

# Effects of Core Histone Tail Domains on the Equilibrium Constants for Dynamic DNA Site Accessibility in Nucleosomes

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The N and C-terminal tail domains of the core histones play important roles in gene regulation, but the mechanisms through which they act are not known. These tail domains are highly positively charged and are the sites of numerous post-translational modifications, including many sites for lysine acetylation. Nucleosomes in which these tail domains have been removed by trypsin remain otherwise intact, and are used by many laboratories as a model system for highly acetylated nucleosomes. Here, we test the hypothesis that one role of the tail domains is to directly regulate the accessibility of nucleosomal DNA to other DNA-binding proteins. Three assays are used: equilibrium binding by a site-specific, DNA-binding protein, and dynamic accessibility to restriction enzymes or to a non-specific exonuclease. The effects of removal of the tail domains as monitored by each of these assays can be understood within the framework of the site exposure model for the dynamic equilibrium accessibility of target sites located within the nucleosomal DNA. Removal of the tail domains leads to a 1.5 to 14-fold increase in position-dependent equilibrium constants for site exposure. The smallness of the effect weighs against models for gene activation in which histone acetylation is a mandatory initial event, required to facilitate subsequent access of regulatory proteins to nucleosomal DNA target sites. Alternative roles for histone acetylation in gene regulation are discussed.

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## Introduction

Each of the four core histone proteins of the nucleosome has ~15–45 amino acid residue positively charged N-terminal “tail” domains that are conserved throughout evolution and are of particular significance because they are the sites for numerous post-translational modifications known to be essential in chromatin function. Among these modifications, histone acetylation is of special interest because it is closely linked to gene activation. Each of the core histone proteins can be acetylated *in vivo* on multiple lysine residues within the N-terminal domains. Many gene-regu-

latory proteins have recently been found to encode histone acetylases or deacetylases, or to act in combination with other proteins that themselves are histone acetylases or deacetylases (Grunstein, 1997; Kuo & Allis, 1998; Struhl, 1998; Turner, 1998; Widom, 1998b; Workman & Kingston, 1998; Wolffe & Hayes, 1999).

The mechanisms through which histone acetylation contributes to gene regulation are not known. Recent evidence suggests that one role of histone acetylation and deacetylation is to control interactions with other proteins that in turn influence gene regulation. It has also been suggested that the N-terminal domains may act directly at the level of individual nucleosomes to control the ability of site-specific regulatory proteins to bind to nucleosomal DNA target sites. One intriguing idea suggested in earlier studies is that histone acetylation could provide an effective all-or-none character to the ability of site-specific DNA-binding proteins to bind to their target sites in nucleosomes: DNA target sites in non-acetylated nucleo-

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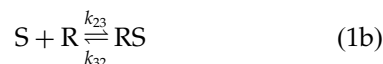
Abbreviations used: SBTI, soybean trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride.

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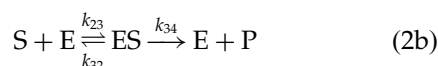
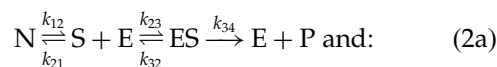
somes may be effectively inaccessible, whereas those same sites in acetylated nucleosomes may be readily accessible to regulatory protein binding (Lee *et al.*, 1993; Godde *et al.*, 1995; Ura *et al.*, 1997). Another study does not support this idea, suggesting instead that acetylation may enhance regulatory protein binding to nucleosomal DNA but that the effects are modest quantitative ones rather than all-or-nothing in character (Vettese-Dadey *et al.*, 1994).

The "site exposure" model for the dynamic behavior of nucleosomes provides a framework for understanding and quantitative analysis of a wide range of phenomena concerning the interactions of DNA-binding proteins with DNA target sites in nucleosomes (Polach & Widom, 1995, 1996; Protacio *et al.*, 1997; Widom, 1998b). These phenomena include equilibrium binding of site-specific proteins to nucleosomal DNA target sites, kinetics of restriction endonuclease digestion of nucleosomal recognition sequences, and kinetics of non-specific exonuclease digestion inward from the ends of the nucleosomal DNA. Within the context of the site exposure model, these disparate experiments are all seen to be equivalent, linked by their requirement for DNA to be partially uncoiled off the surface of the histone octamer prior to DNA sites becoming accessible to a binding protein or nuclease.

Let N represent a nucleosome in its native (most prevalent) conformation. The site exposure model supposes the existence of a transient state S of the nucleosome, at equilibrium with N, in which the nucleosome has transiently uncoiled some of its DNA, such that a DNA target site that previously was inaccessible is now freely accessible to a binding protein R or nuclease E. These can bind to make a complex RS or ES, the latter of which can go on to do catalysis to yield products E and P. We suppose that sufficient DNA is uncoiled so as to make the subsequent steps on the site-exposed nucleosome equivalent to those on naked DNA. Thus:



for equilibrium binding to a nucleosomal target or naked DNA, respectively, and:



for nuclease digestion of nucleosomal or naked DNA, respectively.

Real nucleosomes *in vitro* behave as though such uncoiling processes are occurring constantly yet transiently, in a rapid pre-equilibrium. The uncoiling is non-dissociative; evidently, one side of the DNA remains bound while the other side is exposed. At present, we cannot distinguish whether the detailed mechanism of site exposure is a literal partial uncoiling from one end or a net short displacement of the histone octamer, although available data favor the former hypothesis. As a consequence of this behavior, sites that are buried even within the middle of the nucleosome are nevertheless constantly but transiently accessible. The equilibrium constants for site exposure  $K_{eq}^{conf} (= k_{12}/k_{21})$  decrease from the end of the nucleosomal DNA to the middle. The apparent equilibrium binding affinity for proteins to nucleosomal target sites, and the observed rate constants for digestion of nucleosomal DNA, are reduced from their values on naked DNA by a factor equal to the equilibrium constant corresponding to this site exposure process,  $K_{eq}^{conf}$ . For further discussion of the site exposure model, see Polach & Widom (1998) and Widom (1998a).

The equilibrium constants for site exposure are small, ranging from  $\sim 10^{-2}$ – $\sim 10^{-5}$  for sites located near the outside or the center of the nucleosome, respectively. Thus, for a real situation *in vivo* in which the concentration of free regulatory protein may be low, the actual level of equilibrium binding occupancy achieved on native nucleosomes may be small. The question then arises of whether modifications to the core histone tail domains could substantially increase  $K_{eq}^{conf}$ , such that acetylation would effectively be a mandatory initial event in gene activation.

Nucleosomes in which the tail domains have been removed altogether, by digestion with trypsin, are used by many laboratories as an analog for the possible properties of highly acetylated nucleosomes (e.g. see Lee *et al.*, 1993; Vettese-Dadey *et al.*, 1994). Trypsin removes the N-terminal regions of each core histone that extends beyond the edge of the nucleosome, and the shorter but analogous C-terminal tail of histones H2A (but not that of H3) (Böhm & Crane-Robinson, 1984; Luger *et al.*, 1997a). These N-terminal domains contain all of the lysine residues that are subject to regulated acetylation *in vivo*. Such "tailless" nucleosomes are known to remain otherwise intact *in vitro* (Ausio & van Holde, 1986; Dumuis-Kervabon *et al.*, 1986; Hayes *et al.*, 1991; Luger *et al.*, 1997b). Remarkably, deletions of the N-terminal tail domains made for one core histone type at a time even suffice to allow viability of yeast (Schuster *et al.*, 1986; Kayne *et al.*, 1988; Mann & Grunstein, 1992), i.e. do not detract from gross functioning of nucleosomes even *in vivo*. Yeast cells containing these deletions do give rise to subtle phenotypes. These subtle phenotypes in turn are mimicked by the natural acetylation of tail-domain lysine residues and by mutation of these to residues such as glutamine (which themselves may be mimics of acetyl lysine)

(e.g. see Megee *et al.*, 1990; Thompson *et al.*, 1994); and see Imhof & Wolffe (1998). It is for these reasons that tailless nucleosomes are frequently studied as an analog for the possible effects of acetylated histones.

Here we use such tailless nucleosomes to test the hypothesis that one role of the tail domains is to directly regulate the accessibility of nucleosomal DNA to other DNA-binding proteins. Three distinct assays are used to measure the effects of removal of the tail domains on  $K_{eq}^{conf}$ : one based on equilibrium binding by the DNA-binding domain of the site-specific DNA-binding protein GAL4 (subsequently referred to as GAL4 (1-147)), and two based on dynamic accessibility to restriction enzymes or to a non-specific exonuclease. The results of these three disparate experiments are consistent with each other and show that removal of the tail domains leads to a significant but small increase in the position-dependent equilibrium constants for site exposure within the nucleosome.

## Results

### Preparation and purification of native and tailless nucleosomes

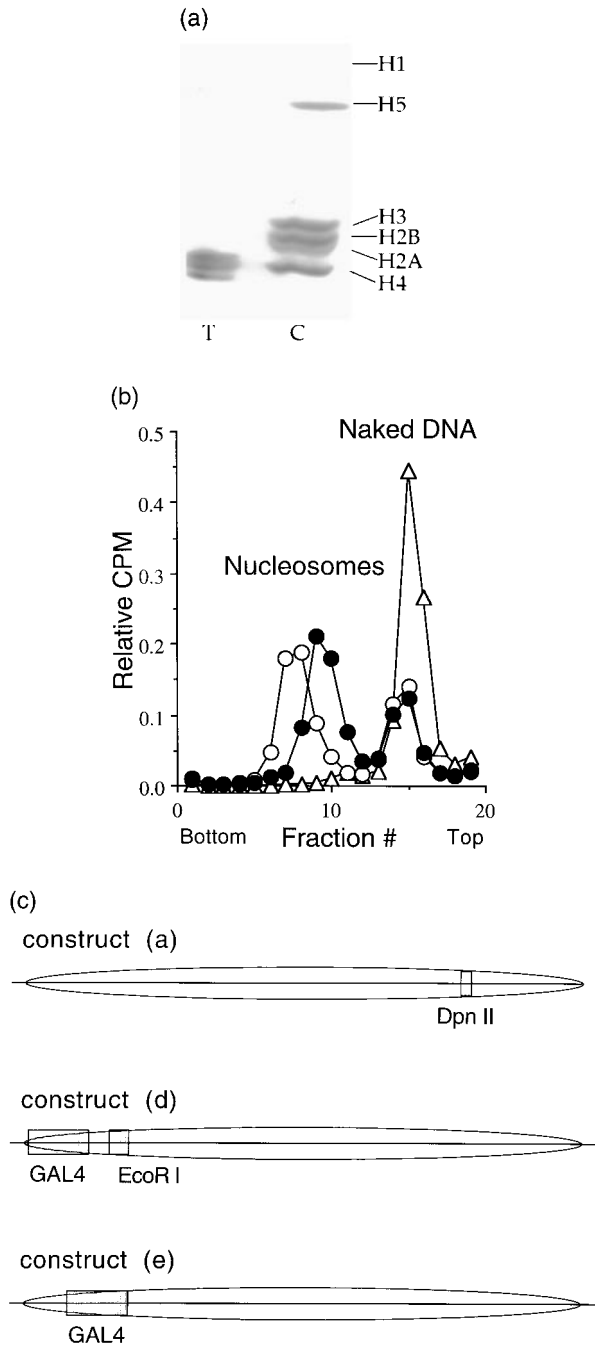
Nucleosomes were assembled onto purified DNA using standard histone octamer exchange procedures. Long H1- (and H5-) depleted chromatin (henceforth referred to as H1-stripped chromatin) served as the histone octamer donor. Tailless nucleosomes were prepared in the same fashion, except the histone octamer donor was long chromatin that had been further treated with trypsin to cleave off the core histone tail domains. A Coomassie-stained protein gel of the native and tailless histones is shown in Figure 1(a). As expected, the four tailless core histones are resolved into only three bands (Luger *et al.*, 1997b). Three different radiolabeled DNA constructs were used, all derived from the well-studied natural nucleosome positioning sequence within the sea urchin 5 S RNA gene (Simpson & Stafford, 1983; FitzGerald & Simpson, 1985). The different DNA constructs are illustrated by Figure 1(c). Construct (a) was described in our previous studies (Polach & Widom, 1995). It is 150 bp in length, with a predominant nucleosome position at residues 4-150. In the present study, we take advantage of the fact that this template includes a target site for the restriction enzyme *DpnII*, located at residues 118-121 on the DNA, i.e. 29-32 bp inward from the right-hand end, for a nucleosome positioned at the known predominant site. Two other constructs, (d) and (e), incorporate sequence changes to introduce target sites for the GAL4 protein and for the restriction enzyme *EcoRI*. (These changes lie outside the region of the 5 S positioning sequence that is responsible for this sequence's nucleosome positioning power (FitzGerald & Simpson, 1985). These constructs also extend the length to 155 bp, adding another 5 bp beyond the end of the preferred

nucleosome location.) Construct (d) incorporates a site for GAL4 at residues 5-21 (i.e. 2-18 bp inside the nucleosome for nucleosomes positioned on residues 4-150) and a site for *EcoRI* at residues 26-31 (i.e. 22-27 bp inside the nucleosome). Construct (e) has a site for GAL4 at residues 15-31 (i.e. 12-28 bp inside the nucleosome).

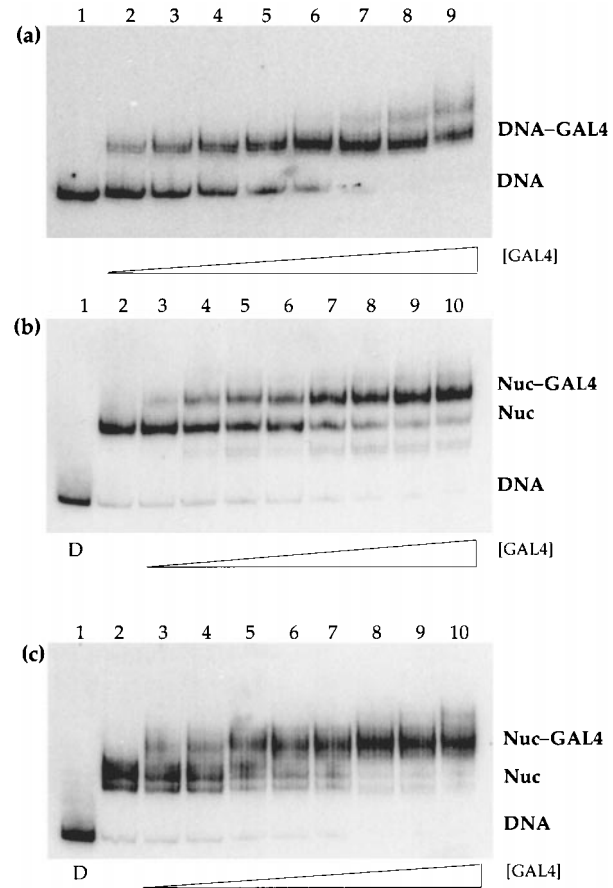
Reconstituted nucleosomes were purified away from the long chromatin histone donor and from any non-nucleosomal products on sucrose gradients. Representative sucrose gradient profiles are shown in Figure 1(b). Nucleosomes reconstituted with native histones comigrate with natural nucleosome core particles (Polach & Widom, 1995). We reproducibly detect a small reduction in sedimentation coefficient for the tailless nucleosomes, possibly due to the substantial reduction in histone mass that occurs when the tail domains are removed or to increased dynamic DNA site exposure or both. Native gel analysis of the nucleosomes after purification (lanes 2 of Figures 2(b) and 3(b)) reveal the expected mobility shift. Nucleosomes reconstituted with trypsinized histones reproducibly reveal a diminished ability to discriminate between alternative nucleosome positions, migrating on high-resolution native gels as a set of two or three closely spaced bands (e.g. lanes 2 of Figures 2(c) and 3(c)). Multiple nucleosome positions can be detected and resolved by native gel electrophoresis even with DNA as short as 146 bp, and with both native and tailless histones (Flaus *et al.*, 1996; Luger *et al.*, 1997b), hence it is not unusual to see such behavior with the longer DNAs utilized in the present study. Quantitative analysis shows the gradient-purified samples to be largely free of contaminating naked DNA (typically 1-3%).

### Equilibrium binding assays

To simplify the analysis of the binding titrations, all of the radiolabeled DNA constructs, whether used as naked DNA or in nucleosomes, are present in binding reactions at concentrations much less than the anticipated dissociation constant for binding by GAL4 (1-147). Consequently, the concentration of free protein is comparable to the total concentration, which is known accurately. Naked DNA and native or tailless nucleosomes were titrated with increasing concentrations of GAL4 (1-147). The resulting products were resolved by native gel electrophoresis and quantified. The results for DNA constructs (d) and (e) are illustrated in Figures 2(a) and 3(a), respectively. Multiple shifted bands are observed, presumably arising from cooperative binding of one or more additional GAL4 (1-147) molecules adjacent to a specifically bound one. Such behavior is routinely observed (e.g. Vettese-Dadey *et al.*, 1994), and can be expected to vary with the particular DNA sequence and experimental conditions. We measure the average or apparent affinity, by monitoring the fraction of unbound DNA (or,



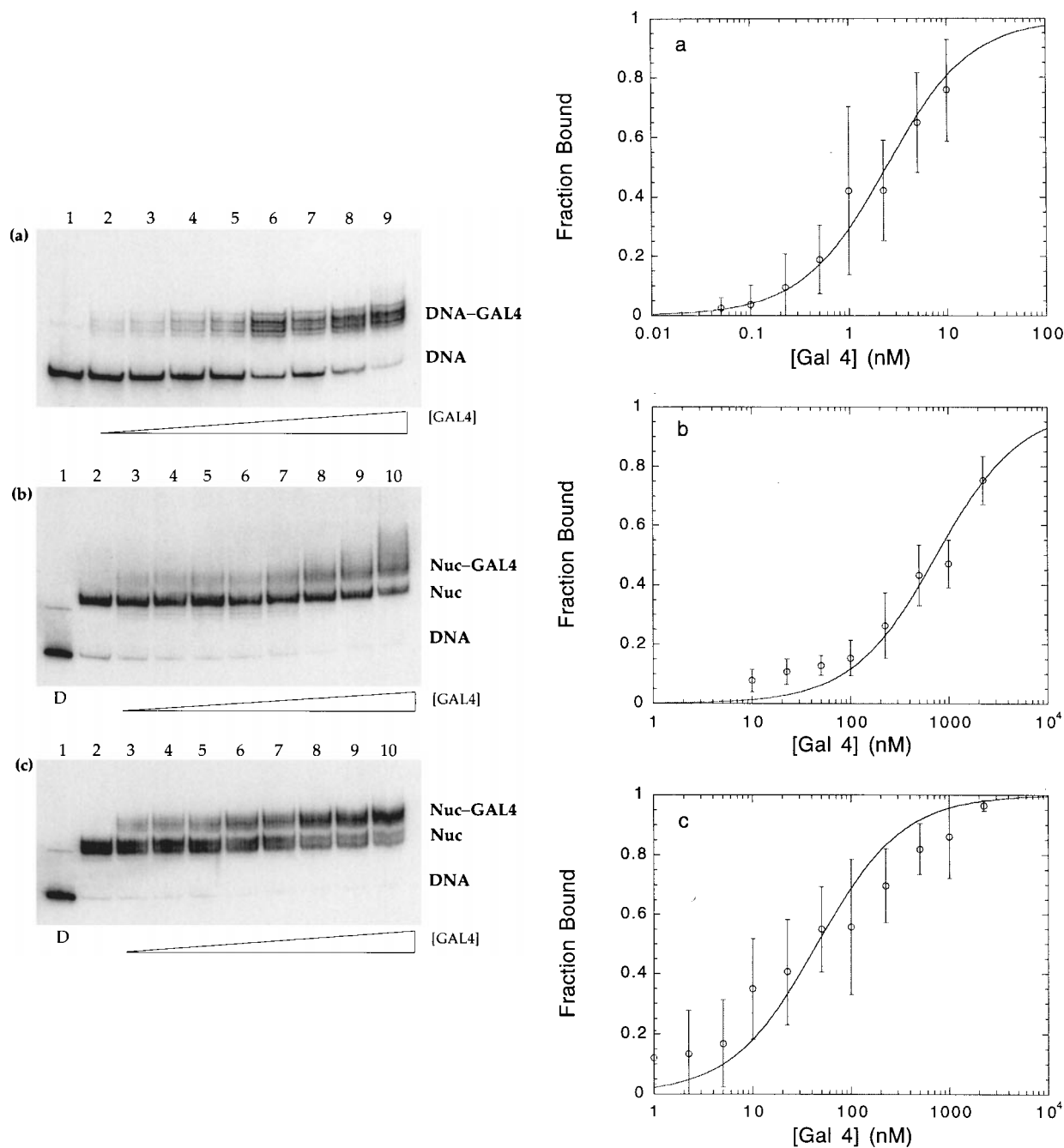
**Figure 1.** Preparation and purification of histones and reconstituted nucleosomes. (a) Coomassie-stained SDS-PAGE analysis of the "tailless" histone octamer (T) compared to native histones in (H1 and H5-containing) long chromatin (C), included here as a standard. The band corresponding to histone H2A in the long chromatin standards stained pink and was not well recorded by the digital camera (hence its fainter appearance). Capillary electrophoresis with UV detection confirms that the four core histones are in fact present stoichiometrically. Fully tailless core histones are expected to yield only three bands in SDS-PAGE (Luger *et al.*, 1997b). (b) Sucrose gradient purification of reconstituted native (○) and tailless (●) nucleosomes compared to naked DNA (△). The graphs show the relative concentration of radiolabeled DNA tracer in each fraction. (c) Schematic of the three different DNA constructs used. The DNA (150 or 155 bp) is illustrated as a line. The major



**Figure 2.** Equilibrium binding titrations on construct (d) for (a) naked DNA, (b) native nucleosomes, and (c) tailless nucleosomes. The Figure shows phosphorimages obtained after native gel electrophoresis. Increasing concentrations of GAL4 protein are added to tracer concentrations of naked DNA, or to native or tailless nucleosomes. (a) DNA: lanes 1-9 correspond to [GAL4] = 0, 0.1, 0.225, 0.5, 1.0, 2.25, 5.0, 10.0, and 22.5 nM, respectively. (b) and (c) Native and tailless nucleosomes: lanes 1 (D) are naked DNA with no GAL4 protein included as mobility standards. Lanes 2 are reconstituted nucleosomes with [GAL4] = 0, included as mobility standards and to allow an assessment of the quality of the purified reconstituted nucleosomes. Lanes 3-10 correspond to [GAL4] = 1.0, 2.25, 5.0, 10.0, 22.5, 50.0, 100.0, and 225.0 nM, respectively.

equivalently, the fraction of DNA in the set of all shifted products) as a function of GAL4 (1-147) concentration. The data obtained from several such titrations on construct (e) are superimposed in Figure 3(a) (right panel), and are summarized quantitatively in Table 1.

nucleosome position is indicated by an ellipse; the locations of *Dpn*II and *Eco*RI cleavage sites and the GAL4 protein-binding sites are illustrated with shaded rectangles.



**Figure 3.** Left: Equilibrium binding titrations on construct (e) for (a) naked DNA, (b) native nucleosomes, and (c) tailless nucleosomes. Other symbols and conditions are as described for Figure 2. Right: Corresponding quantitative analyses. The fraction of nucleosomes having bound GAL4 is plotted as a function of [GAL4] in nM. Datapoints and error bars correspond to mean  $\pm$  one standard deviation from three to five independent experiments. Curves represent the results of non-linear least-squares fits (see Materials and Methods), and were determined using the original datapoints (i.e. without prior averaging of the data, which was done only to simplify the graphs). Note that the GAL 4 concentration range in (a) (naked DNA) differs 100-fold from that in panels (b) and (c), native and tailless nucleosomes, respectively. Both the gel images themselves and the data extracted from them reveal a significant but small increase in the occupancy by GAL4 protein throughout the titration range for the tailless nucleosomes compared to the native nucleosomes, which corresponds to a significant but small increase in affinity.

The results obtained with native nucleosomes are shown in Figures 2(b) and 3(b). At the highest concentration of GAL4 (1-147), additional supershifted products are detected, presumably arising from the non-specific binding of further GAL4 (1-147) molecules as occurs on the naked DNA. We

measure the average affinity of GAL4 (1-147) for the nucleosomal DNA target site by monitoring the fraction of unshifted nucleosomes (or the fraction of nucleosomes in the set of all supershifted products) as a function of GAL4 (1-147) concentration. The small amount of contaminating naked

**Table 1.** Equilibrium binding studies on native and tailless nucleosomes

Sample	Region probed (approx. bp from nucleosome end)	$K_d$ (nM)	$K_{eq}^{conf}$	$K_{eq}^{conf}$ (tailless)/ $K_{eq}^{conf}$ (native)
DNA construct (d)	1-17			
DNA*		0.69 ± 0.11		
Native+		6.6 ± 0.8	1.0 (±0.2) × 10 <sup>-1</sup>	1.5 ± 0.2
Tailless+		4.4 ± 0.4	1.6 (±0.3) × 10 <sup>-1</sup>	
DNA construct (e)	11-27			
DNA+		2.4 ± 0.4		
Native#		750 ± 67	3.2 (±0.6) × 10 <sup>-3</sup>	14 ± 3
Tailless#		54 ± 10.	4.5 (±1.2) × 10 <sup>-2</sup>	

Single fits to combined data from (\*) two, (+) three, or (#) five independent titrations, respectively.  $K_d$ , best-fit dissociation constant and corresponding error for fits to combined datasets.  $K_{eq}^{conf}$ , equilibrium constant for site exposure and corresponding error, obtained from measurements of  $K_d$  on naked DNA and native or tailless nucleosomes. The ratio of  $K_d$  for tailless and native nucleosomes yields the fold-enhancement of binding affinity arising from removal of the trypsin-sensitive tail domains.

DNA also binds GAL4 (1-147) during the titration; these components are omitted for the analysis. The data obtained from several such titrations on construct (e) are superimposed in Figure 3(b) (right panel), and are summarized quantitatively in Table 1. The ratio of apparent binding constants obtained for binding to nucleosomes *versus* to naked DNA yields the apparent equilibrium constants for site exposure. The results for constructs (d) and (e) are in accord with expectation (Polach & Widom, 1995, 1998) and, in particular, show the anticipated decrease of equilibrium accessibility with increasing distance inside the nucleosome.

The results obtained with tailless nucleosomes are illustrated in Figures 2(c) and 3(c). As discussed above, in the absence of any GAL4 (1-147), the tailless nucleosomes migrate as a set of two (construct (d)) or three (construct (e)) closely spaced bands, consistent with occupancy at several predominant positions. Consistent with the gel images themselves, detailed analysis of the intensities of these bands throughout the titrations shows that for both constructs the tailless nucleosomes corresponding to the different bands titrate similarly within experimental error. Therefore, for subsequent analyses these bands were analyzed together; that is, integration regions were chosen

so as to contain the entire set of un-shifted nucleosomal bands. Data from several such experiments are superimposed in Figure 3(c) (right panel) and are summarized quantitatively in Table 1.  $K_{eq}^{conf}$  values are larger for the tailless nucleosomes than for native nucleosomes by 1.5(±0.2)-fold and 14(±3)-fold for constructs (d) and (e), respectively. Thus, as judged by this assay, removing the core histone tail domains increases the accessibility of DNA target sites, but this is a modest quantitative effect rather than an all-or-nothing effect.

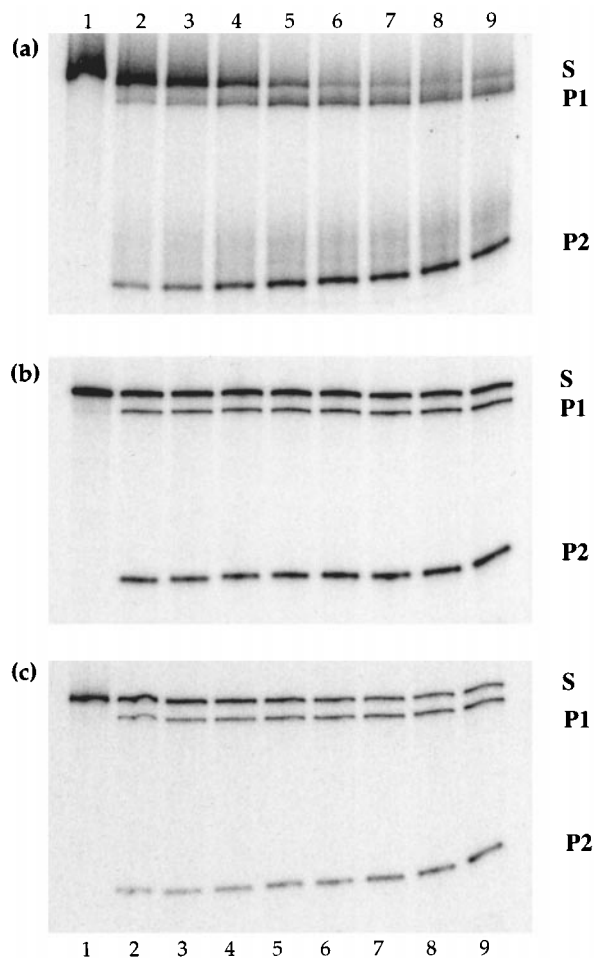
### Nuclease digestion kinetic assays

If this conclusion about the role of the core histone tail domains in governing access to nucleosomal DNA target sites is correct, then according to the site exposure model, comparable effects should be manifested on the observed rate constants for digestion by nucleases. We therefore carried out studies using two restriction enzymes, probing internal sites directly, and using exonuclease III, probing accessibility inward from the nucleosomal DNA ends. Representative results are illustrated in Figures 4, 5, and 6 for *EcoRI*, *DpnII*, and exonuclease III, respectively, and are summarized quantitatively in Table 2. With *EcoRI* as the probe (for a site

**Table 2.** Nuclease digestion kinetic studies on native and tailless nucleosomes

Experiment	Region probed (approx. bp from nucleosome end)	$K_{eq}^{conf}$ (×10 <sup>-4</sup> )	$K_{eq}^{conf}$ (tailless)/ $K_{eq}^{conf}$ (native)
<i>EcoRI</i> digestion, construct (d)			
Native	22-27	1.5 ± 0.4	2.2 ± 1.2
Tailless		2.5 ± 1.0	
<i>DpnII</i> digestion, construct (a)			
Native	29-32	3.9 ± 2.1	4.7 ± 1.8
Tailless		2.1 ± 1.8	
ExoIII digestion, construct (d)	0	n.d.	7.5
	10	n.d.	5.4

$K_{eq}^{conf}$  values (averages ± one standard deviation) from multiple independent experiments.  $K_{eq}^{conf}$  for each independent experiment are obtained from ratios of best-fit rate constants for digestions on native or tailless nucleosomes and naked DNA, scaled by the enzyme concentrations used (see the text). The ratio  $K_{eq}^{conf}$  (tailless)/ $K_{eq}^{conf}$  (native) reported is the average (± one standard deviation) of values obtained from the independent experiments (not the ratio of the average values reported in the Table). It yields the fold-enhancement of DNA site accessibility arising from removal of the trypsin-sensitive tail domains. Additional studies with exonuclease III agree qualitatively but were not analyzed quantitatively.



**Figure 4.** Kinetics of digestion by restriction endonuclease *EcoRI* on construct (d) for (a) naked DNA, (b) native nucleosomes, and (c) tailless nucleosomes. The Figure shows phosphorimages obtained after denaturing gel electrophoresis. (a) Digestions on tracer concentrations of naked DNA; lanes 1-9 correspond to samples removed after 0, 0.5, 1, 2, 4, 8, 12, 16, 20 minutes of digestion with 10 units ml<sup>-1</sup> *EcoRI*. (b) and (c) Digestions on native and tailless nucleosomes, respectively; lanes 1-9 correspond to samples removed after 0, 1, 2, 4, 8, 16, 32, and 64 minutes of digestion with 10,000 units ml<sup>-1</sup> *EcoRI*. The 150 nt substrate DNA (S) is cleaved over time to yield products of length 119 nt (P1) and 27 nt (P2). All reactions were carried out at 37°C.

located 22-27 bp inside the nucleosome), removal of the tail domains increased  $K_{eq}^{conf}$  from 1.2- to 3.9-fold in four independent experiments, with an average of 2.2-fold. With *DpnII* (probing a site 29-32 bp inside the nucleosome), removal of the tail domains increased  $K_{eq}^{conf}$  from 3.4-fold and 6.0-fold (average 4.7-fold). With exonuclease III, loss of the 147 nt full-length band (probing  $K_{eq}^{conf}$  at the ends of the nucleosome core particle DNA) was increased 7.5-fold with removal of the tail domains, while loss of the 137 nt band (probing invasion beyond 10 bp

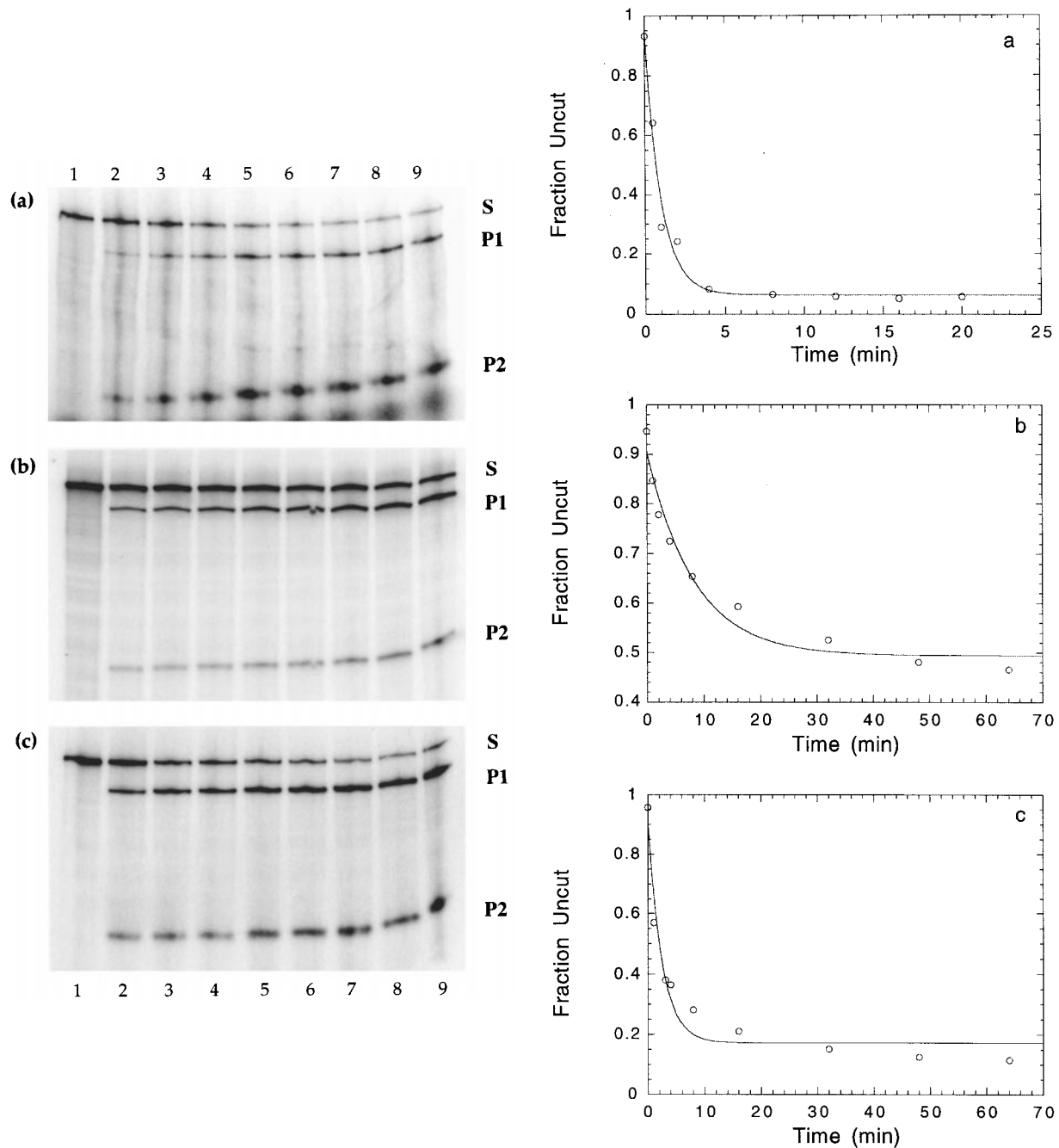
inside the nucleosome) was enhanced 5.4-fold. Additional studies with exonuclease III were in qualitative agreement with these findings, but were not analyzed quantitatively.

## Discussion

The effects of removal of the tail domains as monitored by each of the three assays can be understood within the framework of the site exposure model for the dynamic equilibrium accessibility of target sites located within the nucleosomal DNA. All three experiments point to the same conclusion: removal of the tail domains leads to a significant but small (1.5 to 14-fold) increase in the position-dependent equilibrium constants for site exposure ( $K_{eq}^{conf}$ ).

The smallness of the effect weighs against models for gene activation in which histone acetylation is a mandatory initial event, required to facilitate subsequent access of regulatory proteins to nucleosomal DNA target sites. As described in the Introduction and in our earlier studies (Polach & Widom, 1995, 1996), the values for  $K_{eq}^{conf}$  obtained for native nucleosomes are all small, especially for sites further inside the nucleosome. Thus it seemed likely that the tail domains could be acting essentially in an all-or-nothing fashion to suppress the accessibility of the nucleosomal DNA, and that removal or acetylation of the tail domains would greatly enhance this accessibility. And indeed earlier qualitative studies had reported such all-or-nothing effects on the binding of various transcription factors (Lee *et al.*, 1993; Godde *et al.*, 1995; Ura *et al.*, 1997). However, our finding that removing the tail domains altogether increases  $K_{eq}^{conf}$  only 1.5 to 14-fold is not consistent with such an all-or-nothing character to the expected consequences of acetylating the tail domains. These new findings are supported by results from three independent quantitative assays.

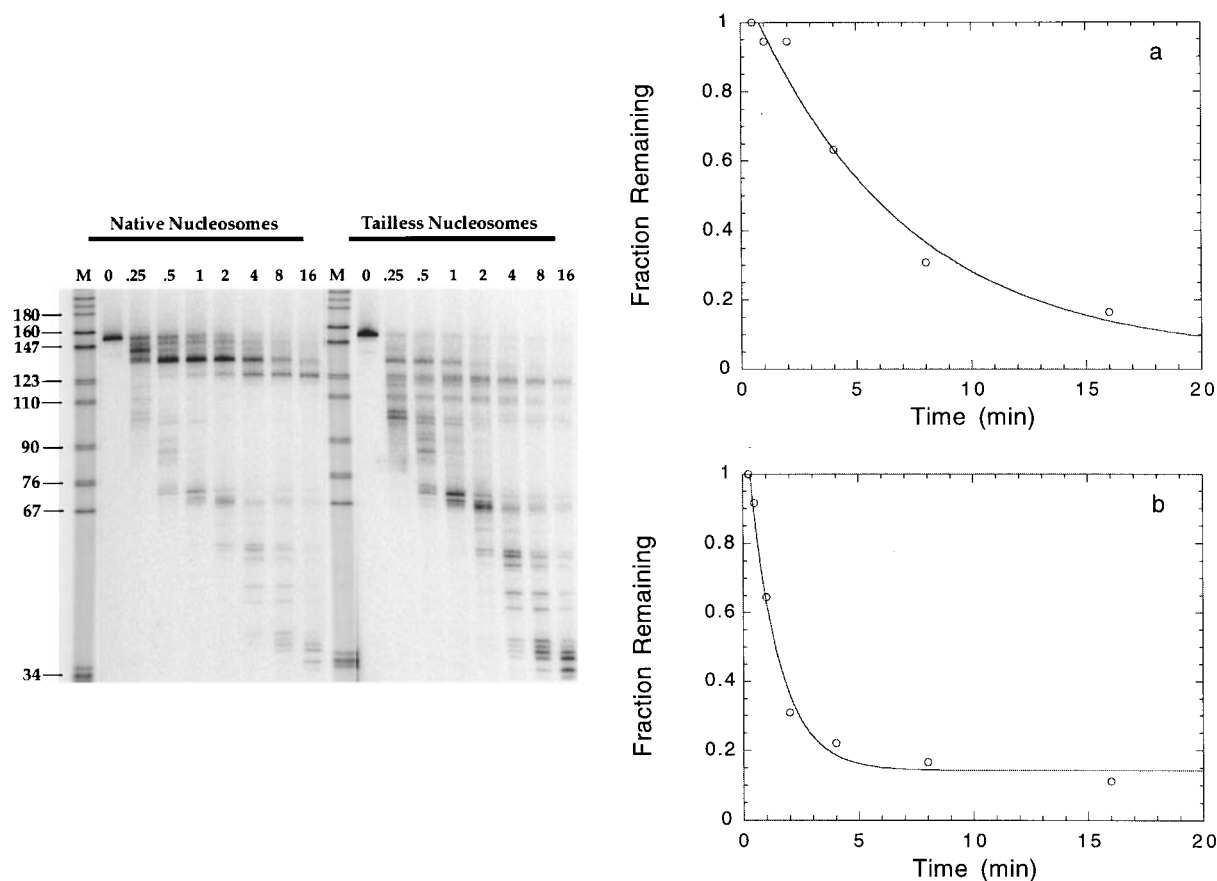
We find that removing the core histone tail domains reduces the ability of the histone octamer to discriminate between nucleosome positions. The predominant positioning remains the same in the tailless nucleosomes (Protacio & Widom, 1996; Protacio *et al.*, 1997; R.U. Protacio & J.W., unpublished results), but there is a modestly increased population of alternative positions. Since the accessibility of nucleosomal DNA target sites is dependent on their location within the nucleosome (Polach & Widom, 1995; Anderson & Widom, 2000), the small changes in accessibility ( $K_{eq}^{conf}$ ) that are revealed in the present study could be, at least partly, attributable to indirect effects of changes in the distribution of nucleosome positions rather than direct effects of the removal of the tail domains on DNA accessibility. Thus a major conclusion of this study is that the effects of deletion of the tail domains is, at most, a modest increase in the time-averaged accessibility of nucleosomal target sites. Actually, though, the available data



**Figure 5.** Left: Kinetics of digestion by restriction endonuclease *DpnII* on construct (a) for (a) naked DNA, (b) native nucleosomes, and (c) tailless nucleosomes. The Figure shows phosphorimages obtained after denaturing gel electrophoresis. (a) Digestions on tracer concentrations of naked DNA; lanes 1-9 correspond to samples removed after 0, 0.5, 1, 2, 4, 8, 12, 16, 20 minutes of digestion with 10 units  $\text{ml}^{-1}$  *DpnII*. (b) and (c) Digestions on native and tailless nucleosomes, respectively; lanes 1-9 correspond to samples removed after 0, 1, 2, 4, 8, 16, 32, and 64 minutes of digestion with 5000 units  $\text{ml}^{-1}$  *DpnII*. The 150 nt substrate DNA (S) is cleaved over time to yield products of length 116 nt (P1) and 30 nt (P2). All reactions were carried out at 37 °C. Right: Corresponding quantitative analyses from one such experiment. The fraction of substrate remaining (i.e. not yet cleaved by the restriction enzyme) is plotted as a function of time. Curves represent the results of non-linear least-squares fits (see Materials and Methods).

suggest that the effects that are observed are not primarily attributable to changes in positioning. As summarized in Results, the GAL4 (1-147) binding experiments allow resolution of the binding behavior of the distinct positioning isomers, and analysis of the isomers individually did not support a

significant difference between them. Thus the significant (although modest) differences that are observed between the native *versus* tailless nucleosomes are unlikely to be attributable to changes in nucleosome positioning of a fraction of the nucleosomes.



**Figure 6.** Left: Kinetics of digestion by exonuclease III on native nucleosomes compared to tailless nucleosomes (construct (d)). The Figure shows phosphorimages obtained after denaturing gel electrophoresis. Samples removed prior to addition of exonuclease III (0) or after 0.25, 0.5, 1, 2, 4, 8, 16 minutes of digestion at 37 °C. A high concentration of exonuclease III (1000 units ml<sup>-1</sup>) is used to digest most of the native nucleosomes from the full-length (155 nt) substrate to a relatively strongly protected ~145 nt species (the core particle length as sensed by the exonuclease) within the first time-point. (There is also a transient accumulation of a longer intermediate.) At subsequent timepoints, the 145 nt species is digested further to a more strongly protected ~137 nt species, and from there to even smaller products. This allows the decays of the 147 nt and subsequently formed 137 nt species to be followed separately. Under the identical conditions, digestion of the tailless nucleosomes proceeds significantly (but only ~fivefold) more quickly. M, size standards (*MspI* digests of the plasmid pBR322, New England BioLabs). Right: Quantitative analyses of digestion kinetics of the 137 bp intermediate from one such experiment on native and tailless nucleosomes, (a) and (b), respectively. The fraction of substrate remaining (i.e. not yet cleaved by the restriction enzyme) is plotted as a function of time. Initial timepoints (during which significant amounts of precursor to the 137 nt product remain) are omitted from the analysis. Curves represent the results of non-linear least-squares fits (see Materials and Methods).

### Are tailless nucleosomes an appropriate model system?

The present study was designed to ask whether the histone N-terminal domains might ordinarily act to make nucleosomal DNA effectively inaccessible to DNA-binding proteins, in which case histone acetylation might abrogate this repression. In the simplest mechanisms through which histone acetylation could abrogate tail domain-induced repression (e.g. the tail domains pack over the nucleosomal DNA so as to prevent site exposure processes from occurring, and tail domain acetylation disrupts this packing), the effects of acetylation could never exceed the effects of removing the tail domains altogether. However, a formal possibility is that particular patterns of histone

N-terminal domain acetylation could have an even more pronounced effect on the structure and dynamics of individual nucleosomes than does removing the tail domains altogether.

We and others who have utilized this model system in the past cannot rigorously exclude this possibility but consider it unlikely. A possible mechanism of this type is if acetylated tail domains might themselves bind to and occlude part of the “helical-ramp” DNA-binding surface formed by the core histone globular domains, thereby destabilizing the wrapping of the DNA. If such intramolecular binding required a specific pattern of tail domain acetylation, then effects arising from such specifically acetylated states could differ from those of simply deleting the tail domains altogether.

Two lines of evidence argue against this possibility. First, no such interaction has been detected despite biochemical studies *in vitro* and genetic ("two-hybrid") experiments *in vivo*, both of which have successfully detected other interacting partners of the tail domains. Second, as mentioned in the Introduction, many cases are now known in which the effects of large histone N-terminal deletions, mutation of one or more histone N-terminal lysine residues to glutamine, or natural histone acetylation yield similar phenotypes in living yeast cells. In those cases where the effects differ, the data implicate interactions between histone tail domains and other, non-histone proteins (Grunstein, 1997; Kuo & Allis, 1998; Struhl, 1998; Turner, 1998; Widom, 1998b; Workman & Kingston, 1998; Wolffe & Hayes, 1999). Thus at this time there is no basis for supposing that, at the level of a single nucleosome, tail domain acetylation should have an effect greater than that of simply deleting the domains. In particular, for the present study, the tailless histones provide a useful and appropriate model system.

### Alternative roles for histone acetylation

If a role of histone acetylation is not to govern in an all-or-nothing manner the subsequent ability of regulatory proteins to bind to nucleosomal DNA target sites, what alternatives remain? Several alternatives have been suggested; we note that these are not mutually exclusive. (i) The results of the present study leave open the possibility that acetylation, prior or subsequent to regulatory protein binding, may have an important role of modestly increasing affinities through modest increases in  $K_{eq}^{conf}$ . (ii) Acetylation may act at higher levels of chromatin folding (Fletcher & Hansen, 1995; Tse *et al.*, 1998), destabilizing the condensed chromatin fiber, thereby decreasing the free energy penalty that otherwise must be paid to allow regulatory protein binding, concomitantly increasing the apparent affinity. (iii) A chief role of acetylation may be to regulate the binding of other (non-histone) proteins to the histones in chromatin through direct protein-protein contacts between these other proteins and specifically acetylated (or deacetylated) histone N-terminal domains. In each of these examples, it is important to recognize that even few-fold changes in affinity can be biologically important, and also that relatively small effects may combine to yield a large dynamic range of gene regulation.

## Materials and Methods

### Preparation of H1- (and H5-) depleted native and trypsinized chromatin donor

Long chicken erythrocyte chromatin was prepared as described (Widom, 1986; Lowary & Widom, 1989). To remove histones H1 and H5, the chromatin was brought to 0.65 M NaCl in TE buffer (10 mM Tris, 1 mM EDTA,

pH 8.0) and loaded onto a Sephacryl S-200 sizing column (5 cm × 45 cm) running at 1 ml min<sup>-1</sup> in 0.65 M NaCl in TE buffer. Peak fractions (that were also free of detectable H1 and H5) were located by SDS-PAGE, pooled, concentrated on Centrprep 10 filters (Amicon), and dialyzed into 25 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. This material, in which the core histone tail domains remain intact, was used as the histone octamer donor in exchange reactions to generate labeled nucleosomes containing intact tails, and further served as the starting point for preparation of the tailless histone octamer donor.

Tailless histone octamer donor-chromatin was prepared by digesting the long H1 and H5-depleted chromatin at 1 mg ml<sup>-1</sup> in 50 mM Tris-HCl (pH 8.0) with 0.4 μg ml<sup>-1</sup> TPCK-treated trypsin for two hours at 37°C. The digestions were quenched with a 20-fold molar excess of soybean trypsin inhibitor (SBTI). The resulting tailless chromatin was concentrated (and purified away from the small peptide products of trypsin digestion) on centricon 100 filters and exchanged into 0.5 × TE buffer containing 0.5 mM PMSF and 0.008 mg ml<sup>-1</sup> SBTI. SDS-PAGE analysis of the two chromatin stocks reveals them to be free from residual contaminating H1 and H5, and shows the complete conversion of the intact core histone proteins to the tailless histone proteins.

### Reconstitution and purification of nucleosome core particles

Nucleosome core particles were reconstituted with radiolabeled DNA using the exchange method (Shrader & Crothers, 1989, 1990; Widlund *et al.*, 1997; Thåström *et al.*, 1999), with the long H1 and H5-depleted chromatin, having or lacking the core histone tail domains, serving as the histone donor. Exchange reactions were carried out as follows: 100 ng of radiolabeled probe was added to a solution of 1 mg ml<sup>-1</sup> donor chromatin in 1.0 M NaCl, 0.01 mg ml<sup>-1</sup> SBTI, in a 25 μl reaction. The reactions were incubated at 37°C for 30 minutes, then the NaCl concentration was reduced by stepwise addition of dilution buffer (0.5 × TE buffer, 5 mM NaCl, 0.01 mg ml<sup>-1</sup> PMSF, 0.01 mg ml<sup>-1</sup> benzamidine hydrochloride and 0.01 mg ml<sup>-1</sup> SBTI) to a final [NaCl] of 0.8 M, 0.6 M, 0.4 M, 0.2 M, and 0.1 M NaCl, with 30 minute incubations for each step at 37°C. After the final incubation the solutions were exchanged into dilution buffer on a Centricon 30 filter.

Reconstituted nucleosome core particles were purified away from the histone octamer donor chromatin, residual naked radiolabeled DNA and any non-nucleosomal aggregates on 5%-30% (w/v) sucrose gradients in 0.5 × TE buffer 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 Čerenkov counting (Figure 1(b)). Fractions containing the reconstituted nucleosomes were pooled and concentrated on Centricon 30 filters and exchanged into 0.5 × TE buffer, 5 mM NaCl for storage.

### Purification of GAL4-DNA-binding domain

Expression of the GAL4 DNA-binding domain was induced from *Escherichia coli* strain XA-90 containing plasmid pTGH21 encoding residues 1-147 of GAL4 under the control of the tac promoter. Overnight starter cultures (5 ml of LB + ampicilin, 50 μg ml<sup>-1</sup>) were inoculated with scrapes from freezer stocks and incubated at

37°C. Overnight cultures were used to inoculate 1 l cultures (LB + ampicillin, 50 µg ml<sup>-1</sup>), which were incubated at 37°C. Protein production was induced by the addition of 1 mM IPTG when growth reached  $A_{600} = 0.8$  (measured in an HP 8452A spectrophotometer without dilution). Cells were harvested at three hours post-induction to yield 4.4 g of cells. Cells were resuspended in 50 ml of buffer A (20 mM Hepes (pH 7.5), 10 mM β-mercaptoethanol, 10 µM zinc acetate, 1 mM PMSF, 20 µg ml<sup>-1</sup> leupeptin and pepstatin) and lysed by sonication. After removal of cell debris by centrifugation at 10,000 rpm for 30 minutes, the DNA was precipitated by addition of polyethyleneimine to 0.5% (w/v). The resulting supernatant containing the GAL4 (1-147) was treated with ammonium sulfate, which was added slowly while stirring on ice, to a final concentration of 0.233 g ml<sup>-1</sup> (41% saturation) and incubated on ice for one hour. The precipitated protein was collected by centrifugation at 10,000 rpm (Beckman JA-20 rotor) for 30 minutes. The protein pellet was resuspended in 50 ml of buffer A and dialyzed overnight against 1 l of buffer A to lower the conductivity of the solution. The overnight dialysis resulted in formation of a precipitate in the sample, which was separated by low-speed centrifugation and discarded. The soluble GAL4 (1-147) was then purified on a 2 ml DEAE-cellulose column equilibrated in buffer A, 50 mM NaCl (GAL4 (1-147) flows through). This purified protein was collected and concentrated to yield apparently homogeneous GAL4 (1-147) at 54.1 µM.

### Binding assays

Binding assays for naked DNA and reconstituted native or tailless nucleosome core particles were performed in separate 10 µl reactions for each titration point, each containing buffer, radiolabeled nucleosomes, and an appropriate amount of GAL4 (1-147). Buffer conditions for each binding reaction were as follows: 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 2 mM DTT, 5% (v/v) glycerol, 200 µg ml<sup>-1</sup> bovine serum albumin. Each reaction was incubated at 23°C for 30 minutes prior to loading onto native 5% polyacrylamide gels running in 1/3 × TBE (30 mM Tris-borate, 0.66 mM EDTA). Appropriate dilutions of GAL4 (1-147) were made on ice in binding buffer just prior to use. Gels were prerun in 1/3 × TBE at 15–20 V cm<sup>-1</sup> for one hour and samples were then loaded with the gel running. Gels were dried and bands corresponding to various species (free DNA, DNA-GAL4 (1-147), nucleosomes, and nucleosome-GAL4 (1-147)) were quantified by phosphorimager. The analysis is complicated by the presence of multiple bands corresponding to related species, having two distinct origins. (i) Binding of GAL4 (1-147) (even to naked DNA) leads to a set of several shifted products. This is found frequently, and presumably arises from cooperative binding of one or more additional GAL4 (1-147) molecules adjacent to a specifically bound one. We measure the average or apparent affinity, by monitoring the fraction of unbound DNA (equivalently, the fraction of DNA in the set of all these shifted products taken together) as a function of GAL4 (1-147) concentration. (ii) In the absence of any GAL4 (1-147) protein, the tailless nucleosomes migrate as a set of two or three closely spaced bands, consistent with occupancy at sets of predominant positions. Consistent with the gel images themselves (see Results), detailed analysis of the intensities of these bands throughout the titrations shows that for both constructs the tailless nucleosomes corresponding to the different bands titrate similarly

within experimental error. Therefore, these bands too were grouped together. The integration regions needed for quantitative analysis are chosen correspondingly; e.g. free DNA, the set of all DNA-GAL4 (1-147) species, the set of unshifted nucleosomes, or the set of all super-shifted nucleosome-GAL4 (1-147) complexes. Background values scaled for the integration area were obtained from appropriately positioned regions of each gel track and were subtracted from the integrals measured for each band. In cases where there were multiple shifted products, quantification included the entire set of shifted products; background values were obtained from boxes of equivalent size. The fraction of DNA (or nucleosomes) having bound GAL4 (1-147) was calculated as (shifted counts)/(shifted + unshifted counts) for each protein concentration examined. This definition is insensitive to variations in gel loading. For each class of template (naked DNA, and native or tailless nucleosomes), the quantitative results for several independent titrations, each having many titration points, were plotted together and fit by non-linear least-squares to the binding equation:

$$\text{fraction bound} = 1/(1 + (K_d/[GAL4 (1-147)]))$$

to yield the best-fit apparent  $K_d$  and its corresponding uncertainty, using the computer program KaleidaGraph (Synergy Software). These values for  $K_d$  were then used to calculate equilibrium constants for site exposure:  $K_{eq}^{conf} = K_d(\text{DNA})/K_d(\text{Nuc})$  (Polach & Widom, 1995). The ratio of  $K_{eq}^{conf}$  for native *versus* tailless nucleosomes yields the fold-enhancement of site exposure attributable to removal of the tail domains.

### Restriction enzyme assays

The basis of the kinetic analysis of the restriction enzyme digestions is described elsewhere (Polach & Widom, 1995). In the present study, reconstituted native or tailless nucleosomes were digested with restriction enzymes as described. Two restriction enzymes (*EcoRI* and *DpnII*) were used, and digestions were performed at 37°C instead of elevated temperatures. We seek primarily the ratio of  $K_{eq}^{conf}$  for native *versus* tailless nucleosomes, for which parallel digestions on naked DNA are not needed; nevertheless, in most cases parallel digestions were carried out on naked DNA, allowing direct determinations of  $K_{eq}^{conf}$  separately for the native and tailless nucleosomes. The specific buffers used for each enzyme are: *DpnII*, 100 mM NaCl, 50 mM bis-Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT; *EcoRI*, 50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 0.025% (v/v) Triton X-100. At various times during the digestion, 10 µl aliquots were removed and quenched with formamide gel-loading buffer. Samples were digested with proteinase K (25 µg ml<sup>-1</sup>) at 37°C for one hour and analyzed on denaturing 8% polyacrylamide gels in TBE, 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. The remaining uncut substrate DNA (S) and the two products (P1 and P2) are resolved simultaneously. The fraction of uncut DNA remaining at each time was calculated as: fraction uncut = (counts in S)/(counts in S + P1 + P2). This definition is insensitive to variations in gel loading. Data analysis was complicated by two features of the results. (i) Primarily with *EcoRI*, the initial digestion timepoint for nucleosomal templates exhibited a modest but anomalously large extent of

digestion. This cannot be attributed to the small percentage of contaminating naked DNA because it often exceeded that amount (which was separately measured by native gel electrophoresis and phosphorimager analysis). We attribute it instead to a small fraction of the nucleosomes dissociating to yield naked DNA when the nucleosomes are introduced into the actual reaction conditions. We address this issue by fitting only the kinetics subsequent to this initial "burst" of digestion. (ii) A fraction of the nucleosomes are never digested; for both restriction enzymes used, this fraction is greater for the native nucleosomes than for the tailless nucleosomes. We subsequently traced this behavior to a fraction of the nucleosomes being insoluble in the digestion buffer as measured by an ultracentrifuge-based sedimentation assay (J. Anderson & J.W., unpublished results). This behavior is actually to be expected, since it is in accord with previous studies on the solubility of native and tailless nucleosomes (Schwarz *et al.*, 1996). We addressed this feature of the data by allowing the baseline to float in the non-linear least-squares fits. For these reasons, the digestion kinetics for each digestion separately were analyzed by non-linear-least squares fits (using KaleidaGraph) to the equation: fraction uncut =  $(a_0 + a_1 \times \exp(-a_2 \times t))$  ( $t$  = time). The ratio of values  $a_2$  (scaled appropriately by the enzyme concentration used (Polach & Widom, 1995)) for nucleosomes *versus* naked DNA (when measured) yields the equilibrium constant for exposing that site ( $K_{eq}^{conf}$ ) in the (native or tailless) nucleosomes. The ratio of  $K_{eq}^{conf}$  for native *versus* tailless nucleosomes (or, equivalently, the direct ratios of the rate constants  $a_2$  for native *versus* tailless nucleosomes, scaled by their enzyme concentrations) yields the fold-enhancement of site exposure attributable to removal of the tail domains. The non-linear least-squares fitting procedure yields both the rate constant  $a_2$  and the corresponding uncertainty. These uncertainties were propagated (Bevington, 1969) to yield the estimated uncertainty in the final measured ratios of rate or (ultimately) equilibrium constants.

As a check on the appropriateness of this analysis, we carried out some analyses using fits to a double exponential, yielding two rate constants. Importantly, however, using the double exponential analysis we obtained for both rate constants independently, and for their amplitude-weighted average, results that are quantitatively similar and qualitatively equivalent to those obtained with the single exponential analysis as regards the dependence of  $K_{eq}^{conf}$  on the presence or absence of the histone tail domains. We therefore focussed only on the single exponential analysis, since this approach has fewer adjustable parameters and allows a clear physical interpretation.

### Exonuclease III assays

The basis of the kinetic analysis of the exonuclease III digestions has been described elsewhere (Protacio *et al.*, 1997). DNA was singly end-labeled at either end and reconstituted with native or tailless histones. Digestions on naked DNA and native or tailless nucleosomes (50 nM total DNA) were carried out in 100  $\mu$ l reactions in 50 mM Tris-HCl (pH 8.0), 5 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol with 1000 units ml<sup>-1</sup> of exonuclease III. Digestion was initiated by mixing equal volumes of two solutions, one containing substrate and the other enzyme, both preheated to 37°C. After variable reaction times, 10  $\mu$ l samples were removed and quenched with an equal volume of formamide containing 0.1% (w/v)

xylene cyanol. Each aliquot was then digested with proteinase K (25  $\mu$ g ml<sup>-1</sup>) at 37°C for two hours prior to electrophoretic analysis. The full-length (155 nt) DNA is rapidly digested to a (relatively) protected  $\sim$ 145 nt core particle length, which in turn is digested to another protected pause site at 137 nt. This allows the decay of the 147 and subsequently formed 137 nt species to be followed separately. Only data obtained after essentially all detectable precursor has been digested are utilized in the kinetic analyses, so that the decays can be approximated as simple first-order processes. The quantitative (background-subtracted) band intensities were analyzed by non-linear least-squares fits (using KaleidaGraph) to the equation: fraction uncut =  $(a_0 + a_1 \times \exp(-a_2 \times t))$ . The ratio of values of  $a_2$  for native *versus* tailless nucleosomes, scaled by their enzyme concentrations, yields the fold-enhancement of site exposure attributable to removal of the tail domains.

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