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Differential repression of transcription factor binding by histone H1 is regulated by the core histone amino termini

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In order to investigate the interrelated roles of nucleosome cores and histone H1 in transcription repression, we have employed a purified system to analyze the function of H1 in the repression of transcription factor binding to nucleosomes. H1 binding to nucleosome cores resulted in the repression of USF binding to nucleosomes. By contrast, H1 only slightly inhibited the binding of GAL4-AH, indicating that H1 differentially represses the binding of factors with different DNA-binding domains. H1-mediated repression of factor binding was dependent on the core histone amino-terminal tails. Removal of these domains alleviated H1-mediated repression and increased acetylation of these domains partly alleviated repression by H1. H1 binding assays suggest a less stable interaction of histone H1 with the core particle in the absence of the amino termini.

Key words: chromatin/histone H1/nucleosome/transcription factor

Introduction

Gene activation in eukaryotic chromatin must involve a sequential unfolding of multiple layers of chromatin structure as regulatory and general transcription factors gain access to enhancer and promoter elements (reviewed in Kornberg and Lorch, 1991; Felsenfeld, 1992). Numerous studies over the past decade have investigated these pathways by analyzing differences in chromosomal proteins associated with transcriptionally 'active' sequences versus total genomic sequences or specific sequences which are transcriptionally 'inactive'. Two of the changes in chromatin composition which have emerged from these studies include both a reduction in the linker histone H1 content and an increase in the acetylation levels of the amino-terminal 'tails' of the core histones in actively transcribed regions of chromatin (reviewed in Csordas, 1990; Turner, 1991; Ausio, 1992; Zlatanova and van Holde, 1992).

Histone H1 is thought to primarily bind to DNA in the nucleosome at the pseudodyad and at the linker DNA as it enters and leaves the nucleosome core particle (Noll and Kornberg, 1977; Simpson, 1978; Allan *et al.*, 1980; Boulikas *et al.*, 1980; Staynov and Crane-Robinson, 1988). H1 binding to multiple DNA duplexes appears to be achieved through two different DNA-binding domains (Ramakrishnan *et al.*, 1993). The globular domain of histone H5 (an H1 variant) contains one DNA-binding domain which is related to that of catabolite gene activator protein and HNF-3 forkhead (Clark *et al.*, 1993), and a second less-well defined domain (Ramkrishna *et al.*, 1993). H1 binding is thought to stabilize the nucleosome and facilitates the folding of nucleosome arrays into 30 nm chromatin fibers (Thoma *et al.*, 1979; Allan *et al.*, 1981; reviewed in van Holde, 1988). Protein DNA cross-linking experiments with cellular chromatin have indicated a reduction of H1 cross-linking (~50%) with actively transcribed genes (Karpov *et al.*, 1984; Nacheva *et al.*, 1989; Dimitrov *et al.*, 1990; Kamakaka and Thomas, 1990; Dedon *et al.*, 1991; Postnikov *et al.*, 1991; Bresnick *et al.*, 1992). Thus, while H1 is not totally depleted, these studies suggest a reduction in its affinity for chromatin in these regions.

The amino-terminal domains of the core histones are not essential for the formation of the nucleosome core (composed of DNA and an octamer of two each of the core histones H2A, H2B, H3 and H4). These domains appear to be located on the surface of the nucleosome and are the sites of numerous post-translational modifications (reviewed in Bohm and Crane-Robinson, 1984; Turner, 1991). Fractionation of cellular chromatin by a variety of approaches has demonstrated a strong correlation between sequences for 'active' genes and increased acetylation of lysine residues in the amino-terminal domains (Allegra *et al.*, 1987; Johnson *et al.*, 1987; Ridsdale and Davie, 1987; Hebbes *et al.*, 1988, 1994; Tazi and Bird, 1990). Acetylation in these domains of H3 and H4 reduces the change in linking number of DNA per nucleosome core observed *in vitro* (Norton *et al.*, 1989, 1990). In addition, these domains have been implicated in the folding of nucleosomes into 30 nm fibers both independently and via histone H1 (Allan *et al.*, 1982; Annunziato and Seale, 1983; Perry and Annunziato, 1989; Ridsdale *et al.*, 1990; Perry and Annunziato, 1991; Garcia-Rameirez *et al.*, 1992; reviewed in Ausio, 1992). Thus, these studies have suggested important functional interactions between the core histone amino termini and the linker histone H1.

In vitro transcription studies have illustrated an enhanced degree of transcription regulation *in vitro* (i.e. dependence of transcription on regulatory factors) resulting from suppression of basal transcription by either nucleosome cores or the subsequent binding of histone H1 (Workman *et al.*, 1988, 1990, 1991a; Laybourn and Kadonaga, 1991; Croston *et al.*, 1992; Lorch *et al.*, 1992; Sandoltzopoulos *et al.*, 1994; reviewed in Workman and Buchman, 1993;

Paranjape *et al.*, 1994). While these functional studies have clearly implicated histones in transcription control, they have not clarified the interrelationships between nucleosome cores and H1 in transcription repression. Moreover, transcription analysis is complicated by the potential for histone repression at several possible rate-limiting steps prior to transcription initiation (reviewed in Kornberg and Lorch, 1991; Workman and Buchman, 1993).

The function of the nucleosome core in transcription regulation has also been investigated by the direct analysis of transcription factor binding. These studies have illustrated several principles of regulatory transcription factor interactions with nucleosome cores (reviewed in Adams and Workman, 1993; Svaren and Horz, 1993). These include: (i) differential affinity of different factors for their recognition sites on nucleosome cores (Pina *et al.*, 1990; Archer *et al.*, 1991; Taylor *et al.*, 1991; also see Li *et al.*, 1994; Svaren *et al.*, 1994); (ii) differential affinity of factors for sites at different locations within nucleosome cores (Li and Wrange, 1994; Li *et al.*, 1994; Vettese-Dadey *et al.*, 1994); (iii) co-operative binding of factors to nucleosome cores in response to repression from the core histone amino termini (Taylor *et al.*, 1991; Vettese-Dadey *et al.*, 1994); (iv) stimulation of factor binding by histone-binding proteins (Chen *et al.*, 1994); (v) stimulation of factor binding by the SWI/SNF complex (Côté *et al.*, 1994a; Kwon *et al.*, 1994; Imbalzano *et al.*, 1994); and (vi) stimulation of factor binding by increased acetylation of the core histone amino-terminal domains (Lee *et al.*, 1993).

In this report, we extend these direct binding studies to an analysis of H1 function in the repression of transcription factor binding. We demonstrate for the first time direct inhibition of factor binding by the association of H1 with nucleosome cores. The contribution to repression of factor binding by H1 is less than that of the nucleosome core (Juan *et al.*, 1993) and differentially inhibits the binding of different factors. The binding of H1 to form a chromatosome significantly repressed the subsequent binding of USF to a nucleosome, but only slightly inhibited GAL4-AH binding. In addition, the extent of H1 repression of factor binding was dependent on the amino-terminal domains of the core histones and was alleviated if these domains were removed by proteases, and partially alleviated by increased acetylation of the amino termini.

Results

To investigate the function of histone H1 in repression of transcription factor binding, we have utilized an approach which employs purified donor cellular nucleosome cores, purified H1 and purified transcription factors (USF and GAL4-AH). Labeled DNA fragments which are long enough to form a chromatosome (i.e. >167 bp) were reconstituted into nucleosome cores by octamer transfer from donor nucleosome cores at 1 M salt (reviewed in Rhodes and Laskey, 1989; Côté *et al.*, 1994b). Following dilution to 0.6 M salt, H1 was added and the mixtures were diluted to 0.1 M salt to form chromatosomes (a nucleosome core bound by H1) (see Materials and methods for details). Previous studies analyzing H1 reconstitution onto nucleosome cores have often utilized the 5S repeats from sea urchin or *Xenopus*. While not readily apparent *in vivo* (Engelke and Gottesfeld, 1990; Chipev and Wolffe, 1992), the ability of these sequences to position a nucleosome core to preferred specific translational frames *in vitro* has proven useful for analysis of H1 binding. These studies have indicated that H1 binding alters the distribution of positions of the nucleosome (Meersseman *et al.*, 1991) and may bind asymmetrically (Hayes and Wolffe, 1993). However, to analyze the binding of regulatory factors for protein coding genes, nucleosomes containing different sequences were required. The probe DNAs used for factor binding analysis in this study were derived from pBEND vectors (but do not contain bent DNA; Kim *et al.*, 1989) and do not demonstrate translational positioning of the nucleosomes. However, nucleosome cores occupied a complete 146 bp of the fragments (Vettese-Dadey *et al.*, 1994). Moreover, as observed with the 5S nucleosome (Hayes and Wolffe, 1993), chromatosome formation on non-positioning sequences required 'linker DNA' (>167 bp), does not occur on shorter fragments which will readily reconstitute nucleosome cores (i.e. 150 bp) and results in chromatosome mobility shifts and micrococcal nuclease digestion intermediates (Juan *et al.*, 1993).

Removal of the core histone amino-terminal tails without degrading histone H1

Several studies have suggested that the formation of higher-order chromatin structures is dependent on both the core histone amino-terminal tails (and their degree of acetylation) as well as the linker histone, H1 (Allan *et al.*, 1982; Annunziato and Seale, 1983; Perry and Annunziato, 1989, 1991; Ridsdale *et al.*, 1990; Garcia-Rameirez *et al.*, 1992). These studies suggest important functional interactions between the core histone amino termini and histone H1. To examine whether the core histone amino termini influence H1-mediated repression of transcription factor binding, we utilized our previous protocol for tryptic removal of the amino termini and nucleosome reconstitution (Vettese-Dadey *et al.*, 1994) that avoids previously described problems resulting from the proteolysis of subsequently added transcription factors (Hayes and Wolffe, 1992; Lee *et al.*, 1993). The protocol as adapted for chromatosome reconstitution is shown in Figure 1. Nucleosome cores were treated with trypsin to remove the core histone amino termini, followed by the addition of an excess of trypsin inhibitor. All of the following steps to transfer the trypsinized histone octamers onto probe DNA and the binding of H1 were performed in the presence of an excess of trypsin inhibitor. Importantly, the control samples (Intact Nucleosome Controls) and the subsequently added H1 were also exposed to the same amounts of protease, but subsequent to the addition of inhibitor. The added trypsin inhibitor effectively prevented proteolysis of the control core histones and subsequently added transcription factors (Vettese-Dadey *et al.*, 1994). Figure 1A illustrates the effect of this treatment on the core histones. The prior addition of trypsin inhibitor prevented the degradation of histones in the control nucleosome samples through the reconstitution and binding reactions (compare lane 3 with histone standards in lane 2). By contrast, exposure of the trypsinized samples to the protease prior to the addition of inhibitor (lane 4) resulted in digestion of the core histones to smaller

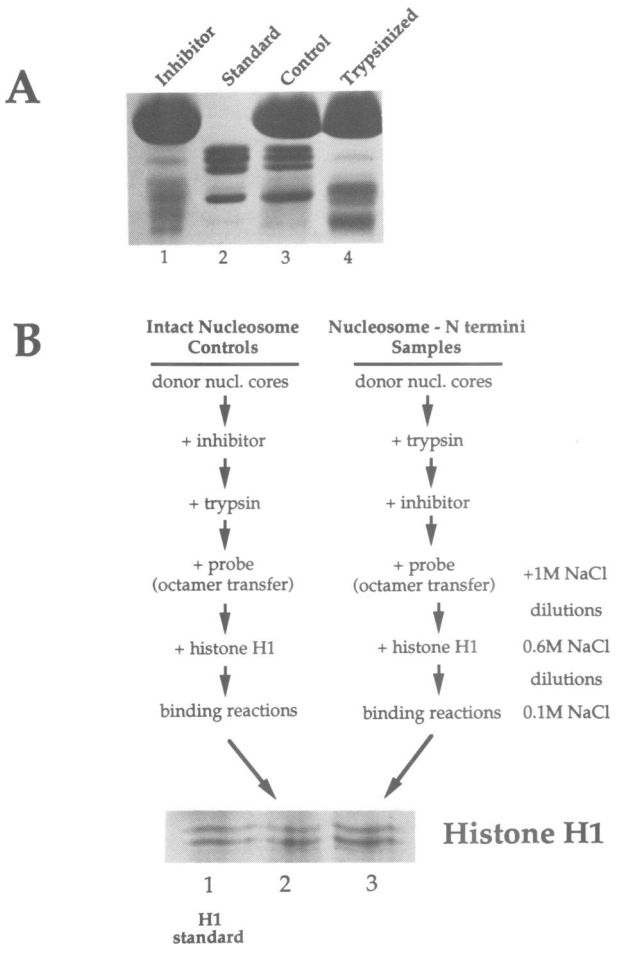

Fig. 1. Protocol and controls for proteolytic removal of the core histone amino termini which does not degrade subsequently added histone H1. (**A**) Proteolysis of core histones in nucleosome cores. Nucleosome cores were exposed to trypsin prior to (Trypsinized; lane 4) or after the addition of trypsin inhibitor (Control; lane 3) in the protocol shown in (B) and the core histones were resolved by SDS–PAGE following subsequent 'mock' binding reactions. Note that the core histones are trimmed by the trypsin only if present prior to the inhibitor. Lane 2 contains core histone standards and lane 1 contains only typsin inhibitor also present in lanes 3 and 4. The background staining in lanes 3 and 4 is a result of the trypsin inhibitor present (compare with lane 1). (**B**) Histone H1 is not degraded during reconstitution and binding reactions. The amino-terminal domains of the core histones were removed by trypsin digestion, followed by an excess of trypsin inhibitor. The intact nucleosome control samples were exposed to the same concentrations of trypsin and inhibitor, but the inhibitor was added prior to the protease which prevented any proteolysis of these histones (A). Both the trypsinized and control nucleosomes were used as histone donors in subsequent octamer transfer reactions at 1 M salt onto labeled probe DNA (see Materials and methods). The reactions were serially diluted to 0.6 M salt followed by H1 addition where indicated. Following dilutions to 0.1 M salt, the reconstituted samples were used in subsequent factor-binding reactions. Trypsin inhibitor was included in all transfer, dilution and binding reaction mixtures to eliminate subsequent trypsin activity. The panel below the flow chart of the dilution transfer scheme is the H1 region of an SDS–PAGE gel of the samples resulting from the diagrammed protocols. The gel illustrates that the H1 was not proteolyzed in the subsequent reconstitution and binding reactions whether the inhibitor was present before (lane 2) or after (lane 3) the nucleosome cores were exposed to trypsin. Lane 1 is an equivalent amount of histone H1 which was not exposed to trypsin.

peptides, indicative of removal of the amino termini (Bohm and Crane-Robinson, 1984). Moreover, the H1 was added to both the trypsinized and control nucleosome cores after the addition of trypsin and inhibitor, and was not proteolyzed in the subsequent reconstitution and binding reactions (Figure 1B, compare lanes 2 and 3).

Histone H1 repression of USF binding to nucleosomes is alleviated by removal of the core histone amino termini

The binding of histone H1 to nucleosome cores could inhibit transcription factor binding by directly blocking accessibility of recognition sites at the edge of nucleosomes and the dyad axis (positions of H1 interaction; Staynov and Crane-Robinson, 1988) and/or by further stabilizing the nucleosome core (van Holde, 1988); thus, indirectly increasing the inhibition due to the core histones. However, we have previously reported that H1 binding to nucleosome cores did not further inhibit the binding of GAL4-AH to either five GAL4 sites covering the middle 95 bp of a nucleosome core, or to two sites at the edge of a nucleosome core reconstituted fragment, beyond the inhibition observed from the nucleosome core alone (Juan *et al.*, 1993). To test further for H1 repression, we have analyzed the effect of H1 binding on the subsequent binding of USF and GAL4-AH to a single site on a nucleosome. The DNA probes used in these analysis each contain both a USF and GAL4 site with the orientation of the sites switched by originally inserting an oligonucleotide containing both sites into the same vector in opposite orientations (see Materials and methods). This allows the generation of probes of identical length which only differ in the orientation of the binding sites. Most important for these studies, by using probes from each vector the USF and GAL4 sites can be analyzed at essentially the same location on nearly identical fragments, allowing a comparison of the effects of H1 on the binding of each factor. The fragments (183 bp) were chosen to be long enough to provide a good target for H1 binding (i.e. >167 bp) with the binding sites situated well within 167 bp from either end. This is to ensure that the binding site would be contained within a reconstituted chromatosome regardless of its exact location on the fragment.

The function of the core histone amino termini in the repression of USF binding by histone H1 is illustrated in Figure 2. Increasing concentrations of H1 effectively repressed USF binding to nucleosome cores reconstituted on the one USF site probe (illustrated in Figure 2A) to 10–15% of that observed in the absence of H1 (Figure 2A, lanes 7–12). By contrast, USF binding was much less inhibited by H1 when added to nucleosome cores lacking the amino-terminal tails (lanes 1–6). In the absence of the amino termini USF binding in the presence of 107 nM H1 was >60% of that in the absence of H1. For direct comparison, the effect of H1 binding on GAL4-AH binding was tested on a DNA probe derived from a related construct containing a GAL4 site at the same location as the USF site (illustrated at the top of Figure 2A and B). The effect of H1 on GAL4-AH binding to nucleosomes containing or lacking the core histone amino termini is shown in Figure 2B. At increasing concentrations of histone H1, GAL4-AH binding to intact nucleosome cores was only slightly reduced (lanes 7–12). Moreover, GAL4-

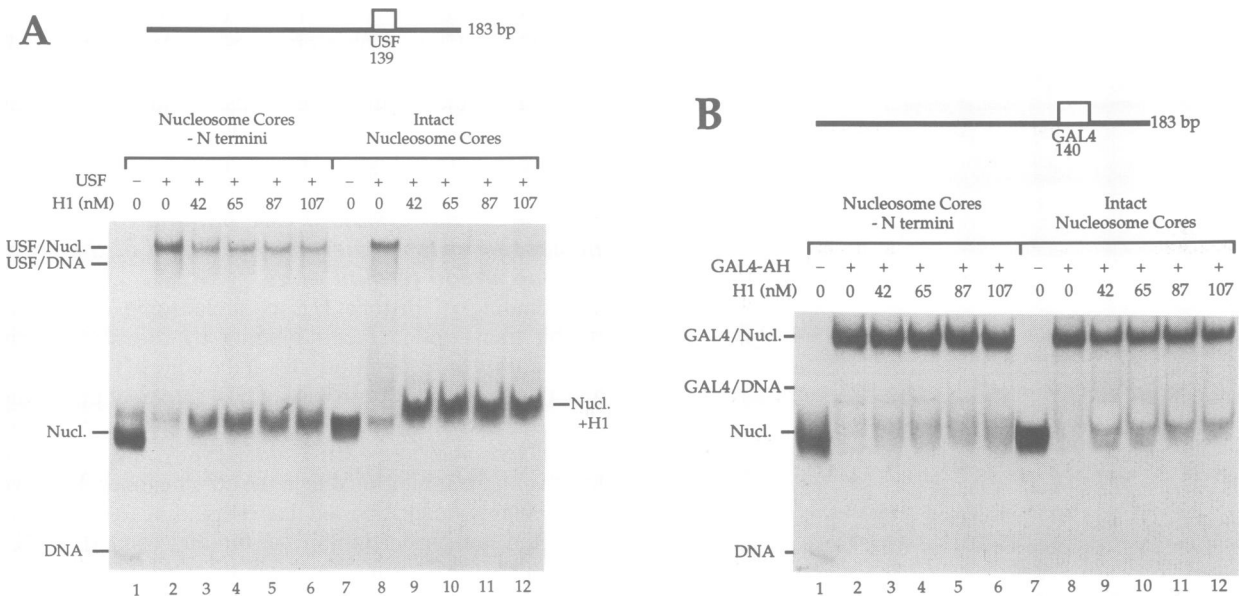

Fig. 2. H1 represses USF binding to a greater extent than GAL4-AH, but requires the core histone amino termini. (**A**) The binding of USF to nucleosome cores lacking the amino termini (lanes 1–6) or to intact nucleosome cores (lanes 7–12) was assayed by gel shift in the absence of histone H1 (lanes 1, 2, 7 and 8) or in the presence of increasing amounts of histone H1 (lanes 3–6 and 9–12). Protein–DNA complexes representing USF bound to naked DNA (USF/DNA) and USF bound to nucleosomes (USF/Nucl.) are indicated. Comparison of the amount of USF/Nucl. complexes in lanes 9–12 versus lane 8 reveals that H1 repressed USF binding to nucleosome cores; however, comparison of lanes 3–6 versus lane 2 shows that this inhibition was greatly reduced when the core histone N-terminal tails were proteolytically removed. The DNA used is outlined above. This 183 bp probe DNA contains a USF binding site located with the center of the binding site as indicated. USF at 50 nM was included in each reaction where indicated. (**B**) Reaction conditions and lane markers are the same as in (A), except that USF was replaced by GAL4-AH and the DNA used contains a GAL4 binding site at the same place. In contrast to USF, the binding of GAL4-AH to intact nucleosome cores was only slightly reduced in the presence of increasing concentrations of H1 (lanes 9–12 versus lane 8). Moreover, the binding of GAL4-AH to nucleosomes without tails was essentially unaffected by H1 (lanes 3–6 versus lane 2). GAL4-AH at 20 nM was included in each reaction indicated.

AH binding to nucleosomes lacking the amino termini was unaffected by the same concentrations of H1 (lanes 1–6). This result further confirms our previous report (Juan *et al.*, 1993) that H1 binding to nucleosomes does not sustantially inhibit the binding of GAL4-AH.

The experiments shown in Figure 2A and B have been repeated numerous times, and the results of three independent repeats of each are plotted in Figure 3. This graph illustrates two important points. First, USF binding is repressed by H1 to a much greater degree than is GAL4-AH binding. This indicates that H1 repression is differentially exerted on different factors with different DNA-binding motifs. This differential repression was also observed at several different GAL4-AH and USF concentrations (data not shown). Second, H1-mediated repression of USF binding is relieved on nucleosomes which lack the core histone amino termini. Thus, these domains influence the extent of repression of factor binding by histone H1. This observation has been confirmed by recent experiments (data not shown) in which we utilized clostripain, a protease which also removes the amino termini of the core histones but does not affect the carboxy termini (Dumuis-Kervabon *et al.*, 1986).

Analysis of H1 repression by DNase I footprinting

To confirm the function of H1 in repression of USF binding to nucleosomes and the role of the core histone amino termini, we have employed DNase I footprinting. A different DNA probe that could be end labeled was used in this analysis and is illustrated in Figure 4A. Figure 4A also illustrates by mobility shift assay that USF binding to this probe was also repressed by H1. Higher concentrations of USF were required to bind the chromatosomes (i.e. form the USF/Nucl. complex; lanes 7–12) than was required to bind the nucleosome core alone (lanes 1–6). Thus, histone H1 repressed the extent of USF binding over a wide range of USF concentrations. The appearance of the higher band (2×USF/Nucl.) may represent the non-specific binding of a second USF dimer or, more likely, the binding of USF tetramers which have been shown to form at the higher USF concentrations (Ferre-D'Amare *et al.*, 1994).

Analysis of USF binding in reactions identical to those in Figure 4A by DNase I footprinting is shown in Figure 4B. At increasing concentrations of USF, binding to the fragment reconstituted with a nucleosome core alone led to a clear footprint at the USF site (Figure 4B; compare lanes 5–9 with lane 3). However, when H1 was also reconstituted onto this fragment, the footprint resulting from USF binding was greatly reduced (lanes 11–15). The footprinting data, therefore, further illustrate the function of H1 in repression of USF binding to nucleosomes. While the nucleosome cores reconstituted on this fragment were not homogeneously phased with regard to DNA sequence, it is interesting to note that H1 binding appeared to result in some protection of the DNA near the center of the nucleosome reconstituted fragment (compare the bottom of lanes 10 and 4). This protection is consistent with the footprinting of H1 at the dyad axis of nucleosomes observed previously (Staynov and Crane-Robinson, 1988). Presumably, the protection observed here is not complete

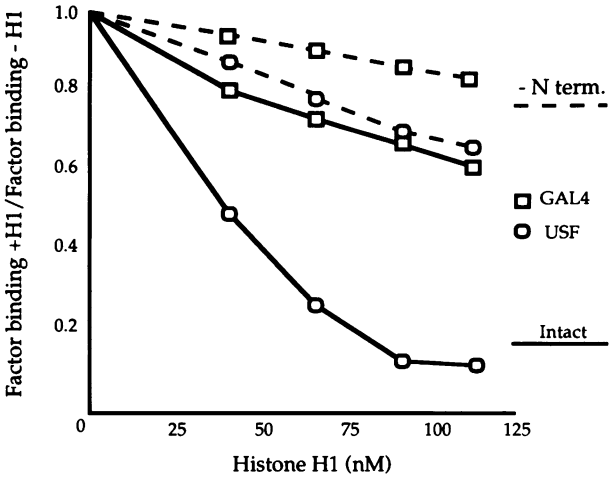

Fig. 3. Graph of the relative inhibition of USF and GAL4-AH binding to nucleosome cores by histone H1. The ratio of USF (circles) and GAL4-AH (squares) binding to nucleosome cores in the presence of H1 versus the absence of H1 is plotted for the H1 concentrations indicated. Repression was assayed on nucleosome cores containing (solid line) or lacking (dashed line) the amino-terminal tails. The plotted data were derived from a quantitation of three independent experiments. The extent of repression was derived as the ratio of factor/nucleosome complexes observed at each H1 concentration relative to that observed in the absence of H1. The plotted values represent the average of the three repeats of each experiment.

because of some heterogeneity in exact nucleosome position.

Repression of USF binding by histone H1, as detected by DNase I footprinting, also required the core histone amino termini (Figure 4C). While H1 binding prevented the USF footprint on intact nucleosome cores (lanes 12–17), the presence of H1 did not prevent USF binding when the core histone amino termini were removed (lanes 6–11). Again, the ability of histone H1 to repress the binding of USF to nucleosome cores was dependent on the presence of the core histone amino termini. It is also interesting to note that the H1-mediated protection of sequences near the center of the nucleosome was reduced when the amino termini were absent (compare lanes 6 and 12), consistent with an altered or decreased interaction of H1 (see Figure 6).

Increased levels of histone acetylation partly relieve H1 repression of USF binding

The data above indicate that the function of H1 in repression of transcription factor binding is regulated by the core histone amino termini. To test further this possibility, we have analyzed H1 repression of USF binding to nucleosome cores containing an enhanced degree of acetylation of the core histone amino termini (Figure 5A) since acetylation of lysine residues reduces the positive charge of the amino-terminal tails and their affinity for DNA (Hong *et al.*, 1993). H1-mediated repression of USF binding to acetylated nucleosome was reduced (lanes 6–10) relative to H1-mediated repression of USF binding to control non-acetylated nucleosomes (lanes 1–5). The extent of H1 repression of USF binding to control nucleosome cores and nucleosome cores with increased acetylation of the amino termini was determined by quantitation of the experiment in Figure 5A and two independent repeats, and is shown graphically in Figure 5B. While repression was observed in both instances, the extent of H1-mediated repression of USF binding was reduced for the acetylated nucleosomes at each of the H1 concentrations tested. Thus, USF binding to the acetylated nucleosomes was less effectively repressed by H1 than control non-acetylated nucleosomes, but not as resistant to repression as nucleosomes lacking the amino termini (Figure 2A). It is important to note in this regard that the acetylated nucleosome cores (prepared from butyrate-treated cells; see Materials and methods) contained a mixed population of acetylated histone species. While considerably more acetylated than the control histones, the acetylation of histones resulting from butyrate treatment was only partial (~50% for H4). This is illustrated in the Triton–acid–urea (TAU) gel of the nucleosome samples used in these experiments shown in Figure 5C. It is reasonable to expect that nucleosome cores containing higher amounts of acetylation would more effectively resist H1 repression of factor binding as for the nucleosome cores lacking the amino termini. Moreover, future experiments may determine whether specific combinations of acetylated sites and/or histone most effectively resist H1 repression.

Function of the core histone amino termini in H1 binding to nucleosome cores

The transcription factor binding studies shown above indicate that H1 repression of factor binding was reduced for nucleosome cores lacking the amino-terminal domains or containing amino termini with higher amounts of acetylated lysines. This suggests that H1 binding to these nucleosome cores was reduced or less stable than that to control nucleosome cores. Previous studies have utilized micrococcal nuclease digestion of H1-reconstituted nucleosomes to indicate that the core histone amino termini are not absolutely required for H1 binding to the nucleosome core (Allan *et al.*, 1982, 1986; Hayes *et al.*, 1995). These experiments illustrated that at least a fraction of the digestion products observed after H1 addition to trypsinized nucleosome cores was 168 bp in length (i.e. chromatosome length). The appearance of this kinetic digestion intermediate suggests that nucleosome cores lacking the amino termini interacted with H1 in solution. However, the decrease in H1 repression of factor binding illustrated above suggests that H1 interactions with the nucleosome cores lacking the amino termini may be less stable or somehow altered. In accordance with this interpretation, the interactions of H1 with nucleosomes lacking the amino termini or with amino termini which are highly acetylated are also unable to bring about condensation of the nucleosome arrays into thick chromatin fibers, an activity observed upon H1 binding to arrays of intact nucleosome cores (Allan *et al.*, 1982; Ridsdale *et al.*, 1990). Thus, while the nuclease digestions indicate H1 interactions, the factor binding studies described above, as well as the previous chromatin condensation experiments, suggest an altered or less stable interaction of H1 with either nucleosomes lacking the amino termini or nucleosomes with acetylated amino termini. In addition, the mobility shift gel shown in Figure 2A indicates a shift in mobility of nucleosome cores (which are not bound by USF) that either contain or lack

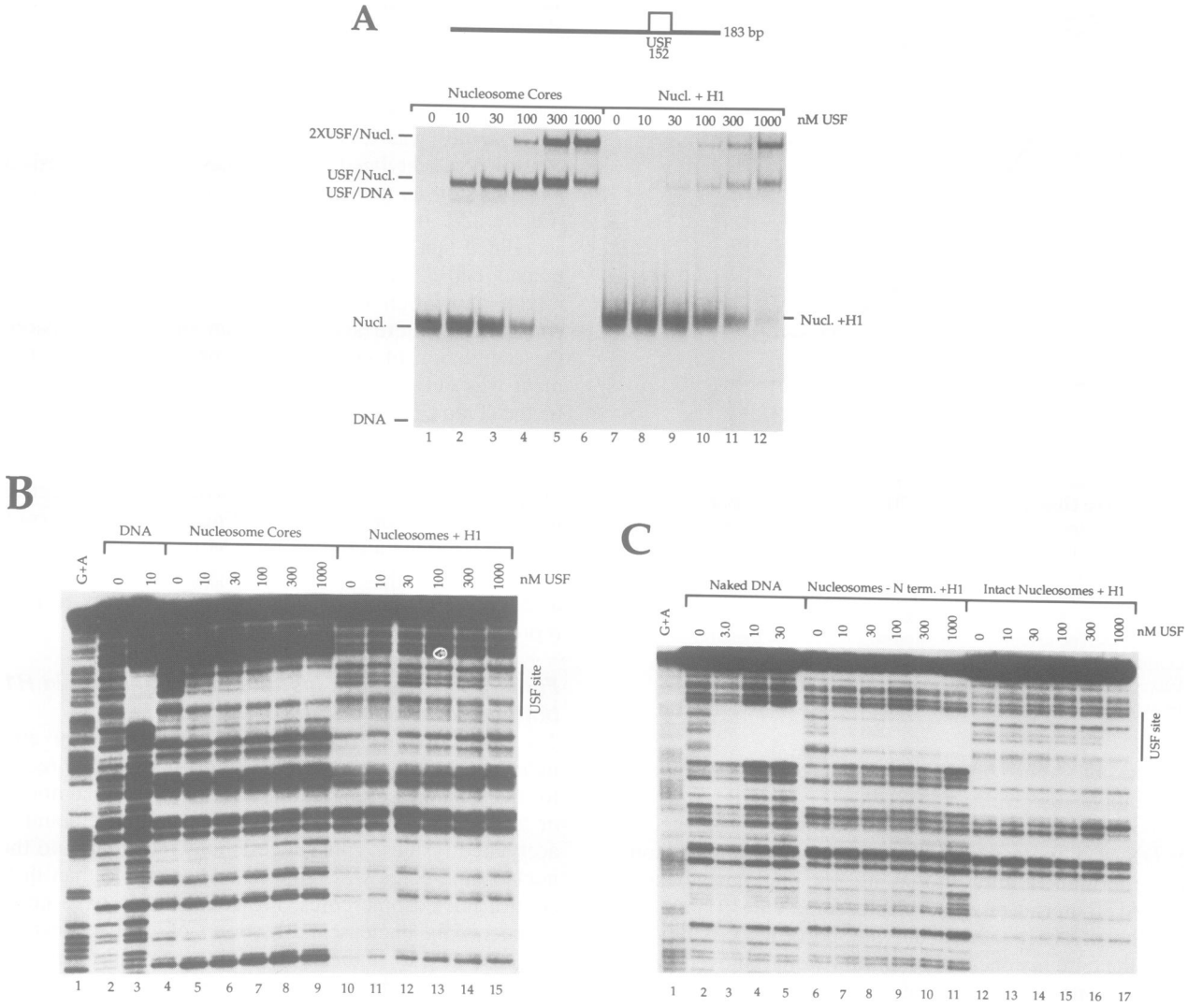

Fig. 4. Analysis of H1 repression of USF binding by DNase I footprinting. (**A**) The 183 bp probe, derived from pGALUSFBEND, containing one USF site 30 bp from one end of the fragment used for footprinting analysis is outlined above. To test H1 inhibition of USF binding on this DNA, nucleosomes were reconstituted with (Nucl. + H1; lanes 7–12) or without (Nucleosome Cores; lanes 1–6) 107 nM histone H1. The amounts of USF added are listed above each lane. Protein–DNA complexes representing USF bound to naked DNA (USF/DNA), USF bound to nucleosomes (USF/Nucl.) and 2×USF bound to nucleosomes (2×USF/Nucl.) are indicated. (**B**) End-labeled probe was mock reconstituted (free DNA; lanes 2–3), reconstituted into nucleosome cores (lanes 4–9) or reconstituted into nucleosome cores + H1 (lanes 10–15), and incubated with the indicated amounts of USF. Binding of USF was assayed by DNase I footprinting. G + A markers are shown in lane 1. The region protected by USF is indicated on the right. (**C**) The same probe was reconstituted with trypsinized nucleosome cores + H1 (lanes 6–11) or with intact nucleosome cores + H1 (lanes 12–17). Free DNA (lanes 2–5) was mock reconstituted as in (B). Samples were similarly incubated with the indicated amounts of USF and assayed by DNase I. G + A markers are shown in lane 1.

the amino termini, suggesting H1 binding in both instances. However, in the absense of the amino termini this apparent binding was less inhibitory to subsequent USF binding.

To explore further a potential effect on the affinity of H1 mediated by the core histone amino termini, we have more closely titrated H1 into binding reactions containing nucleosome core with or without the amino termini. The binding of histone H1 to nucleosome cores to form chromatosomes is apparent by a shift in the mobility of the nucleosome on non-denaturing polyacrylamide gels (Juan *et al.*, 1993). As shown in Figure 6, this mobility shift becomes readily apparent at increasing H1 concentrations with intact nucleosome cores (upper panel; compare even-numbered lanes with the odd-numbered lanes). While the nucleosome core and chromatosome (Nucl. + H1) bands overlap, it is clear that at even the lowest concentrations some of the intact nucleosome cores have become shifted by H1 (lanes 2 and 4). Importantly, this mobility shift was also observed for nucleosome cores lacking the amino termini (lower panel), confirming the suggestion of micrococcal nuclease digestion studies that H1 could interact with nucleosomes lacking the amino termini (Allan *et al.*, 1982, 1986; Hayes *et al.*, 1995). However, the apparent mobility shift of the nucleosome core lacking the amino termini began to occur at higher concentrations of H1 (lanes 8 and 10) than are required for intact nucleosome cores (compare upper and lower panels). This result suggests a reduction in the affinity of H1 for nucleosome cores in the absence of the amino-terminal tails which can account for the reduction in H1 repres-

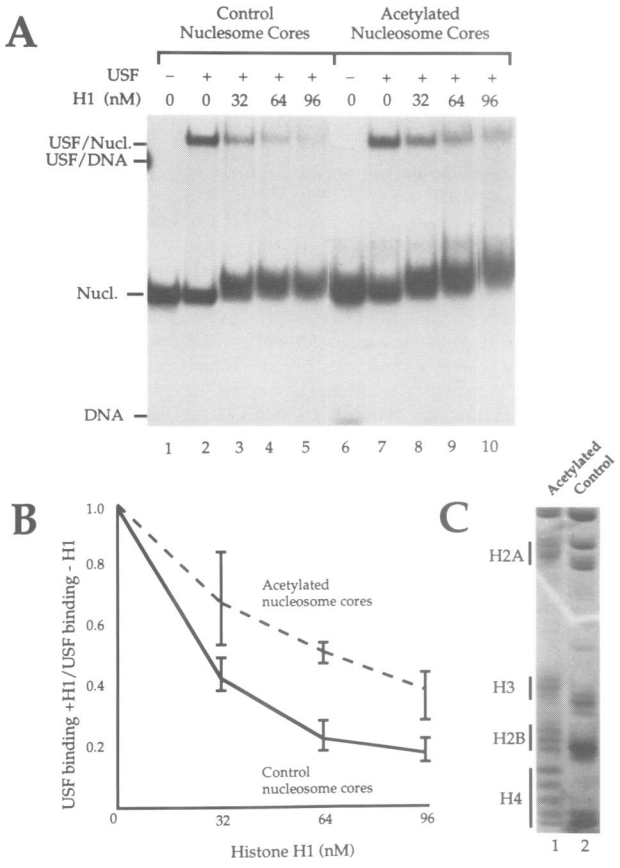

Fig. 5. H1-mediated repression of USF binding is partly alleviated by increased histone acetylation. (**A**) Binding of USF to control nucleosome cores (lanes 1–5) and acetylated nucleosome cores (lanes 6–10) in the presence of the indicated amounts of H1. Each complex was marked the same as in previous figures. Formation of the USF/Nucl. complexes was repressed by H1 to a lesser extent on the nucleosome cores bearing the acetylated core histones than on the control nucleosome cores (compare lanes 8–10 versus lane 7 with lanes 3–5 versus lane 2). (**B**) Graph of the relative repression of USF binding to control nucleosome cores (solid line) or to acetylated nucleosome cores (dashed line) at increasing concentrations of H1. The plotted data results from the quantitation of the experiments shown in (A) and two independent repeats. The ratio of USF binding ± H1 was derived as in Figure 4. The lines are drawn through the average values for the three independent experiments. The cross-bars illustrate the range of the data points. (**C**) TAU gel of the histones from nucleosome cores used in the experiments shown in (A) and (B). Lane 1 is acetylated nucleosome cores from butyrate-treated cells; lane 2 is the control nucleosomes. The bands representing H2A, H3, H2B and H4 are indicated.

sion of factor binding to these nucleosomes (Figures 2, 3 and 4).

Discussion

Repression of transcription factor binding by the nucleosome core is dependent upon the location of the binding site within the core particle. As binding sites approach the edge of the nucleosome core their affinity for transacting factors increases (Li and Wrange, 1994; Li *et al.*, 1994; Vettese-Dadey *et al.*, 1994). A similar nucleosome position effect has been observed for ARS function in yeast (Simpson, 1990). However, the experiments presented in this report indicate that maximum repression of transcription factor binding to nucleosomes requires the binding

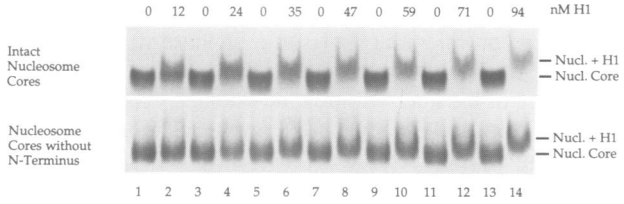

Fig. 6. Histone H1 binding to nucleosome cores containing or lacking the amino termini. Chromatosome mobility shifts of H1 binding to nucleosome cores, reconstituted on the same probe as in Figure 2A, were performed with intact nucleosome cores (upper panel) and nucleosome cores lacking the amino termini (lower panel). Increasing H1 concentrations were added to every other lane as indicated above the panels. The amounts of nucleosome cores and histone H1 were exactly matched in each experiment to provide an indication of the relative affinity of H1 for each type of nucleosome core. While the nucleosome core (Nucl. Core) and the chromatosome (Nucl. + H1) bands overlapped, clearly lower concentrations of H1 shifted the intact nucleosome cores than nucleosome cores lacking the N-termini.

of histone H1. Thus, H1 association with the nucleosome core extends the effective area of repression. The function of H1 in repressing factor binding is most easily interpreted as resulting from the direct interaction of H1 with the ends of nucleosomal DNA, where additional nuclease protection is observed upon H1 binding (reviewed in van Holde, 1988).

H1 repression of transcription factor binding was more strongly exerted on USF than on GAL4-AH. This difference is most likely due to the nature of the DNA-binding domains of these two factors. USF is a b/HLH/Z protein (a member of the Myc-related families) which binds DNA via two α-helices which scissor through the major grove on each side of the DNA helix with substantial fractions of the protein on both sides of the DNA (Ferre-D'Amare *et al.*, 1994, and references therein). By contrast, the majority of the GAL4-AH protein is located on one side of the DNA with binding occuring via bi-nuclear zinc clusters which interact in the major groove (Marmorstein *et al.*, 1992). Differential repression of transcription factor binding by H1 is analogous to the differential repression of transcription factor binding by the nucleosome core (Taylor *et al.*, 1991) and further supports the notion that factors will perform distinct functions with regard to initiating access to enhancer and promoter elements in chromatin (reviewed in Adams and Workman, 1993).

The repression of factor binding by H1 observed in our experiments was dependent on the core histone amino termini. H1-mediated repression was reduced upon removal of the amino termini with trypsin and partly alleviated if the core histones were more highly acetylated on the amino termini. Previous studies have indicated that the core histone amino-terminal tails modulate transcription factor access to binding sites within nucleosome cores (Lee *et al.*, 1993; Vettese-Dadey *et al.*, 1994). These data further implicate these domains in regulating factor access to linker DNA at the edge of nucleosomes via interactions with histone H1. The function of the amino termini in modulating H1 repression of transcription factor binding may be particularly important with regard to co-operative binding of the same or unrelated factors to nucleosomes which can initiate at the edge of the nucleosome (Vettese-Dadey *et al.*, 1994; C.C.Adams and J.L.Workman, unpublished).

The effect of the core histone tails on H1 binding may result from an interaction of some of these domains with the same region of nucleosomal DNA as histone H1 (i.e. at the edge of the nucleosome) and perhaps via interactions with H1 directly. For example, single nucleosome cores containing hyperacetylated H3 and H4 demonstrate increased sensitivity of the 5′ phosphate of the DNA to removal by nucleases, suggesting that the unmodified tails protect the ends of nucleosome core DNA (Simpson, 1978). Moreover, the H3 amino-terminal tail may directly interact with nucleosomal DNA at the end of the nucleosome core and the additional 10 bp protected by H1 binding (Lambert and Thomas, 1986; Hill and Thomas, 1990; reviewed in Turner, 1991). Alternatively, H1 binding may be reduced by subtle changes in nucleosome core conformation which are determined by the core histone tails. Bradbury and colleagues have detected a difference in linking number change per nucleosome core *in vitro* which arises as the result of acetylation of H3 and H4 (Norton *et al.*, 1989, 1990).

Our results are consistent with previous studies indicating that the core histone amino termini participate in the formation of higher-order chromatin structure. The core histone amino termini appear to contribute directly to chromatin folding (Garcia-Rameirez *et al.*, 1992) and are required for H1 to condense nucleosome arrays into chromatin fibers (Allan *et al.*, 1982; Annunziato and Seale, 1983; Perry and Annunziato, 1989, 1991; Ridsdale *et al.*, 1990). This effect may arise from a reduced affinity of H1 for nucleosomes containing acetylated amino termini. Several reports indicate that transcriptionally competent regions of chromatin *in vivo* are partly (but not completely) depleted of histone H1 (Kamakaka and Thomas, 1990; Dedon *et al.*, 1991; Bresnick *et al.*, 1992; reviewed in Ausio, 1992; Zlatanova and van Holde, 1992). H1-depleted chromatin subfractions are enriched in HMGI/Y which has been implicated in H1 displacement (Zhao *et al.*, 1993). H1 depletion, initiated by HMGI/Y or other H1-displacing proteins, may be facilitated and/or maintained by increased levels of core histone acetylation, which appears to occur over domains of less condensed chromatin (i.e. increased DNase I sensitivity; Hebbes *et al.*, 1994). Acetylation would also increase the accessibility of linker DNA to transcription factors by reducing the affinity of the remaining H1, as well as directly enhancing factor binding to the nucleosome cores (Lee *et al.*, 1993; Vettese-Dadey *et al.*, 1994). Such mechanisms may account for the occurrence of specific H4 acetylations (i.e. on lysine 16) in particular regions of the chromosome, and indeed on specific chromosomes, in accordance with their transcription activity (Jeppesen and Turner, 1993; Bone *et al.*, 1994; reviewed in Turner, 1994).

Materials and methods

Preparation of DNA probes

The 183 bp probe DNAs (used in Figures 2, 5A and 6) were generated by *Bam*HI digestion of plasmids pGALUSFBEND or pUSFGALBEND, followed by Klenow incorporation of [^{32}P]dATP. These plasmids were constructed by inserting the 46 bp oligonucleotide (5′-CTAGAC-GGAGGACAGTCCTCCGGTTACCTTCGAACCACGTGGCCGT-3′), containing consensus GAL4 and USF binding sites (underlined) separated by 14 bp, into the *Xba*I site of the pBEND derived vector pTK401 (Kerppola and Curran, 1991) in both orientations.

The 183 bp footprinting probe (shown in Figure 4A) was generated by *Eco*RI digestion of pGALUSFBEND, followed by Klenow labeling with [^{32}P]dATP and subsequent digestion with *Sal*I. The USF site lies 30 bp from the unlabeled end of the fragment. All probes were purified from 8% acrylamide (acrylamide–bisacrylamide, 29:1)–1×Tris–borate–EDTA (TBE) gels.

Purification of nucleosome cores, histone H1, GAL4 derivatives and USF

Nucleosome cores were purified from HeLa nuclei as described previously (Juan *et al.*, 1993; Vettese-Dadey *et al.*, 1994). For hyperacetylated nucleosome cores, nuclei were prepared from cells subjected to 20–24 h treatment with 10 mM sodium butyrate (an inhibitor of histone deacetylase). Sodium butyrate at 10 mM was included in all of the purification steps of the acetylated nucleosomes, as well as parallel preparations of control nucleosomes from cells not treated with sodium butyrate. The degree of acetylation is demonstrated by TAU electrophoresis (see Figure 5C). Samples for the TAU gel were prepared as follows. Protein (25 μg) was pre-mixed with 25 μg/ml protamine sulfate (Sigma), precipitated with 10% trichloroacetic acid and then incubated on ice for 30 min. The resulting protein precipitate was pelleted by centrifugation and washed once with acidified acetone followed by acetone. Pellets were air dried, boiled for 5 min in 0.1 ml of loading buffer (20% sucrose, 1% β-mercaptoethanol, 5% acetic acid, 0.02% pyronin Y). The TAU gel was run as described in Braunstein *et al.* (1993). To obtain nucleosome cores without N-terminal tails, proteolysis with trypsin was performed after nucleosome purification (see below). Histone H1 was purified from HeLa nuclei using a modified method of Stein and Mitchell (1988), avoiding acid extraction. H1-containing fractions from a hydroxylapatite column (Workman *et al.*, 1991b) were pooled and then concentrated with Centriprep-10 concentrators (Amicon) to 3 ml (12 mg protein total). The concentrated sample was applied to a 35 ml Sephacryl-S200 (Pharmacia) gel filtration column (1×45 cm) which was equilibrated and run with buffer containing 0.6 M NaCl, 50 mM $NaPO_4$ (pH 6.8), 0.1% NP40 and 0.2 mM PMSF. H1-containing fractions were detected by SDS–PAGE, pooled, divided into small aliquots and stored frozen at −70°C. The H1 was judged to be >90% pure. The fusion protein GAL4-AH, containing the N-terminal 147 amino acid DNA-binding and dimerization domains of GAL4 and an artificial 15 amino acid putative amphipathic helix, was purified from bacterial strains by the method of Lin *et al.* (1988). The purification of recombinant USF was performed according to procedures described by Pognonec *et al.* (1991).

Nucleosome core and chromatosome reconstitution

Nucleosome core reconstitution was achieved by octamer transfer (Rhodes and Laskey, 1989; Côté *et al.*, 1994b). Probe DNA was mixed with H1-depleted oligonucleosomes (0.2–0.3 mg/ml final concentration) at 1 M NaCl. Following incubation at 37°C for 20 min, the transfer reactions were serially diluted to 0.8 M and then to 0.6 M NaCl with 10 mM HEPES (pH 8.0), 1 mM EDTA, with incubation at 37°C for 15 min at each dilution step. The 0.6 M NaCl dilution mix was divided into aliquots containing 0.5–2.25 mg nucleosome cores and H1 was added in the amounts indicated in the figure legends. Following incubation at 37°C for 25–30 min, the samples were further diluted to 0.4 M and then to 0.2 M NaCl with 10 mM Tris (pH 7.8), 1 mM EDTA, 0.1% NP 40, 1 mM 2-mercaptoethanol, with 25–30 min incubations at 37°C after each dilution. A final 2-fold dilution to 0.1 M NaCl with buffer containing 10 mM Tris (pH 7.8), 1 mM EDTA, 0.1% NP 40, 1 mM 2-mercaptoethanol, 20% glycerol was performed and samples were incubated at 37°C for a final 25–30 min. For experiments utilizing nucleosome cores without tails, dilution buffers also contained 0.2 mg/ml trypsin inhibitor for both trypsinized and control samples. In the experiments using the highly acetylated nucleosome cores, 5 mM sodium butyrate was included in all the dilution buffers for both the acetylated and control samples. Neither trypsin inhibitor nor sodium butyrate at the concentrations used had any effect on nucleosome core reconstitution or H1 binding.

Removal of the amino-terminal tails by trypsin and controls for trypsin inactivation

To remove core histone amino-terminal 'tails', trypsin treatment of H1-depleted nucleosome cores was carried out at room temperature for 5 min at a final concentration of 0.5 mg/ml nucleosome core, 10 mM HEPES (pH 8.0), 1 mM EDTA, 0.03 mg/ml trypsin (Sigma, 10 200 U/mg) in 12 ml reaction volume. The amount of digestion required to remove the amino termini completely was determined previously by

15% SDS–polyacrylamide gels. Following digestion, an excess of soybean trypsin inhibitor (Sigma) was added to a final concentration of 2 mg/ml. The trypsinized nucleosome cores were subsequently used as histone donors in transfer reactions of the histone octamer onto labeled DNA probes. Trypsin inhibitor (0.2 mg/ml) was included in all octamer transfer and binding reaction mixtures to eliminate subsequent trypsin activity. The inactivation of the trypsin was tested directly by control reaction mixtures in which trypsin inhibitor was added prior to the trypsinization step (see Figure 1). These controls illustrated that neither the core histones nor the H1 were degraded by trypsin once the inhibitor was present. To verify further that H1 was intact, the final reaction mixture was acid extracted and proteins were run on a 15% SDS–polyacrylamide gel (see Figure 1B).

Factor binding and electrophoretic mobility shift assays

The final nucleosome dilutions in 0.1 M NaCl were divided for either GAL4-AH or USF binding reactions such that each binding reaction contained 0.3 mg nucleosomal DNA. At this point, the transcription factors were added at the amounts indicated in the figure legends. Binding reactions contained 100 mM NaCl, 0.25 mg/ml bovine serum albumin (BSA), 160 μg/ml trypsin inhibitor, 25 mM KCl, 5 mM HEPES (pH 7.4), 0.5 mM EDTA, 10% glycerol, 2.5 mM 2-mercaptoethanol, 2.5 mM $ZnCl_2$, 0.5 mM PMSF and 0.05% NP 40 for GAL4-AH. For USF, they were the same except an additional 4 mM dithiothreitol (DTT) was also included. The binding reactions were carried out at 30°C for 30 min with a final volume of 27–40 μl, loaded onto 4% acrylamide (acrylamide:bisacrylamide = 29:1)–0.5×TBE gels, and run at 150 V (constant voltage) for 3 h at room temperature. Gels were dried and subjected to autoradiography. In addition, each gel was quantitated using a Betascope blot analyzer (Betagen Corp.).

DNase I footprinting

After nucleosome cores were reconstituted and USF was bound (as described above), the 183 bp end-labeled probes were digested with DNase I (Boehringer Mannheim) at 10–13.3 U/mg DNA, in 30–40 μl binding reactions, for 1 min at room temperature. Mock-reconstituted probe (labeled DNA probe added to the reconstitution reaction at 0.1 M NaCl) were also bound by USF and digested with DNase I, except that 10-fold less enzyme was used. Digestion was terminated with 1×vol. of 20 mM Tris (pH 7.5), 50 mM EDTA, 2% SDS, 0.25 mg/ml yeast tRNA (Sigma) and 200 mg/ml Proteinase K (Sigma). Reactions were then incubated at 50°C for 1–3 h and the DNA precipitated with 0.5 vols 7.5 M ammonium acetate and 3 vols absolute ethanol. DNA pellets were washed with 80% ethanol, dried, and resuspended in 2 μl ddH_2O and 3 μl formamide dye. Samples were incubated at 95°C for 5 min, quenched on ice, and resolved on 8% acrylamide, 8 M urea sequencing gels.

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