig. 8 (lower panel) shows that a similar amount of histone as used with the 159-bp fragment is not able to form a complex with the shorter fragment to the same degree. Furthermore, when histones and factor are added simultaneously, the only protein-DNA complexes formed are IBF complexes.

DISCUSSION

An important issue in the assembly of active chromatin is how a promoter is able to develop and maintain transcriptional competence. The promoters of active genes are occupied by several trans-acting factors and consequently appear to be substantially (and often permanently) nucleosome-free. The achievement of this state would be simple if 1) promoter-binding factors were able to perform their task on a nucleosomal template or 2) they were able to displace nucleosomes from their binding sites. The first mechanism may be theoretically possible but it appears that active promoters are generally devoid of regular nucleosomal structure in vivo. The second mechanism is used in certain genes that respond quickly to an inductive event. However, the nucleosomes to be removed are generally flanked by one or more cis-acting elements, which are not obscured by nucleosomes even in the non-induced state (with the possible exception of murine mammary tumor virus). A cooperative mechanism for nucleosome displacement by several factors (some of which are previously bound) is indicated by the fact that no factor binding to a single site has been observed to effect nucleosome displacement in vitro. If factors flanking a nucleosome are required for removal of that nucleosome, then the question remains as to how those primary fac-
tors are able to bind their sites. Although some trans-acting factors can bind their cognate sites on the surface of a nucleosome (19, 20, 34), the apparently reduced affinity of these complexes formed in vitro may make them physiologically insignificant. Therefore, it is imperative for at least some (if not most) trans-acting factors that they establish binding before nucleosomes are formed over their sites. Furthermore, the mechanism by which this occurs must ensure that each of the thousands of essential genes flawlessly recruit the requisite factors to its promoter in order to maintain cell viability.

We have attempted to address the question of how the properties of trans-acting factors allow them to bind their sites with maximal efficiency in vitro in the presence of an excess of histones. One surprising aspect of IBF binding is its relatively fast off-rate ($t_{1/2} = 20$ min). This does not seem to be a unique property of IBF, however, as the measured off-rate and equilibrium constant are similar to measured constants for many other DNA-binding proteins (55, 56). One advantage of a fast off-rate is that it allows the cell to maintain a more responsive control over the transcriptional activity generated by that factor. The cell may also maintain binding of enhancer-binding factors to their sites by keeping the free factor concentration sufficiently high so that if factor does diffuse away, another would rapidly replace it. It is expected that the rate of dissociation of factor complexes is dependent on the salt concentration. For this reason, factor complexes are much more stable once they are loaded on a mobility shift gel where the ionic strength is much lower (<10 mM).

In contrast to the IBF-DNA complex, the nucleosome formed in our assembly assay seems to be quite stable. This is not surprising, though, in view of both the substantial ionic and hydrophobic interactions that exist between histones and DNA (42). The affinity of histones for DNA indicates that a substantial thermodynamic barrier must be overcome when histones are removed from DNA. The equilibrium constant for H3/H4 tetramer binding has not been reported previously in the literature. Although some have attempted to determine relative affinity using salt dialysis to form nucleosomal complexes, the high ionic strengths used may not be relevant to the physiological situation (33, 57).

The affinity constant measured for the segment of 5 S DNA was slightly higher than that of the 159-bp fragment. Although the 5 S fragment is widely used to phase nucleosomes, this property does not necessarily imply that it also binds nucleosomes with an affinity that is higher than average. Both nucleosome phasing and binding affinity could be important regulatory factors in determining whether a trans-acting factor can bind to a specific site. In fact, Straka and Hörz (58) show that certain DNA fragments, when inserted into the PHOS promoter, can form very stable nucleosomes which hinder inducible activation of the promoter.

It was initially assumed that the association rate constant of histone binding (at low dilution) would be comparable to that of IBF. Most on-rates for trans-acting factors are only limited by diffusion and consequently are very fast (59). In our case, virtually all IBF binding occurs as fast as the gel can be loaded (Fig. 5). Although one might expect the absolute histone on-rate to be similar, nucleosome formation involves an added step of bending the DNA around the histone octamer (38, 60). Since the DNA helix is locally a fairly stiff structure, this presumably does not take place spontaneously. If this step is required to form a stable complex, the association rate constant of histone binding would indeed be slower than that of trans-acting factors.

The observation that an assembly agent is not required to form nucleosomes at low dilution is consistent with earlier experiments that showed nucleosome reconstitution could be achieved at physiological ionic strength by mixing histones and DNA very slowly (61). Apparently, if the free histone concentration is kept low (either by dilution or complexation with an assembly agent) then aggregation does not appreciably inhibit nucleosome formation. The affinity of histones for DNA may be modulated substantially in vivo since they are almost certainly complexed with an assembly agent. Since an abundance of free histones in the cell would probably have deleterious effects, histones are most likely complexed with a transport protein (nucleoplasmmin or N1/N2) that carries them into the nucleus. The data from Smith and Stillman (44) indicate that they are then deposited during replication by CAF-1. Therefore, to determine the thermodynamics of histone deposition in vivo, the affinities of histones for the various carrier proteins will have to be determined. Nonetheless, these interactions are not so strong that they prevent rapid deposition of histones onto DNA at the thousands of replication forks present in a replicating nucleus.

Although certain factors are able to bind their cognate site as it is displayed on the surface of a nucleosome, other trans-acting factors are not able to bind to a nucleosomal template, at least not in a manner that allows them to perform their function (7, 19, 20, 34). Our own results show that when either the factor or histones are prebound to DNA, the complex formed first is not altered when the other protein is added. This argues that IBF does not find its binding site by dissociating nucleosomes. Nor is it likely that IBF becomes bound to the DNA after the histones have spontaneously dissociated at a time when histone synthesis is minimal, as the nucleosome dissociation rate (at least as measured in vitro) is extremely low. Finally, a single site cannot be complexed with both IBF and a histone octamer simultaneously.

**Competitive Factor Binding**

**Extent of Relative Factor/Histone Binding as Determined by Competitive Equilibria**—In trying to understand the mechanism of chromatin assembly in eukaryotic cells, one must take into account the affinities with which histones and trans-acting factors bind to DNA. Histones are thought to bind DNA nonspecifically. In other words, all DNA sequences bind histones with equal affinity. Although this is not strictly true (because certain sequences are known to bind and phase nucleosomes better than others; see Refs. 33, 39, and 57), it is probably safe to assume that most DNA sequences in the eukaryotic genome probably bind histones with affinities that vary no more than an order of magnitude. In contrast, specific DNA-binding proteins bind particular sequences very indiscriminately. The ratio of affinity of specific sites to affinity of nonspecific DNA typically ranges from 4 to 6 orders of magnitude (55). This would seem to account, at least in part, for the exclusive association of promoter regions with trans-acting factors. However, it is obvious that if genomic DNA were saturated with nucleosomes that are quite stable, then no amount of selectivity could allow binding of specific DNA-binding proteins unless they could bind their sites on a nucleosomal surface.

The overall chromatin assembly process can be represented as:

\[
D_n + D_n + H + P \leftrightarrow D_n P + D_n P + D_n H + D_n H
\]

**Reaction 3**

where $D_n$ = specific DNA binding site, $D_n$ = nonspecific DNA, $H$ = histone octamer, and $P$ = specific DNA-binding protein.

This can be broken down into four competing equilibrium reactions, as shown in Equations 1–4.

\[ D_n + P \leftrightarrow D_n P \quad K_c = \frac{(D_n P)}{(D_n)(P)} \] (Eq. 1)

**References**
histones, nonspecific DNA, etc.). Nonetheless, performing such variable is discussed in more detail below). Therefore, it seems ing factors in the nuclear environment. Our calculations sug-
centration of nonspecific DNA in the nucleus (21, 30, 55).

Let $S = \text{ratio of factor's affinity for specific sites to affinity for nonspecific sites.}$ (The assumption is that there are only two classes of sites, nonspecific and specific, each possessing a characteristic affinity for the DNA-binding protein, $K_2$ and $K_1$, respectively.) See Ref. 55 for more information regarding Equation 8.

$$S = \frac{K_1}{K_2}$$

(Eq. 7)

$$\left(\frac{D_P}{D_P^*}\right) = \left(\frac{D_P^*}{D_P}\right)S$$

(Eq. 8)

Dividing Equation 8 by Equation 6 yields Equation 9.

$$\frac{D_P}{D_H} = \frac{D_P^*}{D_H}\left(\frac{D_P}{D_P^*}\right)$$

(Eq. 9)

This simple equation illustrates many of the parameters that can affect factor binding in the eukaryotic nucleus. If the cell requires a trans-acting protein ($P$) to bind a specific site ($D_P$), then it must elevate the $D_P/D_H$ ratio. Using the measured affinity constant of the H3/H4 tetramer and an average affinity constant of a trans-acting factor for its cognate site, it is possible to make rough calculations regarding the thermodynamics of competitive binding in vivo. Obviously, the greatest source of error in these calculations is the estimation of concentrations of the critical components (i.e. trans-acting factors, histones, nonspecific DNA, etc.). Nonetheless, performing such calculations is still valuable because it helps identify the crucial variables that determine the binding success of trans-acting factors in the nuclear environment. Our calculations suggest that enhancer-binding factors cannot establish a totally exclusive interaction with their DNA-binding sites in simple competition with a free pool of undeposited nucleosomal histones. This conclusion is derived from the relatively high concentration of free histones that bind replicating DNA with high affinity, the significant affinity of trans-acting factors for nonspecific DNA, and the great excess of nonspecific sites over specific sites in the nucleus (the magnitude of this important variable is discussed in more detail below). Therefore, it seems unavoidable that either a factor would have to establish itself at its site before nucleosome deposition occurs or there must be some mechanism to displace histone octamers from DNA.

A critical assumption in the calculations described above is the amount of free pool of factor in the nucleus. This, in turn, depends heavily on the concentration of nonspecific DNA that can compete for factor binding. The amount of internucleosomal DNA in the eukaryotic genome is often used to calculate the concentration of nonspecific DNA in the nucleus (21, 30, 55). This is a reasonable assumption because naked genomic DNA is a much better competitor for IBF binding (10-100-fold) than isolated chromatin (data not shown). Even if one conser-

competitive binding in the eukaryotic nucleus. If the cell may compete for factor binding. The amount of internucleosomal DNA in the nucleus (21, 30, 55).

$$D_+ + P \leftrightarrow D_+^*$$

$$K_+ = \frac{(D_+^*)}{(D_+)(P)}$$

(Eq. 2)

$$D_+ + H \leftrightarrow D_+^*$$

$$K_+ = \frac{(D_+^*)}{(D_+)(H)}$$

(Eq. 3)

$$D_+ + H \leftrightarrow D_+^*$$

$$K_+ = \frac{(D_+^*)}{(D_+)(H)}$$

(Eq. 4)

We will assume that histones bind DNA nonspecifically. Therefore,

$$K_+ = K_+$$

(Eq. 5)

$$\frac{(D_+)(H)}{(D_+^*)(H)} = \frac{(D_+^*)(H)}{(D_+)(H)}$$

(Eq. 6)

The Kinetic Window Model for Exclusive Factor Binding—The relative affinities of the histone octamer and IBF would be expected to be very important in determining which will occupy the binding site. However, since these affinities are roughly equal, then other considerations must account for the exclusive association of promoters with transcription factors. At least part of the answer may have to do with the reduced affinity of histones for shorter pieces of DNA (39, 54). This could afford trans-acting factors an advantage during the S phase of the cell cycle when DNA is replicated. As a newly replicated cis-acting element emerges from the replication complex, it must transiently exist as part of a length of naked DNA that is less than 150 bp (at least on the leading strand, the exact conformation of DNA exiting the lagging strand polymerase is unknown). Therefore, the kinetic window model appears to offer a reasonable explanation for the absolute association of factors with their sites to the exclusion of nucleosomes and is consistent with the physicochemical properties of the proteins involved. However, more information on the mechanism of nucleosome deposition during DNA replication will be required in order to ascertain how much time elapses before a nucleosome can bind a newly replicated DNA sequence.

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