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Acetylation of histone H4 plays a primary role in enhancing transcription factor binding to nucleosomal DNA *in vitro*

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Core histones isolated from normal and butyrate-treated HeLa cells have been reconstituted into nucleosome cores in order to analyze the role of histone acetylation in enhancing transcription factor binding to recognition sites in nucleosomal DNA. Moderate stimulation of nucleosome binding was observed for the basic helix–loop–helix factor USF and the Zn cluster DNA binding domain factor GAL4-AH using heterogeneously acetylated histones. However, by coupling novel immunoblotting techniques to a gel retardation assay, we observed that nucleosome cores containing the most highly acetylated forms of histone H4 have the highest affinity for these two transcription factors. Western analysis of gel-purified USF–nucleosome and GAL4-AH–nucleosome complexes demonstrated the predominant presence of acetylated histone H4 relative to acetylated histone H3. Immunoprecipitation of USF–nucleosome complexes with anti-USF antibodies also demonstrated that these complexes were enriched preferentially in acetylated histone H4. These data show that USF and GAL4-AH preferentially interact with nucleosome cores containing highly acetylated histone H4. Acetylation of histone H4 thus appears to play a primary role in the structural changes that mediate enhanced binding of transcription factors to their recognition sites within nucleosomes.

Keywords: chromatin/histone acetylation/nucleosomes/transcription factors

Introduction

In vivo and *in vitro* studies have illustrated clearly that, as suppressors of transcription, the four histones that comprise the nucleosome core (H2A, H2B, H3 and H4) participate in the transcriptional regulation of numerous genes (reviewed in Winston and Carlson, 1992; Svaren and Horz, 1993; Becker, 1994; Owen-Hughes and Workman, 1994; Wolffe, 1994). The core histones undergo several post-translational modifications including ubiquitination, methylation, phosphorylation, ADP-ribosylation and acetylation (reviewed in Matthews and Waterborg, 1985). Of these, the reversible acetylation of ε-amino groups of lysine residues present in the amino-terminal domains, 'tails', of the core histones is the most strongly linked with transcriptional activity (reviewed in Pfeffer and Vidali, 1991; Turner, 1991; Loidl, 1994). Histone acetylation is brought about by two different classes of enzymes for which corresponding genes have been cloned (Kleff *et al.*, 1995; Brownell *et al.*, 1996). Cytoplasmic type-B histone acetyltransferases are thought to acetylate free histones that subsequently are assembled into chromatin, while nuclear type-A histone acetyltransferases are thought to carry out transcription-related acetylation of chromosomal histones (reviewed in Brownell and Allis, 1996).

Several lines of evidence implicate a relationship between histone acetylation and gene activity. For example, in *Tetrahymena*, the transcriptionally active macronucleus contains acetylated histones whereas the transcriptionally inactive micronucleus is deficient (Gorovsky *et al.*, 1973; Vavra *et al.*, 1982; Lin *et al.*, 1989). In addition, acetylation of specific lysine residues on histone H4 may define functional chromatin domains (reviewed in Turner and O'Neill, 1995). The hyperactive X chromosome in *Drosophila* male larvae is more highly acetylated on lysine 16 (H4.Ac16) than female X chromosomes or autosomes (Turner *et al.*, 1992; Bone *et al.*, 1994). By contrast, the inactive female X chromosome in mammals (Xi) is underacetylated (Jeppesen and Turner, 1993). Moreover, in cultured mammalian cells, centric and telomeric heterochromatin are deficient in acetylated H4 relative to euchromatin (O'Neill and Turner, 1995). A biochemical correlation is evident from the chromatography of mammalian and yeast nucleosomes on organomercurial–agarose columns which leads to enrichment of nucleosomes from transcriptionally active genes that also contain highly acetylated histones (Walker *et al.*, 1990). Other fractionation schemes have also shown that chromatin preparations enriched in active genes are also enriched in acetylated histones (Allegra *et al.*, 1987; Ridsdale and Davie, 1987; Ip *et al.*, 1988; Boffa *et al.*, 1990). The direct biochemical link between core histone acetylation and active genes followed from the demonstration that immunoprecipitation of mononucleosomes from chicken erythrocytes with antibodies that recognize all acetylated histones resulted in enrichment of active gene sequences (Hebbes *et al.*, 1988, 1994). Use of the same approach with an antibody specific to acetylated histone H4 (Lin *et al.*, 1989) demonstrated that transcriptional silencing of the yeast mating type cassette and telomere silencing are accompanied by reduced nucleosomal H4 acetylation (Braunstein *et al.*, 1993). The concentration of

acetylated core histones at transcriptionally active loci was also shown by the observation that the chromatin at CpG islands (located at the 5′ end of constitutively transcribed genes and some tissue-specific genes) contains highly acetylated histones H3 and H4 (Tazi and Bird, 1990). Most recently, immunoprecipitations of nucleosomes with antibodies to specific acetylation sites in histone H4 did not reveal an enrichment of transcribed sequences, but did demonstrate the lack of H4 acetylation in heterochromatin (O'Neill and Turner, 1995).

Genetic experiments in yeast show that mutations in the H3 tail result in hyperactivation of genes activated by GAL4 while mutations in the histone H4 tail result in a reduction of the induction of the *GAL1* and *PHO5* genes and a loss of silencing at the mating type loci (Kayne *et al.*, 1988; Durrin *et al.*, 1991). These functions of the H3 and the H4 amino-terminal tails may be mediated in part through direct interactions with regulatory proteins. For example, the amino-terminal tail of H4 is involved in nucleosome positioning by the factors binding the α2 operator (Roth *et al.*, 1992) and binding studies have detected an interaction between the H3 and H4 amino-termini and the Sir3 and Sir4 repressors (Hecht *et al.*, 1995). The amino-terminal tails may also participate in controlling the accessibility of promoter elements in chromatin (Fisher-Adams and Grunstein, 1995).

Biochemical studies implicate the amino-terminal tails in both folding of nucleosomes into higher order chromatin structures (Allan *et al.*, 1982; Annunziato and Seale, 1983; Perry and Annunziato, 1989, 1991; Ridsdale *et al.*, 1990) and in directly controlling transcription factor binding to nucleosomal DNA (Lee *et al.*, 1993; Juan *et al.*, 1994; Vettese-Dadey *et al.*, 1994). While the core histone amino-terminal tails are not essential for nucleosome core formation and stability, these domains appear to play a crucial role in restricting factor binding to nucleosomal DNA. Removal of these domains by proteases has been shown to stimulate the binding of TFIIIA, GAL4-AH and USF to nucleosome cores (Lee *et al.*, 1993; Juan *et al.*, 1994; Vettese-Dadey *et al.*, 1994). Moreover, stimulation of GAL4-AH binding by removal of the amino-terminal tails reduces the apparent cooperativity in the binding of multiple GAL4-AH dimers to nucleosome cores, indicating that cooperative factor binding occurs in response to inhibition from these domains (Vettese-Dadey *et al.*, 1994). In addition, the binding of the basic helix–loop–helix (bHLH) protein, USF, to nucleosomes is inhibited by the binding of the linker histone H1, and this inhibition of USF binding is also dependent on the core histone amino-termini tails (Juan *et al.*, 1994). To test whether core histone acetylation would similarly stimulate transcription factor binding, previous studies employed histones isolated from cells treated with sodium butyrate, an inhibitor of histone deacetylases, which results in an increased level of histone acetylation (i.e. ~50% of sites on H4). Nucleosome cores reconstituted with this more highly acetylated population of histones demonstrated an increased affinity for TFIIIA (Lee *et al.*, 1993) and USF (Juan *et al.*, 1994). Thus, the amino-terminal tails function as repressors of transcription factor binding, but this repression appears to be alleviated by acetylation of lysine residues in these domains.

Here, we use novel immunoblotting approaches with antibodies to acetylated histones to demonstrate directly that nucleosomes bearing acetylated histone H4 are preferentially bound by USF or by GAL4-AH. Importantly, the nucleosome cores with the greatest affinity for USF or GAL4-AH are more highly acetylated on histone H4 than histone H3. Thus, acetylation of histone H4 appears to play a primary role in stimulating transcription factor binding to nucleosomal DNA.

Results

Binding of transcription factors, USF and GAL4-AH, to nucleosomes containing acetylated histones

Nucleosome cores were reconstituted with histones from butyrate-treated cells (hyperacetylated nucleosomes) or untreated cells (control nucleosomes) onto 150 bp DNA probes bearing a single USF or GAL4 site and analyzed for binding of these factors. In Figure 1A, control (lanes 1–6) or hyperacetylated (lanes 7–12) nucleosome cores were transferred onto a probe bearing a single USF site (E-box) located 20 bp from one end. With increasing amounts of USF, binding was observed to both samples of nucleosomes, with a small enhancement of binding to the nucleosomes containing the hyperacetylated histones at each USF concentration. Similarly, a small increase in binding to hyperacetylated nucleosome cores was also evident for GAL4-AH binding to a nucleosome with a GAL4 site 36 bases from the end (Figure 1B).

The data from the gels presented in Figure 1A and B are graphed in Figure 1D and E. There was clearly a moderate stimulatory effect of the 'hyperacetylated' core histones; however, the stimulation of USF and GAL4-AH binding was less than that which was suggested for TFIIIA binding to hyperacetylated nucleosome cores reconstituted on the *Xenopus borealis* 5S RNA gene (Lee *et al.*, 1993). This raises the possibility that there is an inherent difference in the recognition of acetylated nucleosomal binding sites by GAL4-AH and USF relative to TFIIIA. Nucleosomes with an average of only 2–3 acetyl groups per molecule of H4 were reported to be sufficient to enhance TFIIIA binding to nucleosome cores (Lee *et al.*, 1993). Multiple interactions of the nine Zn fingers of TFIIIA (Miller *et al.*, 1985; Churchill *et al.*, 1990) with >40 bp of 5S DNA may allow more permissive recognition of acetylated nucleosomes than is achieved by the two Zn clusters of GAL4-AH dimers (Marmorstein *et al.*, 1992) or the bHLH binding domain of USF dimers (Ferre-D'Amare *et al.*, 1994). Thus, enhancement of nucleosome binding for transcription factors with less extended DNA binding domains than TFIIIA may require more specific or extensive levels of histone acetylation. In this regard, it is important to note that, while the histones from butyrate-treated cells show increased levels of acetylation relative to those from untreated cells, these histones are only partially and heterogeneously acetylated. Figure 1C shows a Coomassie blue-stained Triton–acid–urea gel (TAU) of histones used for the reconstitutions and illustrates that the histones from butyrate-treated cells contained a heterogeneous population of acetylated forms of H3 and H4. Thus, the stimulatory effect of the bulk hyperacetylated histones on USF and GAL4-AH binding might appear small if the most active acetylated species

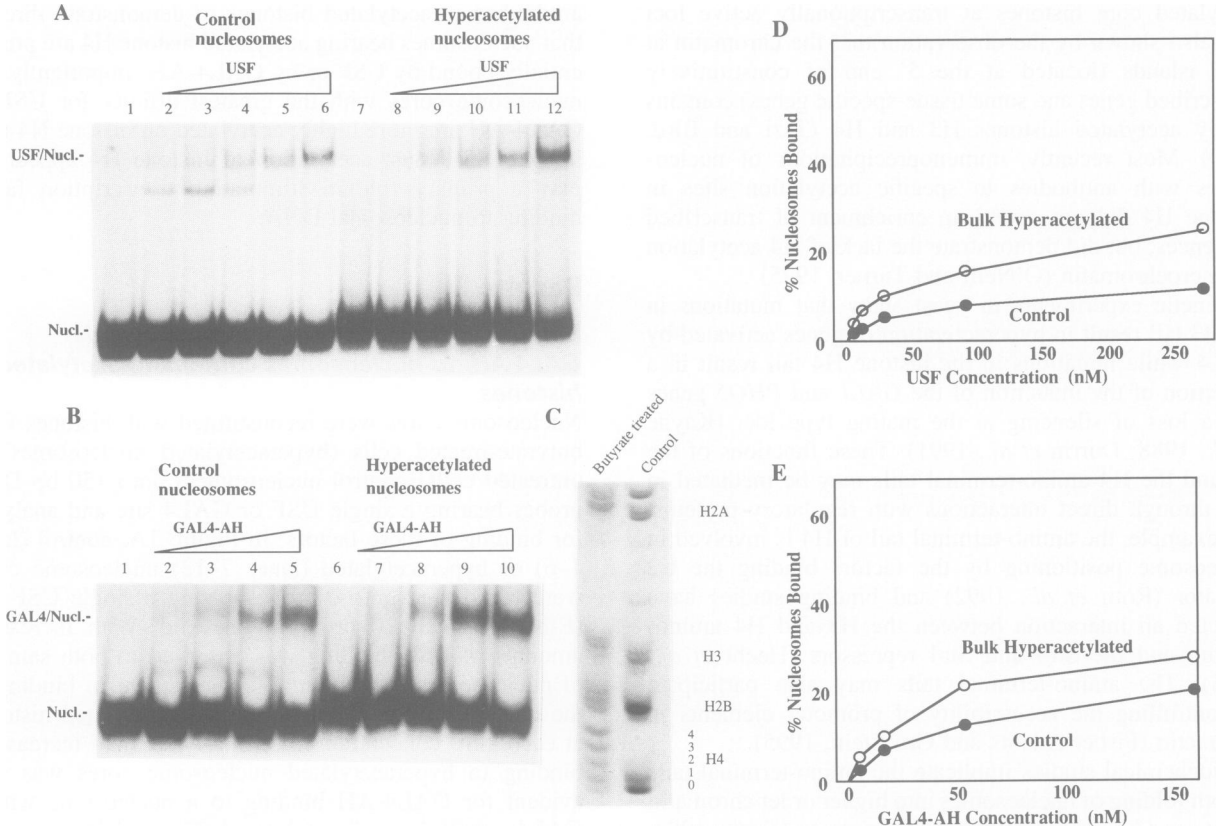

Fig. 1. Binding of USF and GAL4-AH to nucleosome cores with elevated levels of histone acetylation. (**A**) A 150 bp probe DNA derived from the HIV-1 long terminal repeat with a single USF site centered at 20 bp from one end was reconstituted into control nucleosome cores (histones from untreated HeLa cells, lanes 1–6) or hyperacetylated nucleosome cores (histones from butyrate-treated HeLa cells, lanes 7–12) and incubated with increasing concentrations of USF. The mobility of the reconstituted nucleosome cores (Nucl.) and the nucleosome cores with a single USF dimer bound (USF/Nucl.) are indicated. Binding of USF to the nucleosome core was seen for both the control and the hyperacetylated nucleosome cores, with a slight increase in preference for binding to the hyperacetylated cores. The concentrations of USF included in this titration were; 0, lanes 1 and 7; 2.7 nM, lanes 2 and 8; 9 nM, lanes 3 and 9; 27 nM, lanes 4 and 10; 90 nM, lanes 5 and 11; and 270 nM, lanes 7 and 12. (**B**) A similar experiment was performed with a single GAL4 site centered at 36 bp from one end. A 150 bp probe DNA was reconstituted into control nucleosome cores (histones from untreated HeLa cells, lanes 1–5) or hyperacetylated nucleosome cores (histones from butyrate-treated HeLa cells, lanes 6–10) and incubated with increasing concentrations of GAL4-AH. The mobility of the reconstituted nucleosome cores (Nucl.) and the nucleosome cores with a single GAL4-AH dimer bound (GAL4/Nucl.) are indicated. A small increase in binding to hyperacetylated nucleosome cores was also evident for GAL4-AH binding to a nucleosome. The concentrations of GAL4-AH included in the binding reactions were; 0, lanes 1 and 6; 5.3 nM, lanes 2 and 7; 15.9 nM, lanes 3 and 8; 53 nM, lanes 4 and 9; and 159 nM, lanes 5 and 10. (**C**) TAU gel of the histones used in (A) and (B) and subsequent experiments. The butyrate-treated lane (hyperacetylated histones) shows the elevated levels of acetylated forms of the histones as compared with the control histones. Bands representing 0–4 acetyl-lysines on histone H4 are labeled. (**D** and **E**) The data derived from the representative experiments shown in (A) and (B) respectively are presented as a graph of percent nucleosomes bound versus factor concentrations. These graphs illustrate that only a moderate stimulation of USF or GAL4-AH binding was observed with the bulk hyperacetylated nucleosomes.

were only a fraction of the total hyperacetylated histone population. To test this possibility, we examined directly the acetylated forms of H3 and H4 contained in nucleosome cores having the highest affinity for USF and GAL4-AH.

Nucleosomes containing highly acetylated H4 have enhanced affinity for transcription factors

The first experimental strategy is diagrammed in Figure 2. To analyze the histone composition of factor-bound nucleosomes it is necessary that all of the nucleosomes in the reaction mixtures contain binding sites for the transcription factor. Nucleosomes cores of homogeneous sequence therefore were reconstituted with DNA fragments generated by PCR (Workman and Kingston, 1992) and used as substrates for the binding of purified transcription factors. The population of nucleosome cores bound by the transcription factor was separated from the unbound population by electrophoretic mobility shift gels. To determine the distribution of acetylated histone H4 in the factor-bound and the unbound populations of nucleosome cores, the electrophoretic mobility shift gels were blotted directly and simultaneously onto nitrocellulose and DEAE membranes (shift–Western blots). Autoradiography of the DEAE membranes revealed the labeled nucleosomal DNA and thus presented the distribution of total nucleosomes in the bound and the unbound populations. The nitrocellulose membranes were immunostained with antibodies to highly acetylated histone H4, thereby revealing the distribution of nucleosomes containing the highly acetylated H4 forms. The H4-specific antibody used, 'penta' antibodies (Lin *et al.*, 1989), primarily recognizes the tetra- and tri-acetyl forms of H4, only weakly recognizes the di-acetyl forms of H4 and does not recognize the mono-acetyl or unacetylated forms of H4 at all (Perry *et al.*, 1993).

The results of shift–Western blots analyzing GAL4-AH and USF binding to hyperacetylated nucleosome cores are

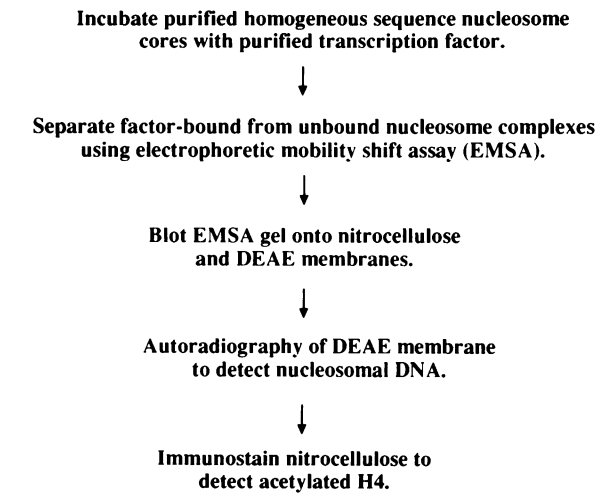

Fig. 2. Experimental strategy for mobility shift–Western experiments to determine the distribution of acetylated histone H4 in factor-bound versus unbound nucleosomes. For experimental details see Materials and methods.

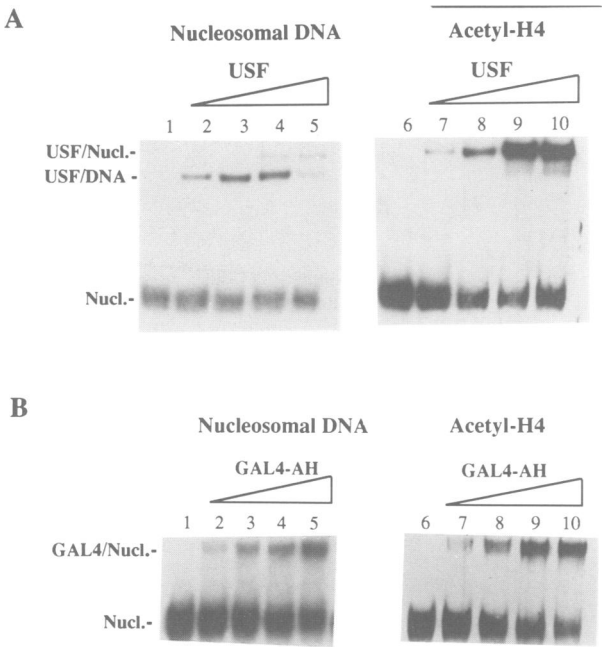

Fig. 3. Preferential binding of USF and GAL4-AH to nucleosome cores containing acetylated histone H4. (**A**) Nucleosome cores were reconstituted using hyperacetylated core histones from butyrate-treated cells. Nucleosome cores bearing a single USF site (E-box) 20 bp from the 5′ end were incubated without (lanes 1 and 6) or with increasing amounts of USF, followed by separation of the bound and unbound nucleosomes on a 6% acrylamide non-denaturing gel. The gel was blotted to nitrocellulose and DEAE membranes. An autoradiogram of the DEAE membrane (nucleosomal DNA) is shown in lanes 1–5 and illustrates the distribution of the total nucleosomal DNA in the bound and unbound fractions. A small amount of free DNA in the nucleosome preparation (not shown) was bound readily by a dimer of USF (USF/DNA; lanes 2–4). With increasing amounts of USF, the USF/Nucl. complex appeared (lanes 2–5). To determine the protein composition in these fractions, a Western blot of the nitrocellulose membrane was performed, and is shown in lanes 6–10 which utilized antibodies to highly acetyled histone H4. Note that USF binds preferentially to the nucleosome fraction which contains highly acetylated H4, relative to the total nucleosomes as seen on the DNA blot. USF concentrations were as follows: 0 (lanes 1 and 6), 2.7 nM (lanes 2 and 7), 9 nM (lanes 3 and 8), 27 nM (lanes 4 and 9) and 90 nM (lanes 5 and 10). (**B**) Conditions as in (A) except that the nucleosome cores bear a single GAL4 site 36 bp from the 5′ end. GAL4-AH bound preferentially to nucleosome cores containing highly acetylated H4 but to a lesser degree than for USF. GAL4-AH concentrations in (B) are as follows: 0 (lanes 1 and 6), 57 nM (lanes 2 and 7), 114 nM (lanes 3 and 8), 228 nM (lanes 4 and 9) and 342 nM (lanes 5 and 10).

shown in Figure 3. Figure 3A illustrates the binding of USF to nucleosome cores reconstituted with hyperacetylated core histones from butyrate-treated HeLa cells. The DNA fragment used was the same as that used in Figure 1A. The left panel of Figure 3A shows the DEAE membrane and illustrates the distribution of total nucleosomal DNA. When USF was included in the binding reactions, the small amount of free DNA in the nucleosome preparation (not shown) was readily bound by a dimer of USF (USF/DNA; lanes 2–4). By contrast, only a very small fraction of the nucleosomal DNA (Nucl.) was bound by USF, resulting in the formation of a supershifted complex (USF/Nucl.). The right panel of Figure 3A shows a Western blot of the nitrocellulose membrane (blotted from the same gel) using the antibody to highly acetylated histone H4 and illustrates the distribution of nucleosomes containing highly acetylated H4. Clearly, a much larger fraction of those nucleosomes containing highly acetylated H4 were bound by USF. This experiment illustrates the substantial preference of USF for binding nucleosomes containing the highly acetylated H4 epitope over the bulk of the hyperacetylated nucleosomes. Figure 3B shows a similar experiment analyzing GAL4-AH binding to a 150 bp nucleosome core containing a single GAL4 site 36 bp from an end which was reconstituted with hyperacetylated histones. As with USF, nucleosomes containing highly acetylated H4 (right panel) were bound preferentially relative to bulk hyperacetylated nucleosomes (left panel).

The results of the experiments shown in Figure 3A and B and two independent repeats of each experiment are graphed in Figure 4. It can be clearly seen that nucleosome cores containing the most highly acetylated forms of histone H4 have a much higher affinity for USF (Figure 4A) and for GAL4-AH (Figure 4B) than the bulk heterogeneously hyperacetylated nucleosomes. Moreover, this effect was much greater than the general stimulatory effect of the bulk hyperacetylated nucleosomes relative to non-acetylated control nucleosomes (Figure 1D and E). The most dramatic difference in affinity between the highly acetylated H4 nucleosomes and the bulk hyperacetylated nucleosomes was observed for USF binding (Figure 4A). We estimate the K_d of USF for naked DNA in our protocols at <5 nM, in agreement with previous measurements (Sawadogo *et al.*, 1988; Pognonec and Roeder, 1991). However, its affinity for non-acetylated nucleosome cores can be reduced by over three orders of magnitude (Adams and Workman, 1994). By contrast, the data in Figure 4A illustrate 50% occupancy of nucleosome cores containing highly acetylated H4 at ~27 nM USF, indicating a K_d in this range. Thus, while dramatically stimulated, the affinity of USF for nucleosome cores containing highly acetylated H4 was not as great as for naked DNA. However, the intranuclear concentrations of USF are estimated to be as high as 500 nM (Sawadogo *et al.*, 1988; Ferre-D'Amare *et al.*, 1994), suggesting that nucleosomes resembling those

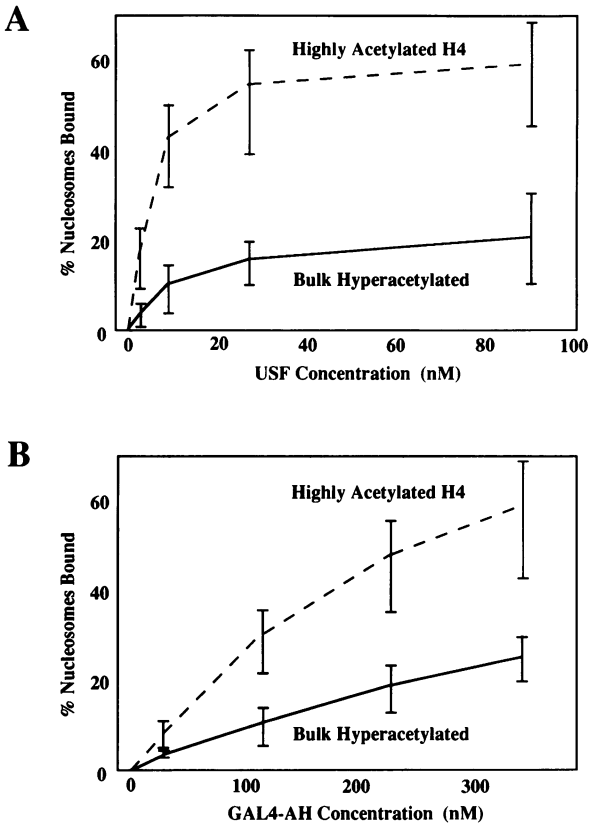

Fig. 4. Graph of the percent nucleosomes bound versus transcription factor concentrations for USF binding at a site 20 bp into the nucleosome core (**A**) and GAL4-AH binding to a site 36 bp into the nucleosome core (**B**). The data were derived from the experiments shown in Figure 3A and B and two independent repeats of each. For each factor, the percentage of the bulk hyperacetylated nucleosomes bound (solid lines), calculated from the DEAE membranes, and percentage of highly acetylated histone H4-containing nucleosomes (dashed lines), calculated from the Western blots, is indicated. The lines are drawn through the average value of the three experiments. The cross bars illustrate the range of data points at each factor concentration.

detected in the shift–Western blots with the acetylated H4 antibody could be easily bound by USF *in vivo*.

We reported previously that the removal of the core histone amino-termini with trypsin stimulated GAL4-AH binding to nucleosome cores (Vettese-Dadey *et al.*, 1994). This stimulation was maximal when the center of the GAL4 site was located between 26 and 40 bp into the nucleosome core (intermediate position). The GAL4 site in the nucleosome cores used to generate the data in Figure 4B was located at a similar position (36 bp into the nucleosome core). The GAL4-AH binding curve to the nucleosome cores containing highly acetylated H4 (Figure 4B) is analogous to that of GAL4-AH binding to nucleosome cores lacking the amino-termini (Figure 7, Vettese-Dadey *et al.*, 1994). Removal of the core histone amino-terminal tails also stimulated GAL4-AH binding to a more inhibited centrally located site (between 60 and 74 bp into the nucleosome core) but to a lesser extent than at the intermediate site (Figure 6, Vettese-Dadey *et al.*, 1994). We have tested the binding of GAL4-AH to a centrally located site 66 bp into nucleosome cores by shift–Western analysis. We also observed enhanced GAL4-

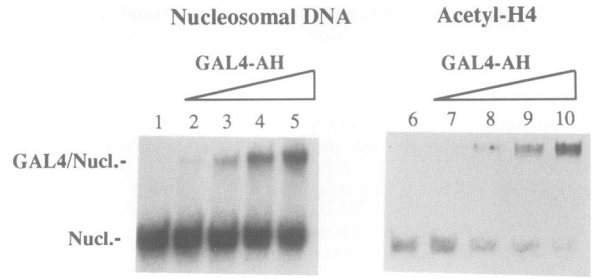

Fig. 5. Preferential binding of GAL4-AH to nucleosome cores containing highly acetylated histone H4 using nucleosome cores containing physiological mixtures of histone acetylation. Conditions were as described in Figure 3B except that the GAL4 site nucleosome cores were reconstituted with histones derived from control (non-butyrate-treated) HeLa cells. The Western blot (right panel) illustrates the distribution of the small amount of highly acetylated H4 present in these histones (Figure 1C). GAL4-AH concentrations were as follows: 0 (lanes 1 and 6), 57 nM (lanes 2 and 7), 114 nM (lanes 3 and 8), 228 nM (lanes 4 and 9) and 342 nM (lanes 5 and 10).

AH binding to nucleosome cores containing highly acetylated H4 at this location, although, as expected, levels of binding were reduced at this position relative to the intermediate position (data not shown). Thus, with regard to GAL4-AH binding, the properties of nucleosome cores lacking their amino-termini mimicked those of the population of nucleosomes containing highly acetylated histone H4.

The increased affinity of transcription factors for nucleosomes bearing highly acetylated H4 was not due to non-specific effects of the butyrate treatment of HeLa cells used to produce the hyperacetylated core histones. Figure 5 illustrates the preferential binding of GAL4-AH to nucleosome cores prepared from 'control' histones derived from cells not treated with sodium butyrate. Thus, these nucleosomes bear only physiological levels of histone acetylation. GAL4-AH preferentially bound the small fraction of nucleosome cores containing highly acetylated histone H4 (right panel) relative to the bulk non-acetylated nucleosomes (left panel).

Acetylated histone H4 has a greater effect on the preferential binding of transcription factors to nucleosome cores than acetylated histone H3

The data in Figure 4 illustrate a substantial enrichment of highly acetylated forms of histone H4 in nucleosome cores having high affinity for USF or GAL4-AH. However, analysis of nucleosomes from 'active' fractions of genomic sequences has illustrated an enrichment of both acetylated H3 and H4 (reviewed in Matthews and Waterborg, 1985; Allegra *et al.*, 1987; Tazi and Bird, 1990). Moreover, acetylation of H3–H4 tetramers has been shown to lead to the change in linking number per nucleosome and thus, presumably, to alterations in nucleosome structure (Norton *et al.*, 1990). Additionally, acetylation of the H3–H4 tetramer stimulates TFIIIA binding (Lee *et al.*, 1993). This raised the possibility that enrichment of highly acetylated histone H4 in the factor-bound population might have resulted from, or required, co-occupancy of the same nucleosome with highly acetylated histone H3.

To determine if acetylated H4, acetylated H3 or both together were responsible for the enhanced binding of USF and GAL4-AH, we employed a second dimension

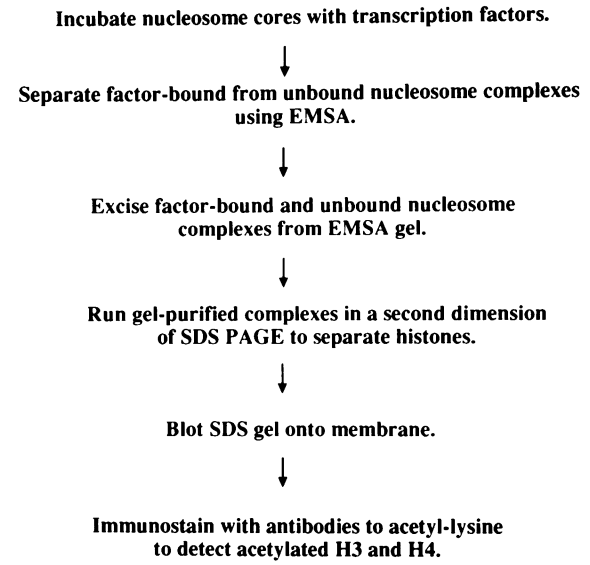

Fig. 6. Experimental strategy for second dimension gel analysis of specific acetylated histones in USF-bound and unbound nucleosomes. For experimental details see Materials and methods.

SDS–polyacrylamide gel followed by immunoblotting with antibodies that recognize both acetylated H3 and acetylated H4. This experimental approach is diagrammed in Figure 6. Homogeneous sequence nucleosome cores, reconstituted with hyperacetylated histones, were incubated with USF or GAL4-AH and then the factor-bound and unbound nucleosomes were separated on native mobility shift gels. The bands representing factor-bound nucleosomes and unbound nucleosomes were excised from the gel, and run on a second dimension of SDS–PAGE. The SDS gels were then blotted to nitrocellulose and probed with an antibody to the epitope of ε-acetyl-lysine (Hebbes *et al.*, 1989) which primarily recognizes acetylated H3 and acetylated H4 in this system. Figure 7A presents the immunoblot of the SDS–PAGE from such an experiment in which USF was bound to nucleosome cores having a USF site 20 bases from one end. The first lane illustrates the presence of acetylated H3 and acetylated H4 in control nucleosomes incubated in the absence of USF, run and excised from the same mobility shift gel. The next two lanes represent the presence of acetylated H3 and acetylated H4 in the unbound and USF-bound nucleosomes excised from the mobility shift gel. The USF-bound nucleosomes contained substantially more acetylated H4 than acetylated H3, whereas the majority of the acetylated H3 remained in the unbound fraction. Figure 7B shows a similar experiment in which the USF site was located 40 bases into the nucleosome core and thus, at a lower affinity position (Adams and Workman, 1995). Whilst a smaller fraction of the total nucleosomes containing highly acetylated histone H4 were bound by USF at this position, the USF-bound nucleosomes were still enriched in acetylated histone H4 relative to acetylated H3. Figure 7C shows the binding of GAL4-AH to nucleosome cores with a single GAL4 site located 36 bp from one end. Enrichment of acetylated histone H4 relative to acetylated H3 was also observed with this different factor, indicating that the preferential stimulatory effect of acetylated H4 was not factor specific.

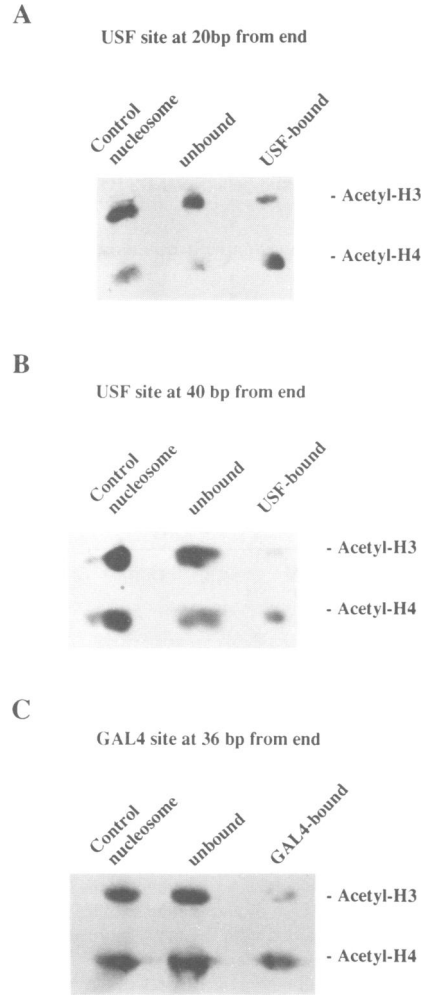

Fig. 7. Acetylated histone H4 is specifically enriched in the transcription factor-bound nucleosomes relative to acetylated histone H3. (**A**) The experimental strategy outlined in Figure 6 was performed with homogeneous sequence nucleosome cores bearing a single USF site 20 bp from one end and reconstituted with histones from butyrate-treated HeLa cells. The second dimension SDS gel was blotted to nitrocellulose and immunostained with the acetyl-lysine antibody which recognizes primarily acetylated forms of H3 and H4. Shown is a Western blot of control, USF-bound and unbound nucleosomes excised from the mobility shift gel. The control nucleosomes were also excised from the same mobility shift gel but were not exposed to USF. Acetylated H4 was enriched specifically in the USF-bound fraction and was depleted in the unbound nucleosome fraction. (**B**) The same experiment as in (A) was repeated, except that the DNA probe contained a single USF site located 40 bp from the end. At this location the affinity of USF is reduced; however, it still preferentially bound nucleosome cores containing acetylated H4. (**C**) The same experiment as in (A) and (B) was performed utilizing GAL4-AH binding to nucleosome cores bearing a single GAL4 site 36 bp from one end. The binding of GAL4-AH also demonstrated a preference for nucleosome cores containing acetyl-H4 relative to acetyl-H3.

The data in Figure 7 indicate that acetylation of histone H4 plays a primary role in stimulating transcription factor binding relative to histone H3. To confirm this result, we have taken another approach to investigate the preferential binding of USF to nucleosomes containing acetylated histone H4. This approach is diagrammed in Figure 8A. Following incubation of the nucleosome cores bearing a USF site at 20 bp from the end with USF protein, the USF-bound nucleosomes were incubated with USF

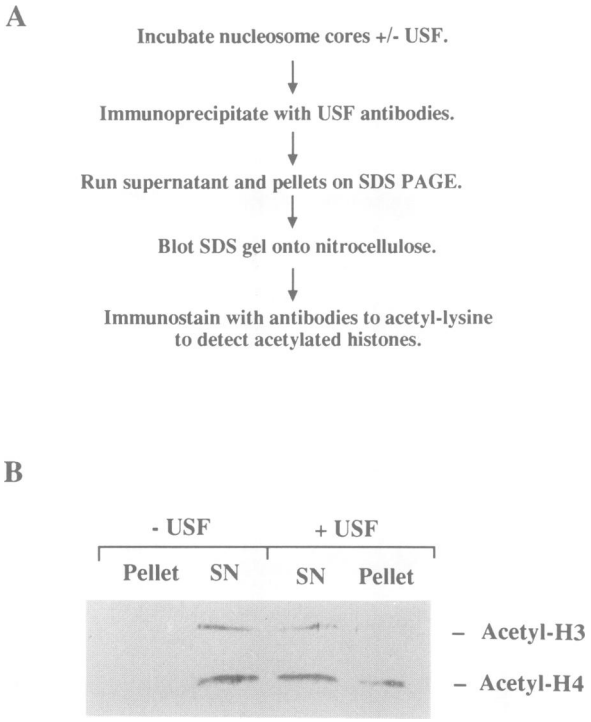

Fig. 8. Immunoprecipitation of USF-bound nucleosome complexes with anti-USF antibody demonstrates enrichment for acetylated H4 over acetylated H3. (**A**) Procedure for immunoprecipitation of USF-bound nucleosomes. Binding reaction mixtures containing or omitting USF were incubated with USF antibody cross-linked to protein A–Sepharose beads. The beads were separated from the supernatant and each was run on an SDS gel and blotted to nitrocellulose. The membrane was immunostained with the anti-acetyl-lysine antibody. For experimental details, see Materials and methods. (**B**) Immunostained nitrocellulose membrane generated as described in (A). In the absence of USF (–USF), neither acetylated histone H3 nor acetylated histone H4 was precipitated by the protein A–Sepharose beads and both acetylated histones H3 and H4 remained in the supernatant fraction. However, in the presence of USF (+USF), the nucleosomes selected by the anti-USF antibody (pellet) specifically contained acetylated histone H4.

antibodies cross-linked to protein A–Sepharose beads. To determine the distribution of acetylated histones H3 and H4, the pellet and supernatants were separated, run on an SDS–polyacrylamide gel and immunoblotted with the antibody that recognizes acetylated H3 and acetylated H4. Figure 8B shows that in the absence of USF protein (–USF), neither acetylated histone H3 nor acetylated histone H4 were precipitated by the USF antibodies and protein A–Sepharose beads, and both remained in the supernatant. However, when USF was added to the reaction and USF–nucleosome complexes were immunoselected, acetylated histone H4 was co-precipitated preferentially. This result further illustrates that the nucleosomes cores bound by USF specifically contained highly acetylated histone H4, confirming the primary role of acetylated H4 in stimulating USF binding. It is also interesting to note that the experiments of Figures 7 and 8 also indicate that acetylated H4 and acetylated H3 do not necessarily cohabit in the same nucleosome cores.

Discussion

The discovery of the post-translational modification of core histones by acetylation (Allfrey *et al.*, 1964) led to the suggestion that the structure and function of chromatin could be altered through an enzymatic pathway. Following in this vein, numerous studies have noted a correlation between histone amino-terminal tail acetylation and transcriptionally active sequences (reviewed in Turner, 1993; Loidl, 1994). A long-standing question regarding the potential roles of histone acetylation in transcription has been whether histone acetylation is at all causal or instead the result of transcriptional activity (Csordas, 1990). The finding that histone acetylation pre-exists transcription at poised but not yet transcribed genes (Hebbes *et al.*, 1988, 1994; Clayton *et al.*, 1993) showed that the modification is not a consequence of transcription but most likely a prerequisite. In accordance with this view, the results presented in this study, as well as earlier reports (Lee *et al.*, 1993; Juan *et al.*, 1994; Vettese-Dadey *et al.*, 1994), suggest that histone acetylation can stabilize the binding of transcription factors to nucleosomal DNA, and might thereby play a role in initiating or in maintaining the accessibility of transcriptional regulatory elements in chromatin.

In this study, we have used novel immunoblotting approaches to demonstrate directly the presence of highly acetylated histone H4 in nucleosome cores that possess the highest affinity for transcription factors USF and GAL4-AH. In addition, we have found that the affinity of USF and GAL4-AH for nucleosome cores was more dependent on the acetylation of histone H4 than of histone H3. While these data do not rule out a smaller stimulatory effect of acetylated H3, they illustrate the primary role of H4 acetylation in stimulating transcription factor binding. The activity of acetylated histone H4 in stimulating transcription factor binding might result from a reduced interaction of the H4 amino-terminus with nucleosomal DNA. Acetylation of lysine residues in a peptide representing the H4 amino-terminus greatly reduced its affinity for DNA (Hong *et al.*, 1993). However, it is interesting to note that USF and GAL4-AH preferentially bound to nucleosome cores containing acetylated histone H4 when their binding sites were at different positions. This observation suggests that the effect of H4 acetylation was not limited to one small region of nucleosomal DNA that might be bound by the H4 tail. This raises an alternative, non-mutually exclusive possibility that acetylated H4 is causal for a conformational change in the nucleosome core which results in an increase in transcription factor affinity. Increased acetylation leads to a reduction of linking number change per nucleosome core *in vitro* (Norton *et al.*, 1989), suggesting an alteration in nucleosome core structure (Bauer *et al.*, 1994). This reduction of linking number change occurs when only H3 and H4 are acetylated (Norton *et al.*, 1990), supporting a crucial role for one or both of these histones in altering nucleosome conformation. It will be interesting to see if the change in linking number can also be attributed specifically to acetylation of histone H4.

Acetylation of histone H4 may play a significant role in the binding of GAL4 and USF to chromosomal DNA *in vivo*. At UAS_{GAL} in yeast, GAL4 is constitutively bound prior to transcription activation unless glucose is present, in which case the accessibility of the UAS is thought to be maintained by a neighboring DNA binding protein, GRF2 (Fedor *et al.*, 1988; Chasman *et al.*, 1990).

Interestingly, GRF2 is not required for the function of UAS_{GAL}, suggesting that GAL4 can access the UAS even in the absence of GRF2 (Chasman *et al.*, 1990). This possibility has been confirmed by Morse (1993), who illustrated that a single GAL4 site occupied by a nucleosome can become bound by GAL4 in yeast. USF is a ubiquitously expressed mammalian transcription factor which binds E-box motifs in multiple cellular and viral enhancers and promoters (Sawadogo and Roeder, 1985; Carthew *et al.*, 1987; Chodosh *et al.*, 1987; Mueller *et al.*, 1988; Navankasattusas *et al.*, 1994; Sirito *et al.*, 1994; and references therein). Interestingly, USF binding *in vivo* can also precede transcriptional activation of inducible genes (see Mueller *et al.*, 1988). Thus, both GAL4 and USF can, in some *in vivo* situations, access enhancers and promoters in chromatin prior to transcriptional induction. The finding here that acetylation of histone H4 enhances the binding of GAL4-AH and USF to nucleosome cores, and previous results indicating that histone acetylation can precede actual transcription (Hebbes *et al.*, 1988, 1994; Clayton *et al.*, 1993), suggest that histone H4 acetylation could participate in poising chromatin for transcription by stabilizing the binding of *trans*-acting factors. Histone acetylation might occur prior to the interactions of the factors with their binding sites in chromatin or occur concurrently. Recent studies suggest that transcription activators may target a region or domain of chromatin for histone acetylation (Brownell and Allis, 1996; Brownell *et al.*, 1996). Acetylation would then, in turn, both stabilize the binding of the activator and enhance the binding of additional transcription factors.

It is becoming increasingly clear that the potential functions of the histone amino-termini in gene transcription can be mediated via direct effects on transcription factor affinity or indirectly as putative targets of repressor proteins (reviewed in Turner and O'Neill, 1995). It is likely that a combination of these effects results in the differential functions of the H3 and H4 amino-termini on different regulatory elements and genes in yeast (Durrin *et al.*, 1991; Mann and Grunstein, 1992; Wan *et al.*, 1995). In this regard, the H4 amino-termini appear to be multifunctional, containing domains involved in both transcription activation and repression (McGee *et al.*, 1983; Kayne *et al.*, 1988; Johnson *et al.*, 1990; Park and Szostak, 1990; Durrin *et al.*, 1991). Multiple functions of the H4 amino-termini might also account, in part, for the apparent occurrence of low levels of acetylated H4 in human euchromatin beyond actively transcribed genes (O'Neill and Turner, 1995). The activity of H4 acetylation in stimulating transcription factor binding might, in part, provide a functional explanation for its enrichment in 'active' chromatin fractions (Allegra *et al.*, 1987; Ridsdale and Davie, 1987; Hebbes *et al.*, 1988; Ip *et al.*, 1988; Clayton *et al.*, 1993) and at CpG islands (Tazi and Bird, 1990), although a much wider distribution reported for histone acetylation in general, for example at the chicken β-globin locus (Hebbes *et al.*, 1994), suggests that histone H4 acetylation may play additional roles. A further distribution of acetylated H4 may also be required in euchromatin to prevent interaction with repressor proteins and heterochromatin spreading, as suggested by studies of mating type and telomere repression in yeast (Johnson *et al.*, 1990; Park and Szostak, 1990; Roth *et al.*, 1992; Braunstein *et al.*, 1993; Hecht *et al.*, 1995; Turner and O'Neill, 1995).

Materials and methods

DNA probe preparation

All three DNA probes were prepared by PCR amplification. A single GAL4 site positioned 36 bp from the 5′ end of a 150 bp fragment (GAL4-36) was generated using the plasmid $G_1$40HSP70CAT and the primers used for amplification of GAL4-36 were HP+48, 5′-CTCGAAAAAGGTAGTGGGAC-3′ and G1-102, 5′-ACGCCAAGCT-TGCATGCCTG-3′. The plasmid, pHXB2DDBc1, used to amplify a single USF site positioned 20 bp (USF-20) or 40 bp (USF-40) from the 5′ end of a 150 bp fragment, contained the human immunodeficiency virus (HIV)-1 5′ long terminal repeat (LTR). The primers used for amplification of USF-20 were HIV-5′B, 5′-GCCGCCTAGCATTTCAT-CAC-3′ and HIV-3′B, 5′-GATCTGAGGGCTCGCCACTC-3′ and for USF-40 were HIV-5′A, 5′-GTTAGAGTGGAGGTTTGACA-3′ and HIV-3′A, 5′-CCCAGTCCCGCCCAGGCCAC-3′. DNA was PCR amplified in numerous repeats so as to generate 20–30 μg of product labeled with [α-^{32}P]dCTP and dATP (3000 Ci/mmol) at a ratio of 1:60 with cold nucleotides. The 150 bp fragments were purified on a native 8% polyacrylamide (acrylamide:bis 29:1), 1× Tris–borate–EDTA (TBE) gel or excised from a 1× TBE 3% low melt agarose gel and digested with β-agarase. The quantity of probe was determined by comparison with a DNA mass ladder (Gibco-BRL) on an ethidium bromide-stained agarose gel. The probe bands were excised from these gels and counted to obtain the specific activity.

Transcription factor purification and nucleosome core reconstitution

GAL4-AH, a fusion protein containing the amino-terminal 147 amino acids of GAL4 (including the DNA binding and dimerization domains) and an artificial 15 amino acid putative amphipathic helix, was purified by the method of Lin *et al.* (1998). The recombinant 43 kDa USF protein was purified by the methods of Pognonec *et al.* (1991). H1-depleted core histones and short oligonucleosomes were purified from HeLa cells (Côté *et al.*, 1995) grown for 20–24 h in the presence (hyperacetylated) or absence (control, with physiological levels of acetylation) of 10 mM sodium butyrate (an inhibitor of histone deacetylase, reviewed in Turner, 1991). All purification steps of the acetylated or control histones and short oligonucleosomes included 2 mM sodium butyrate. The resulting levels of acetylation were analyzed on a TAU gel as described previously (Juan *et al.*, 1994) and are also shown in Figure 1C. Reconstitution with hyperacetylated and control short oligonucleosomes was as described previously (Vettese-Dadey *et al.*, 1994). Homogeneous nucleosome cores were reconstituted by the following procedure: 20–30 μg of PCR-generated, body labeled probe and hyperacetylated or control core histones in a 1- to 2-fold excess were mixed in a final volume of 55 μl with final concentrations of 2 M NaCl, 10 mM HEPES, pH 8.0 and 1 mg/ml bovine serum albumin (BSA), fraction V (Sigma). Nucleosome cores were formed by salt gradient dialysis (Workman and Kingston, 1992) and purified at 4°C on 5–25% sucrose density gradients containing 10 mM HEPES, pH 8.0, 1 mM EGTA, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% NP-40. Fractions were analyzed on a native 6% acrylamide, 0.5× TBE gel. The gel was dried and exposed to Kodak X-Omat film overnight at –80°C with an intensifying screen. Peak fractions containing the nucleosome cores were pooled and stored at –80°C.

Transcription factor binding reactions

Nucleosome cores (20 ng) with a single GAL4 site (GAL4-36) were incubated with increasing concentrations of diluted GAL4-AH (stock 2 mg/ml diluted in 200 μg/ml BSA, fraction V, 10 mM 2-mercaptoethanol, 100 mM KCl, 10 μM $ZnCl_2$, 0.2 mM PMSF). Final reactions were diluted to 20 μl in UG buffer (20 mM Tris–HCl, pH 7.5, 10% glycerol 1 mM EDTA, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1% NP-40) and 200 μg/ml BSA, fraction V, 10 mM 2-mercaptoethanol, 100 mM KCl, 10 μM $ZnCl_2$ and 0.2 mM PMSF were added. USF binding reactions were also diluted to 20 μl (USF stock 2 mg/ml) in UG buffer with addition of 10–100 ng poly(dI–dC) DNA (Pharmacia), 4 mM dithiothreitol (DTT), 100 mM NaCl, 200 μg/ml BSA, fraction V, 0.2 mM $MgCl_2$ and 1 μM $ZnCl_2$. All binding reactions were incubated at 30°C for 30 min and loaded directly onto native 6% acrylamide, 0.5× TBE gels which were run at 200 V (constant voltage) at room temperature.

Western blot analysis and second dimension gel electrophoresis

To detect the level of acetylation of gel-shifted nucleosome cores, we adapted the method of Demczuk *et al.* (1993). This method uses two membranes for transfer of the mobility shift gel; nitrocellulose for the detection of protein and DEAE for the detection of nucleosomal DNA (see Vettese-Dadey *et al.*, 1995 for further details).

Prior to the completion of the mobility shift gel, nitrocellulose (Amersham USB) and DEAE (Schleicher & Schuell) membranes were cut to the dimension of the gel, briefly pre-wet in distilled water according to the manufacturers instructions and equilibrated in Bjerrum and Schafer-Nielson transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.0375% SDS, pH 8.8–9.0) for 15–20 min with gentle shaking. At 4°C, the mobility shift gel was equilibrated with two changes of Bjerrum and Schafer-Nielson buffer containing 0.05% SDS for 5 min.

Blotting was performed in a Bio-Rad plate electrode tank transfer chamber and the blotting cassette was assembled in the following manner. The gel was placed on a piece of wet filter paper followed by the nitrocellulose, filter paper, the DEAE membrane and a final piece of filter paper and was transferred at 4°C in Bjerrum and Schafer-Nielson transfer buffer at 100 V for 2 h. After transfer, the DEAE membrane was wrapped in cellophane and exposed to Kodak X-Omat film overnight at –80°C with an intensifying screen. The nitrocellulose membrane was immunostained and detected using an Amersham USB ECL chemiluminescence kit as follows. The nitrocellulose membrane was placed in phosphate-buffered saline (PBS), 0.1% Tween 20 (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) with gentle shaking for 20 min, then blocked by immersing the membrane in 1% gelatin (Sigma, Swine type A), 0.1% Tween 20 blocking solution for 30–60 min with gentle shaking. The membrane was washed in PBS, 0.1% Tween 20 once for 15 min and twice for 5 min with fresh changes of buffer. Incubation with the primary and secondary antibody was performed for 60 min in 1% gelatin, 0.1% Tween 20. The primary antibodies used for the experiments were either anti-acetylated H4 antibody which recognizes acetylated forms of H4 (Lin *et al.*, 1989) or pan-acetyl antibody which recognizes acetylated forms of H3 and H4 (Hebbes *et al.*, 1988). The secondary antibody was supplied in an Amersham USB kit. The membrane was washed in between the incubation steps once for 15 min and three times for 5 min with fresh changes of wash buffer. The final wash before the detection was for an additional 5 min to help further reduce background. Chemiluminescent detection was performed as per the manufacturer's instructions.

For the second dimension gel, bands of interest were excised from a native 6% polyacrylamide, 0.5× TBE gel. The native gel (17 cm in length) was poured with 0.75 mm spacers and a 15 well comb (Bio-Rad, well size 110 µl). Binding reactions were scaled up 20- to 25-fold and the 6% mobility shift gel was run as described above and exposed to Kodak X-Omat film at 4°C for 4–5 h. The exposed film was used as a guide for removal of the bands to be run in the next dimension on a 17 cm, 5% stacker, 15% separating SDS–polyacrylamide gel with 1 mm spacers and a 10 well comb to accommodate the gel slice from the native gel (well size 229 µl). Gel slices, i.e. control nucleosome core, unbound nucleosome and factor-bound nucleosome (see figure legends), excised from the native gel were placed in 1× SDS–PAGE loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) in tubes for 5 min. Slices were next placed in individual wells of the SDS–polyacrylamide gel, covered with 1× SDS–PAGE loading buffer and run at 200 V. Prior to completion of the gel, nitrocellulose was cut to the area of the histones within the gel and pre-wet with water. Bjerrum and Schafer-Nielson transfer buffer was prepared, as for the standard Western blot (plus 0.0375% SDS), and the second dimension gel, as well as the nitrocellulose, were equilibrated in this buffer with shaking for 15 min. The gel was transferred using the tank transfer system by placing wet filter paper down in the cassette followed by the gel, the nitrocellulose or PVDF (Millipore) and an additional piece of filter paper. Transfer was then in Bjerrum and Schafer-Nielson (plus 0.0375% SDS) transfer buffer at 4°C for 1 h at 50 V. Following transfer, the nitrocellulose or PVDF membrane was immunostained as described above. Quantitation of the bound and unbound complexes was performed on a Laser Densitometer (Molecular Dynamics).

Immunoprecipitation of USF-bound nucleosome complexes

Prior to immunoprecipitation, USF antibody (Santa Cruz) was cross-linked to protein A–Sepharose beads as follows. Fifty µl of USF polyclonal antibody (100 µg/0.1 ml) was mixed on a rotator with 50 µl of protein A–Sepharose beads (swelled to a 50% suspension in PBS) for 1 h at room temperature. Beads were washed twice with 10 volumes of 0.2 M sodium borate pH 9.0. After the second wash, the beads were resuspended in 10 volumes of 0.2 M sodium borate pH 9.0 and solid dimethylpimelidate was added to 20 mM. The beads were mixed on a rotator for 30 min at room temperature and the reaction was stopped by washing the beads in 0.2 M ethanolamine pH 8.0. The beads were stored in PBS with 0.01% merthiolate (Harlow and Lane, 1988). Before use in the immunoprecipitation reaction, beads were pre-incubated for 5 min at room temperature with 2 mg/ml BSA to help eliminate non-specific binding of beads to unbound nucleosomes.

Transcription factor binding reactions were performed as described for the second dimension gel. After the binding reaction, 10 µl of antibody-cross-linked beads were added to the binding reaction and were mixed on a rotator at 4°C for 3 h to overnight. The USF-bound complexes were removed by centrifugation of the beads. Approximately 80–90% of the supernatant was removed and the beads were washed twice before stripping in SDS–PAGE loading buffer. Samples were boiled for 4 min and loaded directly onto a 15/5% SDS–polyacrylamide gel. The gel was run at 200 V for 6 h, Western blotted to nitrocellulose or PVDF and immunostained as described above.

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