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Mechanism of Protein Access to Specific DNA Sequences in Chromatin: A Dynamic Equilibrium Model for Gene Regulation

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We present evidence for a mechanism by which regulatory proteins may gain access to their target DNA sequences in chromatin. In this model, nucleosomes are dynamic structures, transiently exposing stretches of their DNA. Regulatory proteins gain access to DNA target sites in the exposed state, and bind with an apparent dissociation constant equal to their dissociation constant for naked DNA divided by a position-dependent equilibrium constant for site exposure within the nucleosome. A sensitive assay, based on the kinetics of restriction digestion of sites within nucleosomes, reveals this dynamic behaviour and quantifies the equilibrium constants for site exposure. Our results have implications for many aspects of chromatin function. They offer new mechanisms for cooperativity (synergy) in regulatory protein binding and for active invasion of nucleosomes.

Keywords: nucleosome, cooperativity; binding; nucleosome positioning; active invasion

Introduction

DNA sequences that are organized in nucleosomes are largely inaccessible to other proteins. This inaccessibility is due to steric exclusion, a simple and necessary consequence of the proximity of DNA to an impenetrable surface. It applies to DNA that is held on a flat surface such as mica (Rhodes & Klug, 1980), as well as to DNA held on the curved surface of the histone octamer (Lutter *et al*., 1979; Lutter, 1981; Richmond *et al*., 1984; Arents *et al*., 1991; Arents & Moudrianakis, 1993). The recognition of DNA sequences on a nucleosome is further hindered by steric exclusion from adjacent gyres of the DNA on the same nucleosome and from the organization of the nucleosome filament into higher order chromatin structures.

Most DNA *in vivo* is packaged in nucleosomes, but many DNA sequences are critical for biological regulation, and these must be accessible to regulatory proteins at appropriate or specific times. What, then, is the principle that guarantees that regulatory proteins may have access to their DNA target sequences when necessary?

Abbreviations used: GR, glucocorticoid receptor protein; HSF, heat shock transcription factor; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; BZA, benzamidine; BSA, bovine serum albumin; TBP, TATA-binding protein.

The answer to this important question is not known. Current thinking focuses chiefly on three ideas (for reviews, see Felsenfeld, 1992; Kornberg & Lorch, 1992; Wolffe, 1992; Lewin, 1994). (1) There may exist a window of opportunity for regulatory protein binding, coupled to DNA replication. Perhaps regulatory proteins are given an opportunity to bind to DNA prior to chromatin assembly. (2) Chromosomes may be organized much more carefully than has previously been appreciated. Perhaps DNA regions that are critical for regulation are simply never packaged in nucleosomes in such a way as to be inaccessible. (3) Perhaps regulatory proteins may instead have some capability of active invasion, so that steric exclusion does not apply. These three models each have some limitations.

Model (1) has certain well-established counter-examples; most notably, phosphate induction of the yeast *PHO5* gene and glucocorticoid induction of MMTV-LTR transcription (for a discussion of this point, see Schmid *et al*. (1992)). The essential observations are that cells that are prevented from undergoing DNA replication can still switch reversibly between transcriptional states, with corresponding chromatin-structural states, in response to changes in their environment. Thus, model (1) may apply in some instances, but it is not a general solution to the problem of regulatory protein access.

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Model (2) may be questioned on physical grounds. The known free energies that contribute to specifying the positioning of nucleosomes along the DNA (Yao *et al*., 1993) are far too small to guarantee that a particular DNA sequence will be similarly organized in the chromatin of every cell in a large multicellular organism. This point has been appreciated previously by Kornberg & Lorch (1992), who note that the $>10^6$-fold sequence selectivity typical for sequence-specific DNA binding proteins may be an appropriate benchmark. The known free energies of nucleosome positioning fall far short of that required to achieve 10^6-fold selectivity. The prior binding of a sequence-specific regulatory protein might provide the necessary free energy, but this simply replicates the fundamental problem one level up in a regulatory hierarchy. Importantly, observations of precise nucleosome positioning *in vivo* (e.g. Pina *et al*., 1990; Bresnick *et al*., 1991; Shimizu *et al*., 1991) do not address the feasibility of this model. The key issue is the time-averaged and cell-averaged statistical precision of this positioning. Nucleosome positioning may appear to be precise to the level of a single base-pair, but the modest free energies of positioning mean that the same chromosomal location in differing cells will frequently have nucleosomes at differing positions, with probabilities $>10^{-6}$. We do not intend this analysis to imply that nucleosome positioning and the corresponding free energies are without importance; indeed, in the context of the model that we propose below, these free energies and the resulting statistical positioning take on substantial regulatory significance.

Model (3) has not yet been developed to the point of having a definite, testable, proposed mechanism. Nevertheless, it has arisen from studies *in vitro* which show that some regulatory proteins are apparently able to bind to nucleosomal target sequences even though these target sequences are expected to be sterically inaccessible (e.g. Archer *et al*., 1991; Taylor *et al*., 1991; Perlmann, 1992); in some cases, binding may be ATP-dependent (e.g. Cote *et al*., 1994; Imbalzano *et al*., 1994; Kwon *et al*., 1994; Pazin *et al*., 1994; Tsukiyama *et al*., 1994). A major unresolved question with this mechanism is how proteins that are capable of active invasion of nucleosomes know which nucleosomes to invade. An important aspect of our model is that it provides a conceptual framework for the analysis of active invasion.

In this paper, we propose a new mechanism that may contribute to providing access for regulatory proteins to their target DNA sequences. Our hypothesis is that perhaps nucleosomes are dynamic structures, transiently exposing stretches of their DNA, and that regulatory proteins gain access to target sites in the exposed state. Such nucleosome conformational changes have previously been proposed, but only for the very ends of the core particle DNA (McGhee & Felsenfeld, 1980; Shindo *et al*., 1980); the remainder of the nucleosomal DNA has previously been considered inert. Here we report the results obtained using a sensitive assay, based on the kinetics of restriction digestion, which reveal this dynamic behavior for sites throughout nucleosomes and quantify the equilibrium constants for site exposure. These results have several ramifications. They explain numerous observations in the literature, they suggest mechanisms for transcription and replication through nucleosomes and for nucleosome sliding, and most importantly, they offer a new mechanism for cooperativity (synergy) in regulatory protein binding, and provide a framework for the analysis of active invasion.

Model, Assay, and Theory

Model

Our model is illustrated in Figure 1(a). Our hypothesis is that nucleosomes (N) might be dynamic structures, transiently exposing their DNA, such that in the exposed state (S), regulatory proteins (R) may bind as though they were binding to naked DNA. We make the simplifying assumption that sufficient nucleosomal DNA is exposed such that the rates and equilibria for binding to an exposed nucleosomal target sequence or to a naked DNA target sequence are identical. We recognize that, at some level, proximity to the histones (as well as other effects) cannot be ignored (for example, this introduces coupling in the effects of rotational and translational positioning on protein binding; see Discussion). But, as will be seen, interpretation of our new experiment with this simplifying assumption leads to results that are in broad agreement with previously unexplained results in the literature from very different kinds of experiments, suggesting that our simple model captures the essence of the system. With this assumption,

$$\mathrm{N} \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} \mathrm{S} + \mathrm{R} \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} \mathrm{RS} \qquad (1a)$$

for binding to a nucleosomal target, and

$$\mathrm{S} + \mathrm{R} \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} \mathrm{RS} \qquad (1b)$$

for binding to a naked DNA target.

Binding of a regulatory protein to a nucleosomal target sequence will therefore occur with a net free energy change

$$\Delta G^0_{\mathrm{net}} = \Delta G^0_{\mathrm{conf}} + \Delta G^0_{\mathrm{naked\ DNA}}, \qquad (2)$$

in which $\Delta G^0_{\mathrm{naked\ DNA}}$ is the free energy change for process (1b) and $\Delta G^0_{\mathrm{conf}}$ is the free energy cost for site exposure. At present, information is available only for $\Delta G^0_{\mathrm{naked\ DNA}}$, whereas the binding equilibrium is governed by $\Delta G^0_{\mathrm{net}}$.

Expressed in equilibrium constants, a regulatory protein would bind to a nucleosomal target sequence with an apparent dissociation constant

$$K_{\mathrm{d}}^{\mathrm{apparent}} = K_{\mathrm{d}}^{\mathrm{naked\ DNA}} / K_{\mathrm{eq}}^{\mathrm{conf}}, \qquad (3)$$

where $K_d^{\text{naked DNA}}$ is the dissociation constant for naked DNA which is measured in most studies, and K_{eq}^{conf} the equilibrium constant for site exposure,

$$K_{eq}^{conf} = e^{-\Delta G^0_{conf}/RT}. \quad (4)$$

In our model, K_{eq}^{conf} may depend on the position of the target sequence within the nucleosome.

The general resistance of nucleosome core particle DNA to nuclease digestion, and other properties of nucleosomes, suggest that K_{eq}^{conf} might be extremely small; we therefore devised a sensitive assay to detect and quantify this equilibrium (Figure 1(b)). We replaced the regulatory protein with a restriction enzyme (E), and we constructed nucleosomes having a site for E at a known position in the particle. If this conformational equilibrium exists, the restriction enzyme can bind and catalyze cleavage of the substrate to yield products (P), which can be detected by gel electrophoresis and quantified using a phosphorimager. Thus,

$$N \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} S + E \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} ES \overset{k_{34}}{\rightarrow} E + P \quad (5a)$$

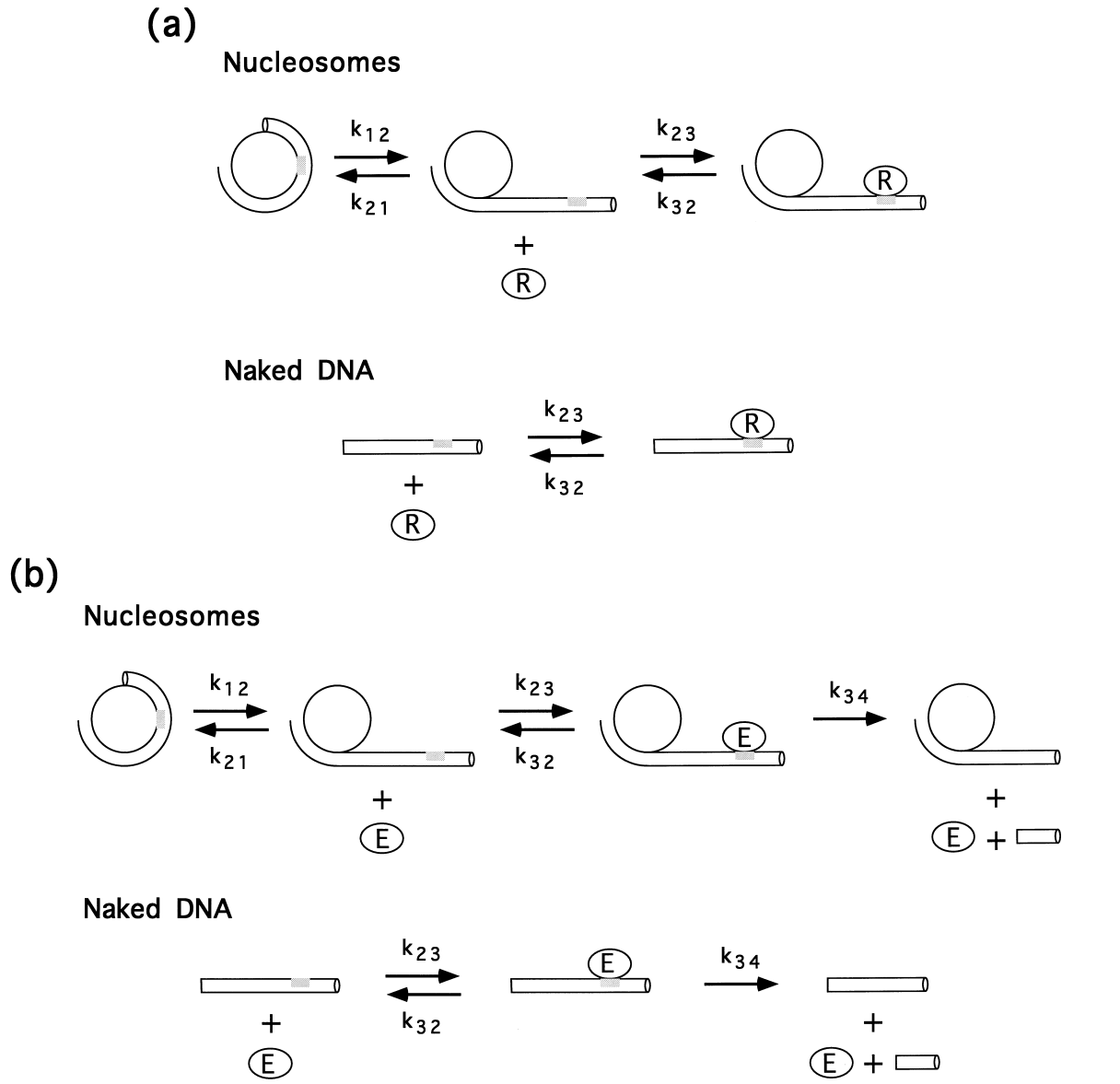

Figure 1. Hypothesis and assay. (a) Hypothetical mechanism for binding of a regulatory protein (R) to a specific DNA target sequence (hatched) on a nucleosome (top) compared to binding to naked DNA (bottom). The Figure illustrates a single nucleosome viewed from above. We do not mean to imply specific structural details for the exposed state or the resulting complex. Our hypothesis is that perhaps nucleosomes are dynamic structures, transiently exposing their DNA through some non-dissociative process such that, in the exposed state, regulatory proteins can bind as though they were binding to naked DNA. The relevant microscopic rate constants are indicated. (b) A sensitive assay to detect and quantify the hypothetical conformational equilibrium. We use a restriction enzyme (E) binding to its recognition sequence (hatched) in place of a regulatory protein; the restriction enzyme can catalyze cleavage of the DNA to yield detectable products. Rates of cleavage for nucleosomal DNA (top) are compared with cleavage of naked DNA (bottom) in identical solution conditions. The relevant microscopic rate constants are indicated.

and

$$S + E \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} ES \xrightarrow{k_{34}} E + P \qquad (5b)$$

for nucleosomes and naked DNA, respectively. The assay is potentially very sensitive because one can carry out the digestions for as long as necessary to detect the conformational equilibrium.

The reader may consider this assay surprising, since the failure to observe restriction digestion is routinely used by others as an assay for nucleosome positioning. In essence, our results establish that the protection against restriction digestion which is afforded by nucleosomal organization of the enzyme's target sequence is quite finite; by quantifying this finite protection, we observe and quantify the non-zero equilibrium constants for site exposure.

Theory

The kinetic analysis of mechanisms such as equations (5) is well established; we follow the derivation from the formally analogous study of Shore *et al*. (1981).

Experimentally, we monitor the loss of reactant nucleosomal DNA (D),

$$(D) = (N) + (S), \qquad (6)$$

which disappears according to a first order rate law with an observed rate constant k_{obs},

$$k_{obs} = \frac{-1}{(D)} \frac{d(D)}{dt}. \qquad (7)$$

Making the steady state approximation for (S) and for (ES), one obtains (Shore *et al*., 1981)

$$k_{obs} = \frac{k_{34}(E)}{K_m} \frac{k_{12}}{k_{12} + k_{21} + \frac{k_{34}E}{K_m}}, \qquad (8a)$$

with

$$K_m = \frac{k_{32} + k_{34}}{k_{23}} \qquad (8b)$$

There are two limiting cases for equation (8a) (Shore *et al*., 1981).

Case I: slow conformational transition

$$\left(\frac{k_{34}(E)}{K_m} \gg k_{21}; \text{ and } k_{21} \ll k_{12} \text{ for small } K_{eq}^{conf} \right). \qquad (9)$$

In this limit, equation (8a) reduces to:

$$k_{obs} = k_{12}. \qquad (10)$$

Case II: rapid conformational pre-equilibrium

As will be seen below, the present kinetic studies obey this opposite limit:

$$(k_{21} \gg k_{23}(E)). \qquad (11)$$

In this limit, equation (8) reduces to

$$k_{obs} = \frac{k_{34}(E)}{K_m} \frac{K_{eq}^{conf}}{1 + K_{eq}^{conf}}. \qquad (12)$$

If K_{eq}^{conf} is small ($\ll 1$), as anticipated, and if the experiment is set up such that (S) $\ll K_m$, so that (E) $\approx$ (E_0), the total concentration of added restriction enzyme, then the observed first order rate constant for loss of reactant nucleosomal DNA (equation (12)) becomes

$$k_{obs}^{nucleosome} = \frac{k_{34}(E_0)}{K_m} K_{eq}^{conf}. \qquad (13)$$

If, in separate experiments, naked DNA is digested under identical solution conditions (but possibly with different (E_0)), the reactant naked DNA will disappear with an apparent first order rate constant given by:

$$k_{obs}^{naked\ DNA} = \frac{k_{34}(E_0)}{K_m}. \qquad (14)$$

Combining equations (13) and (14) yields:

$$K_{eq}^{conf} = \frac{k_{obs}^{nucleosome} / (E_0^{nucleosome})}{k_{obs}^{naked\ DNA} / (E_0^{naked\ DNA})}. \qquad (15)$$

Thus, in this rapid pre-equilibrium limit, we obtain an experimental measurement of K_{eq}^{conf} from the ratio of two observed rate constants scaled by their respective enzyme concentrations, one for nucleosomal DNA targets and one for naked DNA.

The two limits are readily distinguished by the dependence of k_{obs} on (E_0); for limit I, the dependence is zero-order, while for limit II, the dependence is first order. In the present work, limit II applies. We find experimentally that k_{obs} is rigorously first order in (E_0). Moreover, limit II is anticipated on theoretical grounds. The available concentrations of restriction enzyme are unlikely to exceed 100 nM. For a diffusion-controlled encounter between a target DNA sequence and an enzyme active site, one expects $k_{23} \leqslant 10^8\ M^{-1}\ s^{-1}$, hence, we expect $k_{23}\ E_0 \leqslant 10\ s^{-1}$. By contrast, simple theoretical models for the site exposure and recapture process, assuming either an activated or a diffusive process for recapture lead to the expectation that $k_{21} \geqslant 10^5\ s^{-1}$ (J. Widom, unpublished results), in which case $k_{21} \gg k_{23}\ E_0$.

Experimental design

The DNA constructs used in the present study utilize a known nucleosome-positioning DNA sequence from the sea urchin 5 S RNA gene, characterized by Simpson and colleagues (Simpson & Stafford, 1983), incorporating specific base changes to generate restriction sites within the sequence. The majority of this DNA sequence is known not to be essential for its positioning ability, and, with one exception, we have restricted the locations of sequence changes to those regions of the sequence that are not essential for positioning (FitzGerald & Simpson, 1985). The DNA length

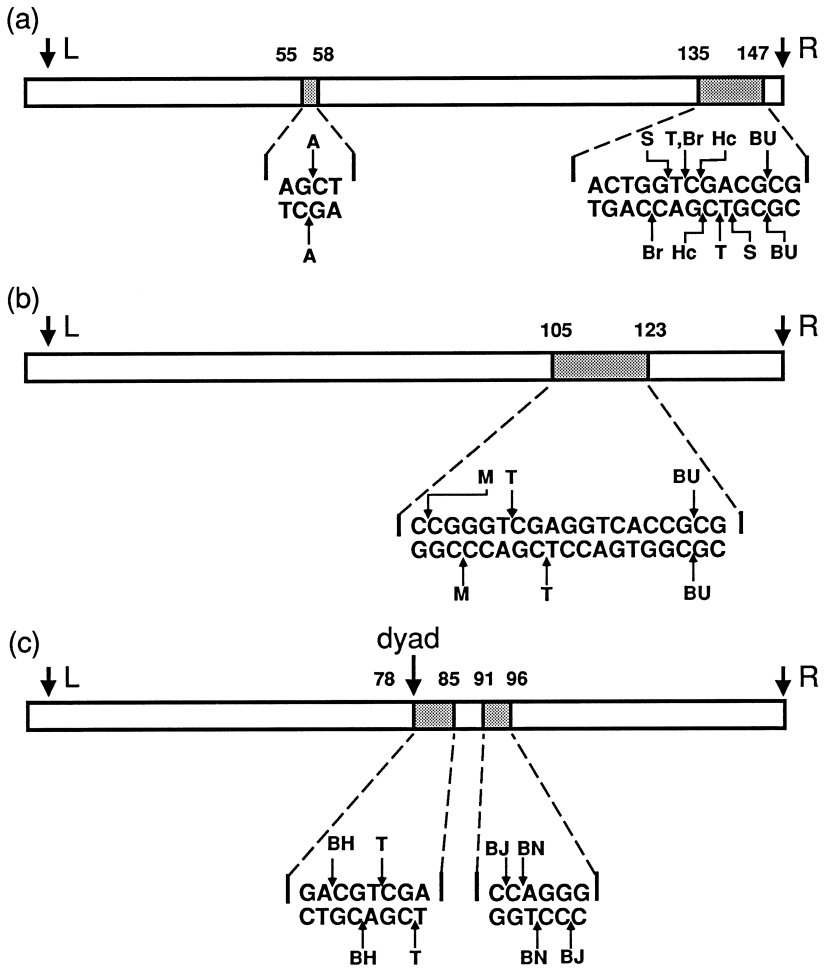

Figure 2. DNA constructs and restriction enzyme sites for kinetic assays. Constructs (a), (b), and (c) are illustrated as rectangles representing the 150 bp long DNA molecules. The histone octamers organize the DNA from positions 5 to 150 (bp), corresponding to the regions between the arrows marked L and R. In construct (c), the location of the dyad is indicated. Sequences for each nested set of restriction sites are shown below the schematics. The positions of key restriction sites in each construct are illustrated. A, *Alu*I; BH, *Bsa*HI; BJ, *Bsa*JI; BN, *Bst*NI; Br, *Bsr*I; BU, *Bst*UI; Hc, *Hin*cII; M, *Msp*I; S, *Sal*I; T, *Taq*$^{\alpha}$I. The numbers above each construct at the left and right ends of the hatched regions indicate the precise locations of those stretches of sequence within the overall 150 bp long sequence (nucleotide 1 is defined as the left end of the DNA sequence).

used in all of these studies is 150 bp, just slightly longer than the protected length in the core particle; consequently, multiple positions of the octamer on the DNA are not anticipated. In addition, we carry out control studies to assess the homogeneity of the product particles and to map the actual location(s) of the histone octamers on the DNA.

The three constructs used in our experiments are illustrated in Figure 2. Together, they span the full range of locations within the core particle, ranging from near the core particle DNA end to the particle's dyad axis of symmetry. An important aspect of these constructs is that, rather than engineering single sites, we nest together sites for multiple enzymes within a short patch of roughly one DNA helical turn. While any one site might be accessible simply because it faces ''out'' (although this is doubtful, on steric grounds), the other sites within that patch will necessarily be protected. By comparing apparent equilibrium constants for site exposure for sites within one patch, we distinguish dynamic exposure of a stretch of DNA from exposure of a single site that inadvertently faces out.

Another aspect of the design of these DNA constructs lies in the choice of restriction enzymes. We chose to use restriction enzymes from thermophilic organisms, so that we could, if necessary, raise the temperature, with the goal of making the nucleosomes artificially more ''dynamic''. Our subsequent results showed this feature of the experimental design to be unnecessary, and certain additional studies were carried out at 37°C.

For the experiments themselves, two parallel reactions were set up in identical solution conditions, one for naked DNA, and one for reconstituted core particles. Optimal (E_0)s that allow the reactions to go to near completion on a 5 to 60 minute timescale are found by trial and error. Typically, (E_0) was 10^2 to 10^3-fold lower for the digestions of naked DNA than for the digestions of particles.

Results

Characterization of reconstituted core particles

The reconstituted nucleosome core particles were prepared by dialysis from 2.0 M NaCl and then purified by sucrose gradient centrifugation. The profile resulting from a typical sucrose gradient is illustrated in Figure 3(a). The reconstituted core particles are well-resolved from naked DNA and from aggregates, and on re-analysis in sucrose gradients, they appear homogeneous. In other studies, we confirmed that they comigrate in

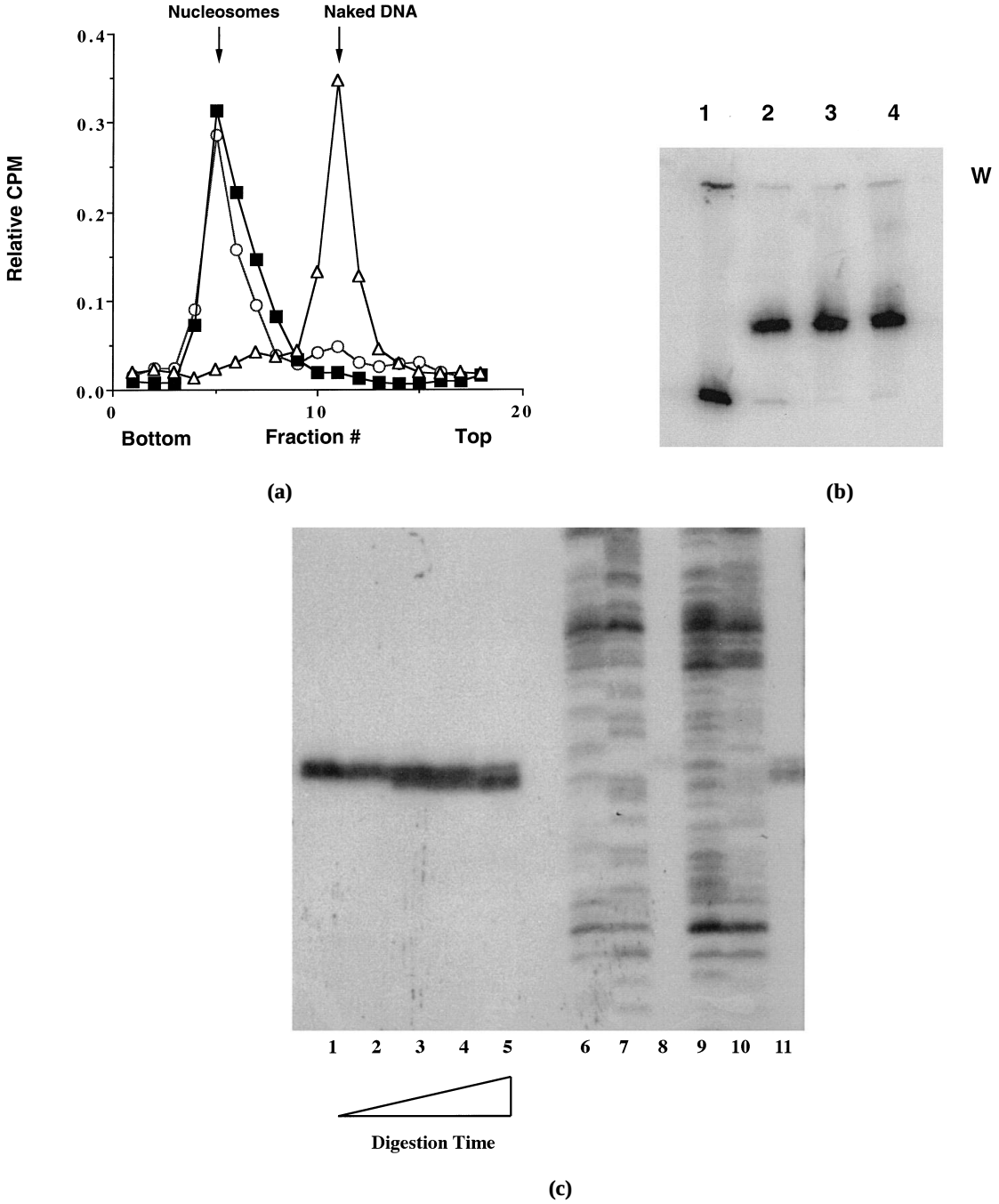

Figure 3. Isolation and characterization of reconstituted core particles. (a) Sucrose gradient purification and re-analysis. Core particles are reconstituted by gradual salt dialysis and isolated from naked DNA and other non-nucleosomal contaminants on 5 to 30% (w / v) sucrose gradients. ○, Preparative run of reconstituted core particles; △, naked DNA only; ■, re-analysis of gradient-purified particles. (b) Native gel analysis. W indicates the bottom of the loading wells. Lane 1, naked DNA; lanes 2 to 4, purified reconstitutes for constructs (a), (b), and (c), respectively. The isolated core particles show the appropriate mobility shift. For a comparison of the relative mobility of the reconstituted core particles compared to the mobility of naked DNA size standards, see Figure 7. Phosphorimager analysis of the gel reveals contamination by free DNA and other non-nucleosomal aggregates to be $< 1\%$. (c) Direct mapping of nucleosome positioning for nucleosomes reconstituted with construct (a). The sample was digested with 0.04 units ml^{-1} (Worthington) of micrococcal nuclease for 0, 1, 8, 16, or 32 minutes (lanes 1 to 5), then extracted and analyzed by sequencing gel electrophoresis and autoradiography. Lanes 6, 7, 9 and 10, which are included as high-resolution size standards, show G, A, T, and C sequencing lanes, respectively, from sequencing reactions on pGEM3Z (Promega) template DNA using the sequencing primer 5'-gttttcccagtcacgac-3' which was end-labeled with ^{32}P prior to sequencing. Lanes 8 and 11 reproduce lanes 1 to 5, respectively. The results are consistent with a distribution of positioning of the entire sample of 0–1 bp. No products having positions differing by, e.g. 3 to 5 bp, can be detected despite the overexposure of the autoradiogram.

sucrose gradients with authentic chicken erythrocyte nucleosome core particles (D. S. Scherl and J. Widom, unpublished results). We used native gel electrophoresis to examine further the quality of the purified reconstitutes (Figure 3(b)). The gels reveal the purified particles to have negligible contamination by free DNA. The relatively sharp bands and the absence of resolved multiple species are consistent with the particles having a unique positioning of the histone octamer on the DNA that is equivalent for all three constructs.

While the native gels suggest that the particles are uniquely positioned, and the absence of additional DNA makes alternative positions unlikely (Dong *et al*., 1990; Pennings *et al*., 1991), we nevertheless carried out studies to map the actual locations of the histone octamers on the DNA in our reconstituted particles. We used micrococcal nuclease in two different procedures to map the actual location of the histone octamers on the DNA in the purified particles. In one procedure, particles which were labeled at both 5' ends with ^{32}P were simply digested with the nuclease, and the products analyzed on sequencing gels. We found that nuclease digestion reduced the size of the DNA from 150 bp to a new resistant length of 147 bp. An example of this experiment for construct (a) is shown in Figure 3(c). The fact that the DNA is still detected means that the nuclease cleavage occurs only on one end. This result demonstrates that nucleosome core particle lengths of DNA are protected in these particles, and it is consistent with the expected wrapping of the DNA, which places one end at the anticipated core-particle end position. If alternative positions had been adopted, leaving a longer end hanging off the particle, shorter protected lengths would have been detected, yet none are visible, even on the overexposed autoradiogram. A different procedure (D. S. Scherl and J. Widom, unpublished results) used Tfl polymerase primer extension to map the sites of cleavage by micrococcal nuclease; a pair of primers complementary to the top and bottom strands near the middle of the DNA template were used. The observed lengths of the primer extension products (data not shown) confirm the expected positioning and they rule out the possibility that significant fractions of the particles have alternative octamer positions. Finally, as will be discussed further below, many of the enzymes used in this study are expected to wrap around their DNA target sites. For these enzymes, no rotational orientation suffices to allow access without some sort of site exposure such as that postulated in this study.

Kinetic assays

Parallel digestions of nucleosomal DNA and naked DNA were set up in identical solution conditions. Reactions are initiated by the addition of enzyme. Aliquots were removed as a function of time during the reaction and quenched, and the products were analyzed on denaturing acrylamide gels and quantified by phosphorimager. An example of such an experiment is shown in Figure 4, which probes a site very near the particle's axis of dyad symmetry (construct (c)). It is clear from the gels themselves that even sites far within the nucleosome are accessible to the restriction enzyme, as evidenced by the digestion products that are produced. Quantitative analysis of the gels (Figure 4(d) and data not shown) confirms that the reactions on the core particles can be followed to near completion, even for these innermost sites: the results that we obtain evidently apply to the majority of the particles present. The results are described by a first-order decay of the substrate, as expected (equation (7)).

Similar data were obtained with this construct in experiments at other temperatures and with the other restriction enzymes. And similarly, digestion data obtained from constructs (a) and (b) revealed that essentially all of these particles, too, were accessible to the restriction enzymes and participated in the reactions.

Equilibrium constants

Equilibrium constants (K_{eq}^{conf}) were calculated from the rates obtained for digestions of naked DNA and reconstituted core particles (equation (15)) from data such as those in Figure 4(c) and (d). Experiments were carried out over a range of temperatures, for each restriction enzyme and for the three different constructs. The results are shown in Figure 5(a) to (c). No systematic temperature dependence is evident; temperature dependences are observed, but with both positive and negative slopes, even for measurements of K_{eq}^{conf} made within single patches of DNA. We conclude that the real temperature dependences are small compared to our experimental error; we therefore calculate averages of these data, shown in Figure 5(d).

Equilibrium constants for site exposure differ significantly from zero for positions throughout the nucleosome core particle. Construct (a) probes sites just inside the end of the core particle. All three restriction enzymes yielded comparable values for $K_{eq}^{conf} \approx 1 \times 10^{-2}$ to 4×10^{-2}. These data are consistent with exposure of a stretch of DNA, rather than an individual outward facing site.

Construct (b) probes sites $\sim$30 to 40 bp in from the end. The values obtained for K_{eq}^{conf} at these sites, $\approx 5 \times 10^{-4}$ to 3×10^{-3}, are substantially lower than those obtained for sites near the end. We conclude that equilibrium constants for site exposure on nucleosomes are position-dependent, decreasing as one moves inward toward the center of the nucleosome. As observed near the particle ends, the different enzymes probing within this region yielded similar values for K_{eq}^{conf}, again suggesting exposure of a stretch of DNA. Moreover, like other enzymes which leave blunt ends after cleavage, *Bst*UI is expected to wrap around roughly three quarters of the DNA circumference at its site (Winkler *et al*., 1993; Cheng *et al*., 1994); the

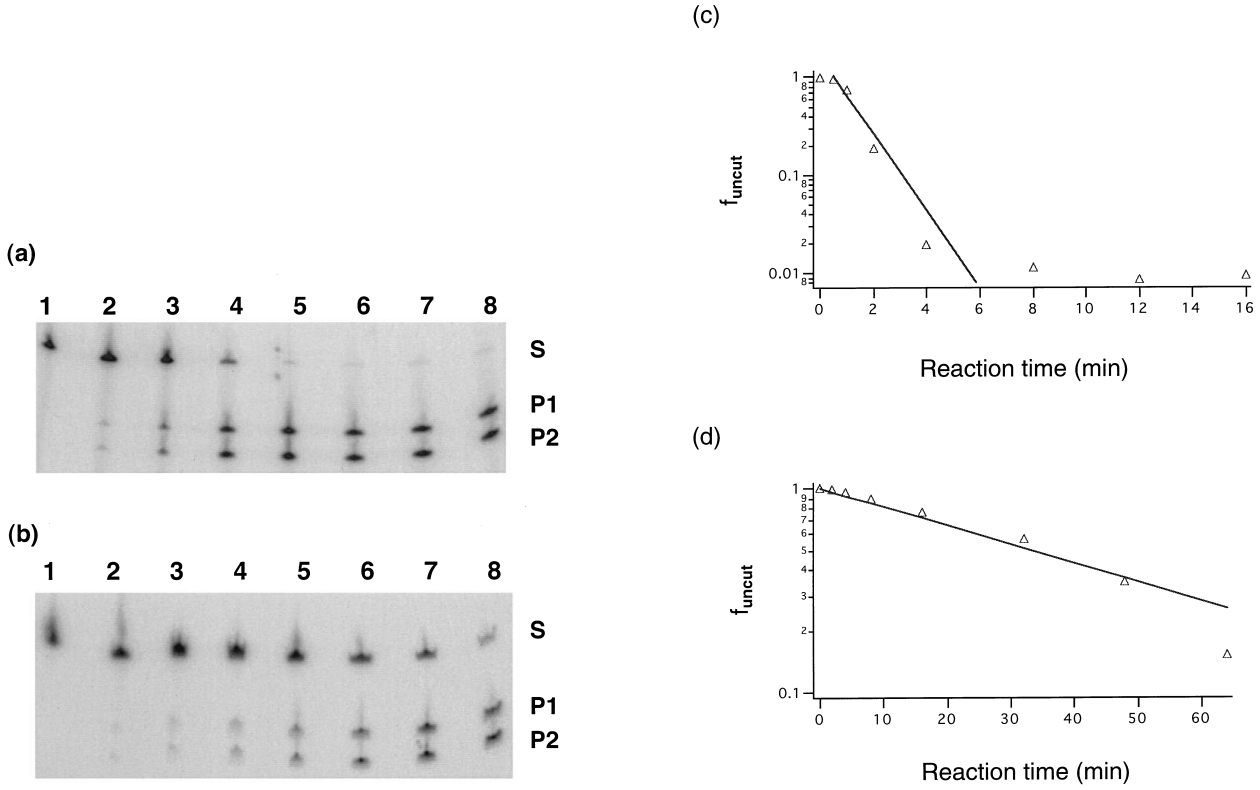

Figure 4. Example analysis of construct (c), probing site-exposure near the particle dyad. (a), (b) Denaturing polyacrylamide gel analysis of the time-course of digestion for construct (c) using the enzyme *Taq*$^{\alpha}$I, which cleaves very near to the axis of dyad symmetry in the reconstituted particles. (a) Naked DNA, digested with *Taq*$^{\alpha}$I at 40 units ml^{-1}. (b) purified nucleosome core particles, digested with *Taq*$^{\alpha}$I at 10,000 units ml^{-1}. Lanes 1 through 8 are obtained from samples removed at 0, 0.5, 1, 2, 4, 8, 12, and 16 minutes for the naked DNA, and at 0, 1, 2, 4, 6, 16, 32, and 64 minutes for the core particles. In each case, the substrate (150 nt, S) is converted over time to two products (82 nt, P1, and 66 nt, P2; the sizes of S, P1, and P2 expected from the DNA sequence are confirmed on other gels, not shown, in which their mobilities are compared to the mobilities of size standards. Size standards are not routinely run on the experimental gels simply to increase the number of timepoints that may be quantified under identical conditions. Alternate lanes of the gel are left empty to optimize the accuracy of the quantification of individual bands.) (c), (d) Quantitative analysis of the time-course of digestion from the data in panels (a) and (b), respectively. The fraction uncut is plotted on a log scale *versus* time. The superimposed lines represent the results of unweighted least-squares fits to single exponential decays.

proximity of additional DNA segments on the nucleosome (Richmond *et al*., 1984; Arents *et al*., 1991; Arents & Moudrianakis, 1993) precludes such binding, whatever the rotational orientation of the site, again suggesting that DNA must be exposed off the surface of the nucleosome prior to binding and cleavage by *Bst*UI.

Construct (c) probes sites close to and over the particle dyad axis of symmetry, over the full range of rotational positions. It is remarkable that non-zero equilibrium constants are readily detected at these sites as well. In these regions, 10 to 20 fold differences are detected in the averaged equilibrium constants measured at closely spaced sites by different enzymes. These may be real differences; alternatively, perhaps the apparent differences between closely spaced sites reflect increased experimental error arising as a consequence of the substantially decreased K_{eq}^{conf}. In any case, the average accessibility of sites in these regions ($\approx 10^{-5}$ to 10^{-4}) continues the progressive decreasing trend for K_{eq}^{conf} with distance inward from the core particle end.

Digestions at 37°C

K_{eq}^{conf} is, at most, only weakly dependent on temperature, so we anticipate that similar conformational states will obtain at 37°C to those observed at 45°C and higher temperatures. Moreover, as will be seen in the Discussion, K_{eq}^{conf} can also be extracted from equilibrium binding studies which have been carried out by us and others at temperatures of 37°C and lower, and the results for K_{eq}^{conf} obtained in this way are in good agreement with the results of Figure 5. Nevertheless, we have taken advantage of restriction sites for *Sal*I, *Hin*cII, *Msp*I, and *Alu*I that are present in our constructs to directly test the behavior at 37°C. The results from two sets of experiments with these four enzymes are summarized in Table 1.

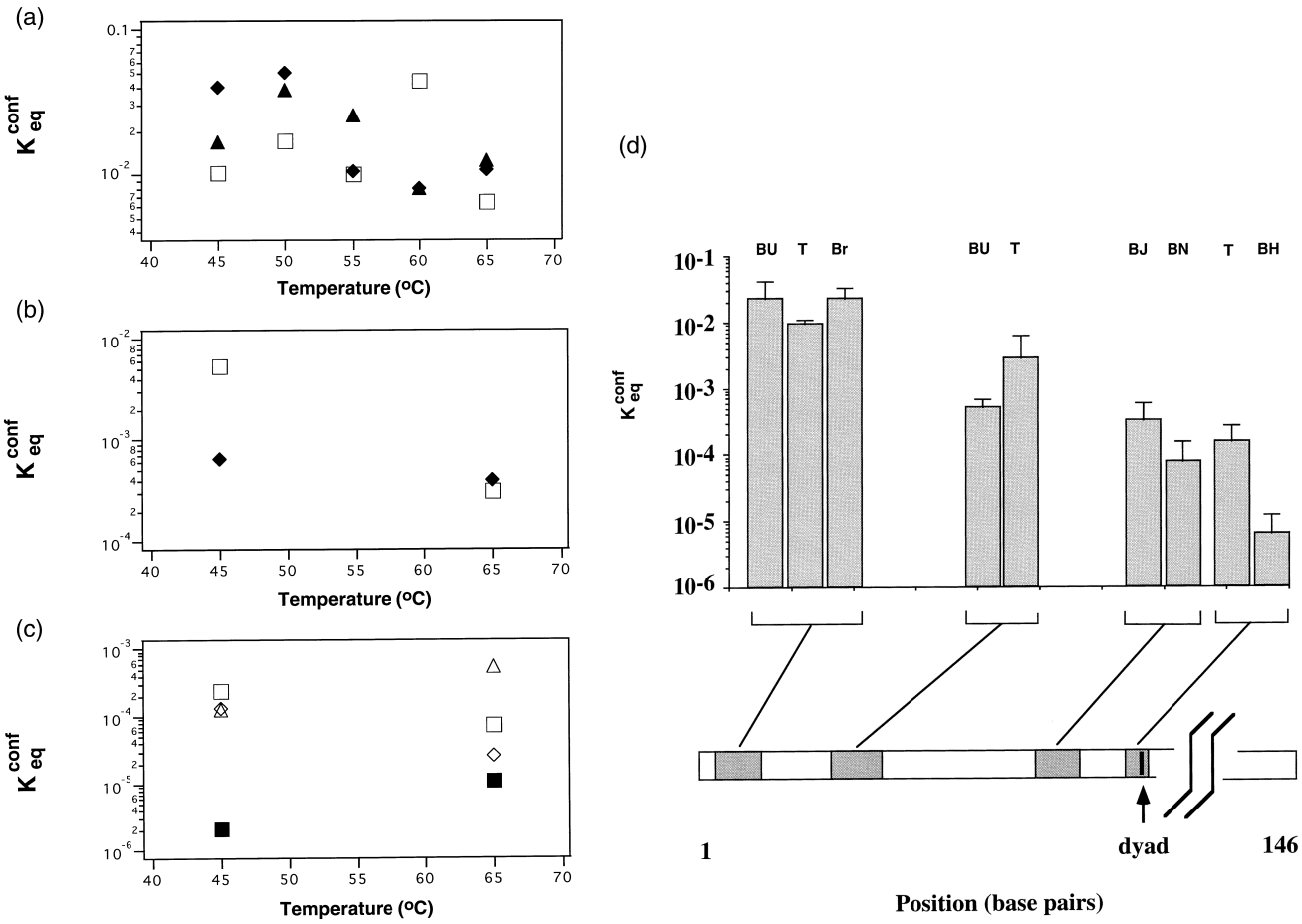

Figure 5. Summary of measured equilibrium constants for site exposure. (a) to (c) Equilibrium constants were measured at positions throughout the core particle, at two or more temperatures in the range 45 to 65°C. The results for constructs (a) to (c) are shown in panels (a) to (c), respectively. ◆, *Bst*UI; □, *Taq*$^{\alpha}$I; ▲, *Bsr*I; ■, *Bsa*HI; △, *Bsa*JI; ◇, *Bst*NI. (d) For each enzyme, for each construct, equilibrium constants obtained at varied temperatures (panels (a) to (c)) were averaged; the means and standard deviations are plotted. The approximate locations of each site in the various core particle constructs are represented beneath the plot. BU, *Bst*UI; T, *Taq*$^{\alpha}$I; Br, *Bsr*I; BJ, *Bsa*JI; BN, *Bst*NI; BH, *Bsa*HI.

The results for *Sal*I and *Hin*cII are in good agreement with the results at higher temperatures from construct (a) which probe sites just inside from the end of the particle. Similarly, the results for *Alu*I are in reasonable agreement with the results at higher temperatures for *Taq*$^{\alpha}$I on construct (b) and *Bsa*JI on construct (c), whose sites bracket the location probed with *Alu*I. Importantly, the results obtained with *Alu*I provide clear evidence that sites well inside a nucleosome (51 to 54 bp inside from the end of the core particle) have substantial dynamic accessibility even at 37°C. Like *Bst*UI (above), *Alu*I also leaves blunt ends after cleavage and hence is expected to wrap around roughly three quarters of the DNA circumference at its site (Winkler *et al*., 1993; Cheng *et al*., 1994); again, we infer that exposure of DNA off the surface of the nucleosome must precede binding and cleavage by *Alu*I.

Table 1. Equilibrium constants obtained from 37°C data

Enzyme	[a]Cleavage site (nt from nearest end of core particle)	[b]K_{eq}^{conf}	[c]Mean of $\log(K_{eq}^{conf})$	[c]Std. deviation of $\log(K_{eq}^{conf})$
*Sal*I	7, 11	6.4×10^{-2}	–1.33	0.52
*Hin*cII	9, 9	2.6×10^{-2}	–1.60	0.11
*Msp*I	43, 45	3.1×10^{-7}	–6.52	0.07
*Alu*I	52	7.1×10^{-3}	–2.19	0.26

[a] Positions of the cleavage sites on both strands, expressed in nucleotides from the nearest end of the DNA positioned on the core particle, which includes bp 5 to 150 of the construct DNA sequences.
[b] Equilibrium constants represent the mean value from two separate experiments.
[c] The uncertainty in K_{eq}^{conf}, which is multiplicative in nature, is most naturally represented as the mean and standard deviation of the logarithm of K_{eq}^{conf}.

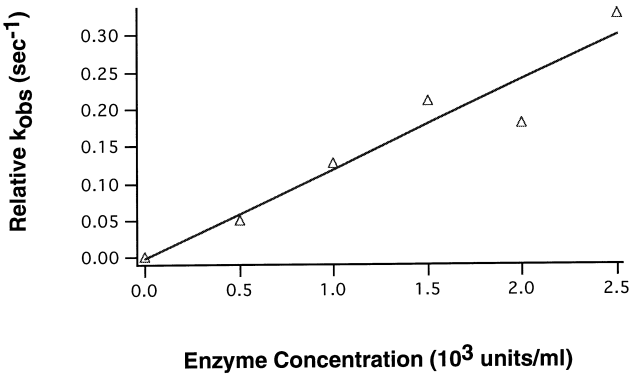

Figure 6. Order of the reaction in (E_0). Core particles reconstituted with construct (a) were digested with increasing concentrations of *Taq*$^{\alpha}$I, and the observed rate constant determined (see Figure 4 (b) and (d) and Materials and Methods). The line represents a least-squares fit of the resulting data. The data are well described by this line, which is straight and passes through the origin, indicating that the reaction is first-order in (E_0).

*Msp*I recognizes a site positioned 43 to 46 bp from the end of the core particle. The results with this enzyme reveal accessibility at that site, also, at 37°C, but with a significantly lower K_{eq}^{conf} than is found for sites both closer to the particle dyad and further out toward the ends at other temperatures. This observation is discussed further below.

Order of the reactions in (E_0)

Our analysis hinges on the system obeying limiting case II of the kinetics. It was therefore important to test the dependence of the observed rates of cleavage, k_{obs}, on the concentration of enzyme added, (E_0). For each restriction enzyme for all three constructs, we observed qualitatively that the apparent rate of the reaction, k_{obs}, depended approximately linearly on the total concentration of enzyme added, (E_0), suggesting that the reactions might be first-order in (E_0). We examined the dependence in more detail for one of the sites. Figure 6 shows the results of quantitative measurement of k_{obs} as a function of (E_0) for digestions of construct (a) reconstitutes with the restriction enzyme *Taq*$^{\alpha}$I. The linearity of the plot, together with the fact that the line passes through the origin, demonstrates that the digestions are first order in [*Taq*$^{\alpha}$I]. An alternative way of analyzing these data is to determine the value of the exponent that relates k_{obs} and (E_0). For any particular small range in (E_0), we may write

$$k_{obs} = a_1(E_0)^{a_2}. \quad (16)$$

The value of a_2 varies between the extreme values of 0 and 1, as the mechanism progresses from limiting case I to limiting case II, or, alternatively, as S is titrated with increasing (E_0). We determined a_2 by plotting ln(k_{obs}) versus ln([E_0]), and obtained the slope $a_2 = 1.07$. These analyses provide firm evidence that the reactions take place in the rapid pre-equilibrium limit (limit II), so ratios of cleavage rates yield the desired equilibrium constants K_{eq}^{conf}.

Stability of the particles during digestions

It is important that the nucleosomes do not simply dissociate when exposed to the digestion conditions. Previous studies revealed that exposure to elevated temperature (50°C) does not lead to detectable changes in nucleosome structure as probed by hydroxyl radical footprinting (Bashkin *et al*., 1993); indeed, the positioning of histone octamers on the *Xenopus* 5 S phasing sequence is maintained to at least 75°C (Bashkin *et al*., 1993), a temperature substantially greater than that probed in our studies.

Most importantly, our results themselves establish that instability of the particles prior to or during the reactions does not contribute significantly to the observed digestion. If dissociation of the particles were substantial and rapid, then low enzyme concentrations, comparable to those used for the naked DNA digestions, should suffice for the particle digestions. This is contrary to our observations: we find that much greater enzyme concentrations are required in order to achieve the digestion of particles on a reasonable timescale. Alternatively, if dissociation were slow, the mechanism would approach limiting-case I (equation (9)), and the reactions would be zero-order in (E_0) (equation (10)); but we find that the observed rate of the reaction is first-order in (E_0), providing formal evidence against this hypothesis. A third possibility, that dissociation occurs on an intermediate timescale, cannot apply to all of our results, since our measured k_{obs} span four orders of magnitude.

We carried out additional control experiments to test the particle's stability directly. Reconstitutes were carried through mock digestions (in digestion buffer but without enzyme) for 15 minutes at 0, 37, or 65°C, then analyzed by native gel electrophoresis. The results are shown in Figure 7. Lane 3 shows the original particles to be free from contaminating naked DNA, as expected. Lanes 4 to 6 reveal the presence of a small amount of naked DNA ($\sim$2% of the total), evidently caused by dilution of the particles into the restriction enzyme digestion buffer. There is no apparent effect of temperature over this range (compare lanes 4 to 6). Importantly, this naked DNA will not significantly affect the kinetic analysis. Its absolute amount is negligible, given our likely experimental error. Also, as pointed out above, naked DNA is digested $\sim 10^2$ to 10^5 times more rapidly than is the DNA in particles; had enzyme been added to these samples in amounts appropriate for digestion of the particles, the contaminating naked DNA would have been digested to completion within the mixing time, and would not contribute to the measured first-order digestion of the bulk of the material.

Remarkably, for construct (a), in which only small fragments of DNA are cleaved from the end, the particles remain intact even after digestion essen-

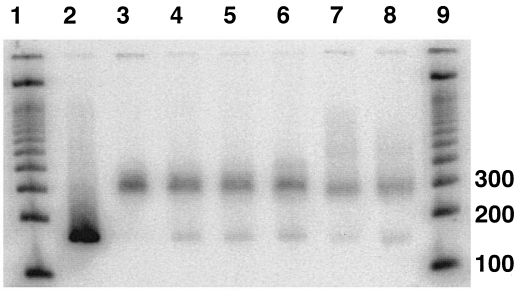

Figure 7. Stability of reconstituted core particles during restriction digests. Core particles reconstituted with construct (a) were subjected to mock digestions or actual restriction digestion and then analyzed by native polyacrylamide gel electrophoresis. Lanes 1 and 9: 100 bp ladder used as size standards. Lane 2: naked DNA. Lane 3: reconstitutes after initial purification, with no additional treatment. Lanes 4 to 6: mock digestions; reconstitutes were incubated in *Taq*$^{\alpha}$I digestion buffer for 15 minutes at 0°C, 37°C, or 65°C, then analyzed on the gel. Lanes 7 and 8: actual digestions; reconstitutes were digested with *Taq*$^{\alpha}$I for 15 minutes at 65°C, and then either cooled on ice and loaded directly on the gel (lane 7), or cooled on ice, quenched with 40 mM EDTA, and then loaded on the gel (lane 8). Although small amounts (2%) of DNA are released upon exposure of the particles to buffer (independent of the temperature over the range 0 to 65°C), the great majority of core particles remain as intact core particles even after digestion with restriction enzyme, which cleaves a short fragment from one end (the short fragment runs off the bottom of the gel). When the reactions are quenched with concentrated EDTA (lane 8), some naked DNA is released from the particles; its increased mobility relative to that of the starting DNA shows it to have been cleaved, as expected. This trace naked DNA, induced by quenching the digestions with EDTA, is of no consequence: in our kinetic analysis, all of the DNA in the particles is extracted prior to being run on denaturing gels.

tially to completion (lane 7). No naked DNA is detected, and the majority of the label migrates as a band with slightly more rapid mobility than that of the original particles. A trace amount of material having lower than native mobility is also apparent.

We conclude from these studies that essentially all of the particles remain intact when exposed to restriction enzyme buffer conditions and elevated temperature, and that the site exposure detected and quantified in our experiments occurs without complete dissociation of the DNA.

Tests for contaminating protease activity

The possibility also exists that the DNA site exposure detected in this study could be facilitated by proteases which may contaminate the restriction enzymes or the histone octamer preparations. To assess this possibility, reconstituted nucleosome core particles were incubated with *Taq*$^{\alpha}$I, *Bst*UI, or *Bsr*I, at 65°C for 60 minutes (our most extensive digestion conditions), and the products analyzed by SDS/gel electrophoresis and Coomassie staining. The core particle concentration was comparable to those used in the determinations of K_{eq}^{conf}, and the restriction enzyme concentrations corresponded to the high end of the concentration range used in the determinations of K_{eq}^{conf}. No traces of proteolysis, which would be expected to produce definite new bands at somewhat lower molecular mass (van Holde, 1989; Wassarman & Kornberg, 1989), could be detected. We conclude that proteolytic action does not contribute to the DNA site exposure detected in this study.

Possible nucleosome sliding

It is possible that nucleosome sliding (Spadafora *et al*., 1979; van Holde, 1989; Meersseman *et al*., 1992) might be responsible for the observed site exposure. This is not a concern *per se*, since sliding might be a means of guaranteeing that regulatory proteins would have access to their DNA target sequences. But in any case, sliding is unexpected in the present studies because of the absence of any unoccupied DNA for the octamer to slide onto. And our results suggest that nucleosome sliding is not the mechanism of site exposure in these studies. The data in Figure 4 show that even sites at the particle dyad axis are readily exposed to restriction enzymes; hence sliding, if it were responsible, must be of a very great magnitude (at least 70 to 80 bp). Particles in which the octamers had repositioned to great but varying extents would be expected to reveal a range of mobilities on native acrylamide gels (Pennings *et al*., 1991), yet Figure 7 reveals no such behavior. We tentatively conclude that sliding does not account for the site exposure detected in the present studies.

Possibility that added restriction enzymes drive or catalyze the site-exposure process

One concern in these studies is raised by the mechanism itself (equation (5a)): if (E_0) is sufficiently high, binding of the enzyme will drive the system toward site exposure, directly altering the apparent equilibrium constants K_{eq}^{conf}. However, our observation of the exponent $a_2 = 1.07$ (equation (16)) provides formal proof that our experiments are carried out in the low (E_0) limit, so that this potential concern does not apply to our results. That this is true is also demonstrated in another way: the equilibrium constants that we measure are explicitly independent of (E_0). By contrast, if we were in the high (E_0) limit, the exponent a_2 would approach the value 0, and the cleavage rate would approach that for naked DNA, or, ultimately, it would approach the rate constant for site exposure, k_{12}. If we were in an intermediate (E_0) range, the exponent would have a value intermediate between 0 and 1, and the measured apparent equilibrium constant would depend explicitly on (E_0), all contrary to our experimental observations. We conclude that the restriction enzymes do not drive the site exposure process at the concentrations used in our studies.

We also tested the possibility that non-specific DNA binding by the restriction enzymes could be responsible for the observed site exposure, even though the kinetic argument outlined above provides formal proof that non-specific binding is not a factor in our studies. We carried out two parallel digestions on reconstitutes. To one sample we added an enzyme (*Taq*$^{\alpha}$I) which has a site in the particle; to the other sample, we added both *Taq*$^{\alpha}$I and a comparable concentration of another restriction enzyme (*Bsm*AI) for which there were no cleavage sites within that construct. Both enzymes were used at concentrations near the upper end of those used for any of the experiments in this study, and, in particular, near the upper end of the (E_0)-dependence data of Figure 6. We obtained identical rates of digestion, and hence identical apparent equilibrium constants, for the two samples. We conclude that non-specific binding by the restriction enzymes is not involved in the mechanism of site exposure in our studies.

Another formal possibility was that interaction of restriction enzyme with DNA on the surface of the nucleosome may influence or facilitate the subsequent dissociation of that DNA off the histone surface. This possibility is excluded by our finding that the equilibrium constants that we measure are explicitly independent of (E_0).

Other assumptions in the kinetic analysis

The results presented above demonstrate that the kinetics are first-order in both (D) and (E_0) and that the reaction obeys the low (E_0) limit of limiting-case II. Two additional assumptions simplify our analysis. One assumption is that $(S) \ll K_m$. This condition is satisfied by construction, for both the naked DNA and the particles. Typical K_ms for restriction enzymes are $\sim$1 to 10 nM. Our naked DNA digestions are set up with $(S) \ll 1$ nM. For the particle digestions, the total concentration of sites, including sites statistically present in the particles that contain carrier DNA, may approach several nM; but these sites are exposed with probabilities of 10^{-2} or less (Figure 5), so that again $(S) \ll K_m$. For particles, the condition $(S) \ll K_m$ is required for limit II to apply, and our demonstration that limit II does apply establishes the validity of the assumption. Another assumption is that $K_{eq}^{conf} \ll 1$; this assumption simplifies the analysis but is not essential (except as required to keep $(S) \ll K_m$ even for the measurements with particles). This result is substantiated by the measured values for K_{eq}^{conf} that we obtain (Figure 5). The final important assumptions in our analysis are that both S and ES (equations (5a), (5b)) are in steady state. The rapid pre-equilibrium, which is demonstrated by the observation that the reactions are first-order in both (D) and (E_0), together with the experimentally determined $K_{eq}^{conf} \ll 1$, are sufficient conditions for the reaction to obey steady state behavior in S. We have not explicitly tested for steady state behavior of ES, but we note that this is a general property of enzyme-catalyzed reactions.

Discussion

The most important conclusion from this study is that the site exposure which we postulated does occur, with substantial values for K_{eq}^{conf}. This dynamic property intrinsic to nucleosomes provides a general mechanism guaranteeing that regulatory proteins may have access to their DNA target sequences. Our data are consistent with, but do not prove, the hypothesis that the mechanism of site exposure is that illustrated in Figure 1.

The binding of individual regulatory proteins made possible by site exposure is expected to occur in accord with equation (3). The occupancy of a site, the fraction of equivalent sites in a population of cells occupied at any instant, or the fraction of time during which any one particular site is occupied, will depend on the location of the site within the nucleosome, the intrinsic affinity of the regulatory protein for naked DNA, and the free concentration of the regulatory protein. No matter where a site is within a nucleosome, and even if the corresponding binding protein has limited affinity or is present in very low free concentrations, the sites will be occupied with a probability greater than zero and, averaged over time, any particular cell will have that site occupied a non-zero fraction of the time.

In some or many cases, a greater level of occupancy at a particular site may be required than can be provided by the binding of a single protein in accord with equation (3). The site exposure mechanism provides two additional routes through which the occupancy may be increased. (1) Binding of proteins to multiple sites within the same nucleosome can occur synergistically (cooperatively), even if the proteins do not ordinarily interact, substantially increasing the occupancy by each protein. (2) A regulatory protein that binds by the site exposure mechanism may recruit additional proteins, which may subsequently act to displace the histone octamer. These ideas are discussed in more detail, below.

Mechanism of site exposure

The simplest physical picture for the mechanism of site exposure is one in which DNA ''unpeels'' in a stepwise fashion starting from the end of the core particle. In this model, all sites may be reached by unpeeling half or less of the core particle DNA, allowing the site exposure process to be non-dissociative. A DNA molecule lying on a surface naturally makes contacts with surface residues every helical turn, when the backbone is oriented so that the minor groove faces in toward the surface. If all such contact sites in the nucleosome represented equivalent protein–DNA binding sites, the free energy of site exposure would increase in steps with every additional $\sim$10 bp of DNA unpeeled, and there would be a corresponding

stepwise decrease in K_{eq}^{conf}. This is consistent with the majority of our data, which show that K_{eq}^{conf} does appear to decrease systematically (although not monotonically) with increasing distance from the end in toward the center.

We emphasize, however, that other, quite different, mechanisms for site exposure are not excluded by our data. This is an important area for further analysis because, as will be discussed below, it bears directly on a new mechanism for synergy (cooperativity) in the binding of multiple regulatory proteins to sites within the same nucleosome.

In the context of the unpeeling mechanism, the primary determinant of K_{eq}^{conf} is the translational location of a target site within the core particle, since, in this model, exposure of the whole DNA region precedes binding. Rotational positioning also plays a role, however, because the length of DNA that must be unpeeled to allow unhindered access of a protein to a nucleosomal target site will depend on the rotational setting of the site, the size and shape of the protein, and possible DNA bending.

Relation to chromatin structure *in vivo*

Chromatin *in vivo* differs in significant ways from the model system investigated in this study. One key difference concerns histone H1, which is a stoichiometric component of most nucleosomes *in vivo*. Histone H1 is sometimes thought to seal two turns of DNA on the core particle, which would prevent our site exposure mechanism. However, studies of chromatin *in vitro* reveal that histone H1 is in free exchange in physiological conditions (Caron & Thomas, 1981). Consequently, the presence of histone H1 *in vivo* is likely to affect the quantitative values of K_{eq}^{conf}, but is not expected to alter the qualitative nature of the site exposure process revealed in this study.

Another significant difference between our model system and chromatin *in vivo* arises from the fact that chromatin *in vivo* exists in very long chains that are organized into higher order structures. If such higher order structures were inert, the proposed site exposure mechanism could not occur. But physical studies of chromatin fragments *in vitro* suggest that higher order chromatin structures are only marginally stable in physiological conditions (Widom, 1986), and that the compact state is in dynamic equilibrium with an extended nucleosome filament state (Widom, 1989). Thus, it seems likely that higher order chromatin structure, like the presence of histone H1, may alter quantitative but not qualitative aspects of our conclusions.

The present studies are carried out in non-physiological solution conditions, including 10 mM Mg^{2+} and 50 or 100 mM NaCl. These conditions are requirements of our assay. Chromatin is considered to be relatively insensitive to specific ion effects (van Holde, 1989; Widom, 1989), but the polyelectrolyte properties of DNA cause the affinities of protein–nucleic acid interactions, and hence the measured values of K_{eq}^{conf}, to vary somewhat with the solution conditions.

The absence of a clear temperature dependence in our results suggests that the elevated temperatures used in many of the measurements in this study do not greatly affect the measured values of K_{eq}^{conf}, consistent with previous hydroxyl radical footprinting studies which showed the basic nucleosomal organization and positioning to be stable to at least 75°C (Bashkin *et al*., 1993). Importantly, our native gel analysis revealed that the particles remain intact even after prolonged incubation at the highest temperature studied, and our data for K_{eq}^{conf} obtained at 37°C are in reasonable agreement with the data obtained at higher temperatures.

We conclude that non-physiological aspects of our model studies will cause our measured values of K_{eq}^{conf} to differ quantitatively from those that obtain *in vivo*, but these differences are not expected to change the qualitative nature of the site exposure mechanism or its consequences.

Relation to earlier studies

Earlier studies which are consistent with our new results were carried out by Linxweiler & Horz (1984). In that study, only small extents of digestion were obtained and a possible requirement for site exposure was not discussed, hence equilibrium constants for site exposure were not provided. The authors estimated that the rate of cleavage at the dyad was greater than 1000× slower than for naked DNA, which is in accord with our observation of $K_{eq}^{conf} \approx 10^{-4}$–$10^{-5}$. For another site located a short distance in from the end of a nucleosome core particle, those investigators detected cleavage at a rate $\approx$50× slower than for naked DNA, in good agreement with our measured $K_{eq}^{conf} \approx 1 \times 10^{-2}$ to 4×10^{-2}.

Relation to equilibrium binding studies

The site exposure mechanism revealed in this study provides a simple and definite mechanism with which previous studies in the literature may be analyzed. Several previous studies have examined the ability of a variety of regulatory proteins to bind to nucleosomal target sequences. However, in the absence of a model for the binding process, the meaning of the results is unclear. The present work provides a natural explanation for this earlier work. We suggest that the observed binding occurs by the site exposure mechanism (Figure 1(a) and equation (1a)). In this case, we predict that the net free energy of binding will be the sum of the free energy of binding to naked DNA and the free energy of the required nucleosome conformational transition; similarly, we predict that the effective, or apparent, dissociation constant for binding will be given by the dissociation constant for binding to naked DNA divided by the equilibrium constant for site exposure measured in the present study (equation (3)).

Table 2. Comparison of measured and predicted dissociation constants

Protein	Approximate K_d for naked DNA	Location of site (bp from end)	Approximate K_d for core particle	Predicted K_d for core particle
GAL4-AH	3×10^{-9} (1)	21	2×10^{-8}[a] (2)	3×10^{-7}
	3×10^{-9} (1)	32	1×10^{-6} (1)	3×10^{-6}
	3×10^{-9} (1)	43	3×10^{-7} (1)	3×10^{-6}
	1.2×10^{-9} (3)	≈60	2.4×10^{-7} (3)	1.2×10^{-5}
	3×10^{-9} (1)	73	3×10^{-5}[a] (4)	3×10^{-5}
TBP	1×10^{-9} (5)	73	$>2\times10^{-6}$ (5)	1×10^{-5}
MerR	2.8×10^{-11} (6)	15	1.5×10^{-9} (6)	2.8×10^{-9}
Max	5.3×10^{-10} (7)	≈73	2.5×10^{-6} (7)	5.3×10^{-6}
Myc[b]	1.4×10^{-9} (7)	≈73	$>2.1\times10^{-6}$ (7)	1.4×10^{-5}
Myc[b]/Max	1.5×10^{-9} (7)	≈73	$>3\times10^{-6}$ (7)	1.5×10^{-5}
Myc[b]–GCN4	1×10^{-9} (7)	~73	1.1×10^{-7} (7)	1×10^{-5}
GR	3×10^{-10} (8)	43	7×10^{-10} (8)	3×10^{-7}

Binding constants measured for several transcription factors to reconstituted mononucleosomes have been compiled from the literature and are presented here. Data taken from the following references: (1) Cote *et al*. (1994); (2) Vetesse-Dadey *et al*. (1994); (3) Taylor *et al*. (1991); (4) Kwon *et al*. (1994); (5) Imbalzano *et al*. (1994); (6), J.W. unpublished results; (7) Wechsler *et al*. (1994); (8) Perlmann (1992).

[a] Values which were calculated by us from data within each paper; all other values were reported by the authors.

[b] Myc in this Table refers to a truncated form of the c-Myc protein. Myc/Max is a heterodimer of truncated c-Myc and Max; Myc-GCN4 is a hybrid protein. Binding data for TBP taken from experiments which include TFIIA in the binding reactions. For Gal4-AH (Taylor *et al*., 1991) and Max (Wechsler *et al*., 1994), the rotational positioning of sites is found not to influence the apparent K_d.

We have collected quantitative or semi-quantitative data available in the literature (together with one unpublished result from our own laboratory) to test these conclusions. This analysis is complicated by ambiguities or uncertainties in the nucleosomal locations of the target sequences and in the measured affinities. For each case, we compare the approximate apparent dissociation constant for binding to a nucleosomal target sequence, to an apparent dissociation constant that we predict using equation (3). The predictions require a measured dissociation constant for binding to a naked DNA target site, which we take from the literature; and they require an estimate of K_{eq}^{conf} which we take from the results of the present study, making the best estimates that we can about the appropriate nucleosomal positions of the target sequences. Variations in the ionic conditions from one study to another, and differences in the conditions between those studies and the present study, will also contribute to discrepancies in this analysis.

The results of this analysis are summarized in Table 2. The deviations that do exist in this comparison must be considered relative to the 10^4 to 10^5-fold differences in affinity that may obtain between a naked DNA site and a site positioned near the nucleosome dyad. Given this benchmark, we consider that, in general, there is good agreement between the predicted and the measured apparent dissociation constants. We conclude that the site exposure mechanism and equations (2) and (3) provide a framework for the analysis and interpretation of these binding studies.

Apparent exceptions and unresolved questions

These binding studies also suggest certain apparent exceptions to our ideas, and one unresolved question. Of the apparent exceptions, we believe that certain of them are not exceptions at all, while others may not be failures of the model *per se*, but rather may represent the consequences of effects that are not included in the limiting case model of Figure 1.

One apparent inconsistency between published binding studies and the predictions of the site exposure model pertains to the glucocorticoid receptor protein (GR; Perlmann, 1992; Li & Wrange, 1993). GR evidently binds to a site near the nucleosome dyad with a much greater affinity than would be predicted if it were necessary for GR to pay the corresponding free energy cost of site exposure. In those studies, the GR sites were rotationally oriented on positioned nucleosomes *in vitro*, such that the sites face outward. The DNA binding domains of GR dimers are small and chiefly contact one face of the DNA (Luisi *et al*., 1991). It is plausible that GR may bind to nucleosomal target sites without a need for further site exposure, when these sites face out. In that case, the apparent K_d for GR binding to a nucleosomal site would approximate that for naked DNA, as observed. It is critical to appreciate, however, that finite free energies of positioning mean that, *in vivo*, some nucleosomes may have alternate positions such that GR sites are buried (see Introduction). Our new results suggest that GR would be able to bind even if its site was buried, but binding in such cases would occur with substantially reduced affinity.

Another set of possible inconsistencies seem not to be inconsistencies at all. The heat shock transcription factor (HSF) is reported as being unable to bind to a nucleosomal target site (Taylor *et al*., 1991). However, only HSF concentrations up to $\approx10^2\times$ (perhaps $10^3\times$) higher than $K_d^{naked\ DNA}$ were investigated. The HSF sites were placed near the nucleosome dyad, so we anticipate that $K_d^{apparent}$ will

be $\approx 10^4$ to 10^5 times greater than $K_d^{\text{naked DNA}}$. Higher HSF concentrations must be investigated before one can safely conclude that binding cannot occur. Interpretation of studies with HSF is further complicated by a trimer $\leftrightarrow$ hexamer equilibrium that is coupled to DNA binding. Similarly, while Max homodimers are able to bind to nucleosomal targets, c-Myc homodimers and c-Myc/Max heterodimers are reported to be unable to do this (Wechsler *et al*., 1994). However, as for HSF, those experiments do not extend to sufficiently high protein concentrations, and the results obtained are in accord with expectation based on the K_d values for naked DNA and our estimates for K_{eq}^{conf}. Indeed, for c-Myc/Max, trace binding is detected, in accord with [c-Myc/Max] $\ll K_d^{\text{apparent}}$. Moreover, values for $K_d^{\text{naked DNA}}$ are in general difficult to measure accurately (and the same applies to our K_{eq}^{conf}), hence these studies may not be probing as close to K_d^{apparent} as anticipated; and for systems approaching K_d^{apparent}, the occupancy of a site at equilibrium is a strong function of the concentration.

The present analysis has emphasized translational settings, but, for the case of TATA-binding protein (TBP) (Imbalzano *et al*., 1994), the ability to bind to a nucleosomal target site (in the presence of TFIIA, SWI/SNF, and ATP) is found to depend on the rotational orientation of the site. We emphasize two important points. First, the rotational orientation of a site is expected in general to be explicitly important, simply because proteins occupy volume, and therefore (in the context of our unpeeling picture) the length of DNA that must be exposed in order to allow binding will depend on the rotational setting of a binding site and the physical size and shape of the protein that needs to bind there. Sites that face ''inward'', and proteins having larger sizes or particular shapes, may require that more DNA be unpeeled in order for binding to be allowed. This extra unpeeling comes at greater free energy cost and leads to an increase in K_d^{apparent}. These effects are presumably present in our own measurements using the restriction enzymes. Second, in the case of TBP, the authors make the plausible suggestion that, for certain rotational settings, the TBP-induced DNA distortion will be greatly hindered by the adjacent histone surface. This is not a failure of our model: rather, it is a clear example of an additional effect that will be important in many cases; in particular, whenever binding of a protein changes the bend of DNA, which is a frequent occurrence.

For the case of Myc–GCN4 homodimers, there appears to be a discrepancy (in the application of our model) compared to the results for Max homodimers binding to the same site (Wechsler *et al*., 1994): organization of the site in a nucleosome near the nucleosomal dyad suppresses the affinity of Max homodimers by $\approx 10^4\times$ in accord with our expectations (Table 2). But binding of Myc–GCN4 fusion protein homodimers to the same site is suppressed by only $\approx 10^2\times$. Why can Myc–GCN4 bind with significantly higher affinity? Three possible explanations (in the context of our unpeeling picture) are as follows. (1) Perhaps $(\text{Myc–GCN4})_2$ is smaller or has a more favorable shape than does $(\text{Max})_2$, such that less DNA needs to be unpeeled. (2) Perhaps there are differences in factor-induced DNA bending, so that the bending resulting from Myc–GCN4 is accommodated in the particle with lower free energy cost. (3) Perhaps the Myc–GCN4 has a favorable free energy of oligomerization and multiple molecules are binding to DNA, in which case the experiment needs to be analyzed in the context of the multi-site cooperative binding model discussed below. Some observations discussed by the authors supports this latter interpretation (Wechsler *et al*., 1994).

The results of the present study also raise an interesting unresolved question concerning the stability of the nucleosome core particle. The problem arises from the mechanism that we envision for site exposure, which is that internal sites are reached by unpeeling from one end. If sites near the nucleosome dyad are exposed with probability (K_{eq}^{conf}) equal to 10^{-4} to 10^{-5} by this mechanism, then, with the square of this probability, both DNA ends might be expected to simultaneously unpeel into the dyad, leading to dissociation. While the timescale of this hypothetical process is not known, its quite moderate probability suggests a fundamental problem for nucleosome stability. Perhaps the unpeeling mechanism of exposure is wrong or misleading; or perhaps the affinity of DNA segments for the histone octamer depends on whether DNA at the other end of the core particle is bound or exposed at any moment. Some experimental and theoretical studies that bear on this important question have been carried out (Yager & van Holde, 1984; Yager *et al*., 1989; Marky & Manning, 1991; 1995), and further studies are needed.

Finally, among all 13 sites that we probed using ten different enzymes, one site (*Msp*I, in construct (b)) had a substantially lower value of K_{eq}^{conf} than did all of the others, despite being flanked on either side by sites having higher values of K_{eq}^{conf}. With the exception of this one site, there appears to be a progressive decrease in site exposure from the end of the core particle in toward the center. It remains to be seen whether this represents a real break in the otherwise consistent trend in the translational dependence to K_{eq}^{conf}. The explanations given above for differences in transcription factor binding apply equally to restriction enzymes, and one or more of them may be operative in this case.

New roles for nucleosome positioning, posttranslational modifications of histones, and particular histone variants

The present study suggests an important new role for the forces and principles that together affect the statistical and time-averaged positioning of nucleosomes along the genome (Yao *et al*., 1993). The analysis outlined in the Introduction leads one to conclude that static and perfect positioning, which

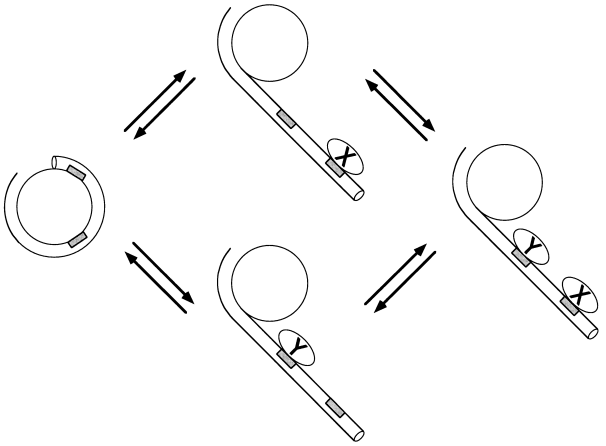

Figure 8. Model for cooperative (synergistic) binding of multiple regulatory proteins. The model depicts a hypothetical case in which the DNA of a particular nucleosome has sites for two sequence-specific DNA-binding proteins, X and Y; X and Y may be two different molecules of the same protein, or two entirely different proteins. The binding of X and Y is linked in a thermodynamic cycle; each facilitates the binding of the other, with no special requirement for additional protein–protein contacts or other specialized properties.

is often implied, is unlikely to occur. Nevertheless, nucleosomes do occur with substantial statistical occupancies at particular locations (Simpson, 1991), and the present study reveals that such positioning causes the effective dissociation constant for binding by regulatory proteins to vary over several orders of magnitude. In this way, even very modest forces, such as particular DNA sequence preferences inherent to histone octamers, which affect the statistical positioning of nucleosomes but which are far too small to confer perfectly precise positioning, nevertheless may have substantial regulatory consequence.

The site exposure mechanism also suggests a natural way that posttranslational modifications of the histones or the presence of particular histone variants (van Holde, 1989; Wassarman & Kornberg, 1989) could be coupled to gene regulation. It is plausible that certain posttranslational modifications of the histone octamer or the presence of certain histone variants may alter the effective rate constants or the dissociation constant for regulatory protein binding. These may be considered to be mechanisms of active invasion (see below). Modifications to histone H1 (or the presence of particular H1 variants) that alter its binding affinity to the nucleosome core may have analogous consequences.

Cooperative binding of regulatory proteins without protein–protein contacts

The site exposure mechanism leads to a surprising possible synergy or cooperativity in the binding of multiple regulatory proteins to sites on a single nucleosome. Such a process is illustrated in Figure 8. The net free energy change is given by

$$\Delta G^0_{\text{net}} = \Delta G^0_{\text{conf}} + \Delta G^0_{\text{X, naked DNA}} + \Delta G^0_{\text{Y, naked DNA}}, \quad (17)$$

where ΔG^0_{conf} is the free energy of the conformational change required to allow binding of both X and Y; $\Delta G^0_{\text{X, naked DNA}}$ and $\Delta G^0_{\text{Y, naked DNA}}$ are the free energies for binding of X and Y, respectively, to naked DNA. In this illustration, binding of protein X may take place with no further conformational transition once protein Y has bound; hence, binding of Y increases the apparent affinity of X for its nucleosomal target site. Conversely, the ability of X to bind facilitates the binding of Y, since at least some of the final cost in ΔG^0_{conf} is already paid. (Note that the two binding events are linked in a thermodynamic cycle.) The important conclusion is that X and Y may act cooperatively (synergistically) even if they do not touch: the binding of one protein radically alters the binding ability of the other. No special properties are required of X or Y: they need only bind DNA for this cooperativity to be manifested. X and Y may be two different proteins, or they may be two molecules of the same protein. One example of this phenomenon in the literature may be the synergistic effects of multiple GAL4 binding sites within a single nucleosome (Taylor *et al*., 1991). This idea provides a natural explanation for the clustering of regulatory protein binding sites in eukaryotic genomes.

This synergy (cooperativity) is a necessary consequence of the site exposure mechanism if, and only if, site exposure occurs simultaneously at multiple sites. If the site exposure process somehow operates in such a way as to expose individual sites while even closely positioned flanking sites are kept occluded, then cooperativity would not be observed.

Mechanisms for active invasion

Existing ideas on active invasion have two limitations: there is a lack of definite mechanisms which can be tested experimentally and there remains the underlying question of how the molecules know which nucleosomes are to be actively invaded. Our site exposure mechanism provides a framework for analysis of the proposed phenomenon of active invasion. We distinguish three different classes of models in which some protein factor ''A'', having a capacity of active invasion, may facilitate the binding or action of a regulatory protein. Several researchers have recently reported that certain proteins or protein complexes are able to facilitate the binding of regulatory proteins to nucleosomal target sequences in an ATP-dependent manner (Cote *et al*., 1994; Imbalzano *et al*., 1994; Kwon *et al*., 1994; Pazin *et al*., 1994; Tsukiyama *et al*., 1994). The requirement for ATP is strongly suggestive of active invasion. At this time, however, there is not enough additional information available to relate these observations to the specific mechanisms outlined here.

Model I: nucleosome displacement

In this model, the site-exposure mechanism (equation (1a)) allows a sequence-specific regulatory protein R to bind at its recognition site in accord with equation (3). Even though the occupancy obtained by R alone may be modest, R may recruit a protein or protein complex A, which acts to displace the histone octamer, leading to the binding of additional proteins and assembly of a long-lived complex. This mechanism solves the problem of how A identifies which nucleosome to invade, and it allows for R having profound actions even if its free concentration is quite low compared to $K_d^{apparent}$ (equation (3)).

Model II: catalyzing site exposure

In this model, factor A acts by catalyzing the site-exposure process, so that the overall process is described by mechanisms such as equations (18a) or (18b):

$$N + A \rightleftarrows NA \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} S + A + R \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} SR \qquad (18a)$$

$$N + A \rightleftarrows NA \rightleftarrows N' + A \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} S + R \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} SR \qquad (18b)$$

In these schemes, A effects active invasion catalytically, increasing both rate constants k_{12} and k_{21} for interconversion of N and S, such that $K_d^{apparent}$ remains unchanged. For example, this is one plausible consequence of posttranslational modification of the histones, yielding N' (equation (18b)). This form of active invasion will be significant only if k_{12} or k_{21} are rate limiting and if the complex SR is kinetically stable; whether this situation obtains *in vivo* or *in vitro* remains to be explored.

Model III: driving site exposure

In model III, factor A acts to drive the site exposure process for the binding of R. Rather than catalyzing site exposure, as in model II, A may change the two rate constants k_{12} and k_{21} unequally, such that $K_d^{apparent}$ for subsequent binding of R is decreased (i.e. the affinity for R is increased).

If A binds DNA and remains in the complex, this form of active invasion is an example of the cooperative (synergistic) binding discussed above (equation (17) and Figure 8). This may be referred to as active invasion, but such terminology obscures the underlying mechanism. It is the dynamic activity of the nucleosome itself that makes this mechanism possible; any protein that binds DNA has the capacity of active invasion in this sense.

Alternatively, A could bind to the histones and remain in the complex,

$$N + A \rightleftarrows NA \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} AS + R \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} ASR. \qquad (19a)$$

In this scheme, A drives site exposure by changing $K_d^{apparent}$, but by binding to the histones rather than to DNA, distinguishing this mechanism from the cooperative binding of equation (17) and Figure 8.

In another model of this class (19b), A could again be an enzyme that catalyzes posttranslational modifications of the histones as in mechanism (18b), but in this case, with the result of changing the two rate constants k_{12} and k_{21} unequally, such that $K_d^{apparent}$ for binding of R is decreased. In another example, *in vivo*, conversion of N to N' could represent the enzymatic removal of histone H1.

$$N + A \rightleftarrows NA \rightleftarrows N' + A \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} S + R \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} SR \qquad (19b)$$

In a rather different mechanism for driving site exposure, A binds DNA, but with a mechanism for subsequently removing it from the complex, perhaps by changing A's state of posttranslational modification, yielding *A.

$$N \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} S_1 + A \rightleftarrows AS_2 + R \rightleftarrows ASR \rightleftarrows *ASR \rightleftarrows SR + *A \qquad (19c)$$

S_1, S_2, and S represent differing states of the nucleosome, with only state S being suitable for binding by R. This mechanism is significant only if the complex SR is kinetically stable. In one extreme example of this model, the histone octamer may be entirely displaced from the DNA; note, though that A has acted prior to the binding of R, in contrast to the mechanism in model I.

Relation to other dynamic properties of chromatin

Finally, we note that our site exposure mechanism may play a role in other dynamic processes of chromatin. The site exposure process detected here provides a mechanism for elongation of RNA or DNA polymerase through chromatin: perhaps polymerases advance through a nucleosome's DNA during moments when DNA segments are released from the surface of the octamer (Kornberg & Lorch, 1992). We further postulate that the site exposure mechanism may play a role in nucleosome mobility (Spadafora *et al*., 1979; van Holde, 1989; Meersseman *et al*., 1992), perhaps representing a first kinetic step.

Conclusion

The present studies with restriction enzymes reveal the existence of a mechanism for site exposure that is intrinsic to nucleosomes. The results imply that any protein that binds DNA has the ability to bind to a nucleosomal target sequence. In particular, there is no need to invoke mysterious properties that are unique to transcription factors, although the quantitative value of $K_d^{apparent}$ may depend on properties particular to each protein, such as its size and shape, and DNA bending, as well as on the rotational and translational positions of the binding sites. The idea of site exposure postulated here and supported by the experimental results provides one answer to an important question, namely, what principle guarantees that

regulatory proteins may have access to their target sites in chromatin? Previously, there were no satisfactory answers to this. The site exposure mechanism allows for cooperativity or synergism in the binding of two or more proteins to sites within a nucleosome, even if these proteins do not touch each other. Finally, the site exposure mechanism provides a conceptual framework for an analysis of active invasion.

Materials and Methods

Preparation of DNA constructs

DNA constructs were prepared by polymerase chain reaction (PCR) using the Simpson phasing sequence (from the 5 S RNA gene of sea urchin; Simpson & Stafford, 1983). PCR primers complementary to the two ends of the desired region of the phasing sequence (nucleotides 14 to 163; Simpson & Stafford, 1983) were used; they included specific base changes to produce the desired restriction sites. Six oligonucleotides were purchased to generate four constructs (nucleotide changes capitalized): LE28, aacttccagggatttataagccgatgac; LE36, aacttGAagggatttataagccgatCacgtcataac; RE36, accgCGTCGACCAGtgcttgacttcggtgatcgga; RE64, aaccgagctctatgctgcttgacttcACGCGGTGACCTCGACcTggtatattcagcatggtatg; RE88, aaccgagccctatgctgcgcgactCcAgtgatcggacgagaaccggtGCattcaCcCtggtatagtcgACgTctcttgcttgatgTTa and RE19, aaccgagccctatgctgcg. For constructs (a) and (b), the template for PCR amplification was the 256 bp *Eco*RI restriction fragment originally described (Simpson & Stafford, 1983), purified by ion-exchange HPLC (D. S. Scherl & J. Widom, unpublished results). Construct (a) was produced using the primer pair (LE28, RE36); construct (b) was produced using the primer pair (LE28, RE64). Construct (c) was produced in two steps. First, an intermediate PCR reaction using the same template and the primer pair (LE28, RE88) was carried out, and the product purified by HPLC (see below); this product was then used as a template with the primer pair (LE36, RE19) to produce construct (c). Oligonucleotides were purchased trityl-on and purified on a C–18 reverse phase HPLC column (Vydac 218TP54) using standard methods. After de-tritylation, these primers were used in PCR reactions consisting of: template DNA ($\approx$50 ng ml^{-1}), Tfl polymerase buffer (50 mM Tris-HCl (pH 9.0), 20 mM ammonium sulfate, 1.5 mM $MgCl_2$), primer oligonucleotides (1 μM each), dNTPs (200 μM each), and Tfl DNA polymerase (1 μl enzyme (Epicentre Technologies) per 100 μl reaction). The annealing temperatures and number of cycles were optimized for each reaction. The desired products were HPLC purified on a Mono-Q HR5/5 anion-exchange column using a linear gradient of 0.65 M NaCl in TE (pH 8.0; room temperature) to 0.8 M NaCl in TE, for 90 minutes, at a flow rate of 0.25 ml/min. The purified material was then concentrated on Centricon 30 filters (Amicon) and resuspended in 0.1 × TE. Typical yields from 5 ml PCR syntheses (50 100 μl reactions) were 100 μg of DNA after HPLC purification.

Reconstitution and isolation of core particles

Construct DNA was radioactively labeled with [γ-^{32}P]ATP prior to reconstitution onto the histone octamer. Each labeling reaction was divided in half for use in naked DNA digestions and reconstitutions. Reconstitution reactions (300 μl) contained 100 ng labeled construct DNA, 19.2 μg chicken erythrocyte core particle DNA, 15.5 μg purified chicken erythrocyte histone octamer (Feng *et al*., 1993), 2.0 M NaCl, in 1 mM Tris (pH 8.0), 0.1 mM EDTA (0.1 × TE), supplemented with protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM benzamidine (BZA). The reactions were dialyzed successively (minimum of two hours per step) into 2.0, 1.5, 1.0, and 0.5 M NaCl, followed by an overnight dialysis into 0.15 M NaCl. All buffers contained 0.1 × TE, 0.5 mM PMSF, 1 mM BZA.

Reconstituted core particles were isolated from free DNA and aggregates on 5% to 30% (w/v) sucrose gradients (in 0.5 × TE, 0.5 mM PMSF, 1 mM BZA) which were spun at 41,000 rpm in a Beckman SW41 rotor for 24 hours at 4°C. Each gradient was fractionated into ~0.5 ml fractions and quantified by Cerènkov counting. Fractions containing the reconstituted material were pooled and concentrated on Centricon 30 microconcentrators and resuspended in 300 μl of 0.1 × TE for storage at 4°C. The yield of reconstituted particles after purification was approximately 50% (of input DNA), measured by Cerènkov counting.

Mapping of reconstituted core particles

The translational phasing of the reconstituted core particles was determined by nuclease digestion with or without primer extension, for constructs (a) and (c). Reconstitutes (1 μg of total DNA) were digested with 0.002 Worthington units of micrococcal nuclease in a 50 μl reaction (240 mM Tris-HCl (pH 7.5), 8 mM CaCl) at 37°C. For each timepoint, 5 μl samples were removed, quenched in 100 μl 0.1 × TE, extracted once each with 100 μl phenol:CIPA (1:1, by vol.); CIPA was chloroform:isoamylalcohol (24:1, by vol.) CIPA, and ether, then dried in a Speedvac. The DNA was resuspended in 0.1 × TE and either analyzed directly on sequencing gels, or used as a template in the primer extension reactions.

Primers specific to central portions of the core particle sequence were used to map the regions protected from micrococcal nuclease digestion (D. S. Scherl & J. Widom, unpublished results). The A primer (atcaagcaagagcctacgacc) anneals to the bottom strand and maps cleavage sites at the right end of the construct; the B primer (ggtcgtaggctcttgcttgat) anneals to the top strand and maps cleavage sites at the left end of the construct (see Figure 2). Primer extension reactions were carried out on the isolated template in 20 μl reactions containing 50 mM Tris-HCl (pH 9.0), 20 mM NH_4SO_4, 1.5 mM $MgCl_2$, 1 μM ^{32}P-labeled primer, 200 μM dNTps, and two units of Tfl polymerase. Extension products were analyzed on 6% (w/v) denaturing acrylamide gels using sequencing lanes as exact size standards.

Restriction digests

Reconstituted core particles and naked DNA were digested with restriction enzymes as follows: construct (a): *Taq*$^{\alpha}$I, *Bsr*I, and *Bst*UI at 45°C, 50°C, 55°C, 60°C, and 65°C; construct (b): *Taq*$^{\alpha}$I and *Bst*UI at 45°C and 65°C; construct (c): *Taq*$^{\alpha}$I, *Bsa*HI, *Bsa*JI, and *Bst*UI at 45°C and 65°C. In later studies, construct (a) was analyzed at 37°C with *Alu*I, *Hin*cII, and *Sal*I, and construct (b) was analyzed at 37°C with *Msp*I. All enzymes were obtained from New England Biolabs, and all restriction digests were carried out using buffers provided by NEB. Glycerol was added to the naked DNA digestions and $MgCl_2$ was

added to the reconstitute digestions to achieve identical final concentrations in the parallel reactions. The concentration of glycerol in the reactions never exceeded 5% (v/v); reactions carried out at 5% glycerol continued the first-order dependence on enzyme concentration observed for digestions carried out at lower glycerol (and enzyme) concentrations (see the text).

The specific buffers used for each enzyme are: *Taq*$^{\alpha}$I: 10 mM Tris-HCl (pH 8.4, 25°C), 100 mM NaCl, 10 mM $MgCl_2$, 100 μg ml^{-1} bovine serum albumin (BSA); *Bsr*I: 10 mM Tris-HCl (pH 7.8, 25°C), 150 mM KCl, 10 mM $MgCl_2$, 100 μg ml^{-1} BSA; *Bst*UI, *Msp*I, *Bsa*JI, *Bst*NI: 10 mM Tris-HCl (pH 7.9, 25°C), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT; *Hin*cII; 50 mM Tris-HCl (pH 7.9, 25°C), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μg ml^{-1} BSA; *Sal*I: 10 mM Tris-HCl (pH 7.9, 25°C), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μg ml^{-1} BSA; *Alu*I: 10 mM bis Tris-propane-HCl (pH 7.0, 25°C), 10 mM $MgCl_2$, 1 mM DTT; *Bsa*HI: 20 mM Tris-OAc (pH 7.9, 25°C), 50 mM KOAc, 10 mM MgOAc, 1 mM DTT, 100 μg ml^{-1} BSA.

At various times during the digestion, 10 μl samples were removed and quenched with 40 mM EDTA. Samples were analyzed on denaturing acrylamide gels, and quantified using the phosphorimager. Background values were obtained from appropriate regions between bands on each gel track and were subtracted from the integrals measured for each band.

Data analysis

The fraction of uncut DNA was calculated as follows. For construct (c), the substrate DNA (labeled S in Figure 4) and the two products (labeled P1 and P2 in Figure 4) are simultaneously resolved. We calculate the fraction uncut as (counts in S)/(counts in S + P1 + P2), after background subtraction. This definition is insensitive to variations in gel loading. For constructs (a) and (b), adequate resolution of all three species on a single gel was difficult to obtain. Instead, the gels were run such that the shorter labeled product strand (analogous to P2) was well-resolved from the other two (analogous to S and P1). We calculate the fraction uncut as ((counts in S + P1) – (counts in P2))/(counts in S + P1 + P2) after background subtraction. This definition, too, is insensitive to variations in gel loading. Control studies with naked DNA revealed two problems. The two ends of the construct DNA are not labeled with identical specific activity. This does not affect the analysis of construct (c), for which all three species are well resolved, but it leads to an artificial non-zero (positive or negative) baseline for constructs (a) and (b). This problem was overcome by obtaining an experimental measure of the baseline, from naked DNA digested to completion. Experimental baselines were obtained for each different enzyme. Data for constructs (a) and (b) were fit by least-squares to exponentials that decayed to the corresponding separately determined baseline, whereas data for construct (c) were fit to exponentials that decayed to zero. A second problem was that the enzymes *Bsa*HI, *Bsa*JI, and *Bst*UI, which are recommended for use at 60°C, were found to lose activity during the course of digestions at 65°C. This problem was overcome by restricting the analysis of the 65°C datasets to early times only, during which the reaction continues its first-order decay.

The rate constant obtained from each exponential decay defined k_{obs}. Equilibrium constants for site exposure were calculated from pairs of observed rate constants according to equation (15).

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