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A Positive Role for Histone Acetylation in Transcription Factor Access to Nucleosomal DNA

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Summary

Acetylation of the N-terminal tails of the core histones directly facilitates the recognition by TFIIIA of the 5S RNA gene within model chromatin templates. This effect is independent of a reduction in the extent of histone-DNA interactions or a change in DNA helical repeat; it is also independent of whether a histone tetramer or octamer inhibits TFIIIA binding. Removal of the N-terminal tails from the core histones also facilitates the association of TFIIIA with nucleosomal templates. We suggest that the histone tails have a major role in restricting transcription factor access to DNA and that their acetylation releases this restriction by directing dissociation of the tails from DNA and/or inducing a change in DNA configuration on the histone core to allow transcription factor binding. Acetylation of core histones might be expected to exert a major influence on the accessibility of chromatin to regulatory molecules.

Introduction

The organization of regulatory DNA elements into precise chromatin structures is important for both DNA replication and transcription in vivo (Simpson, 1991; Felsenfeld, 1992). The mechanisms determining the positioning of histones relative to particular cis-acting elements and the consequences for the association of sequence-specific DNA-binding proteins are presently ill defined. Formation of nucleosomes presents many impediments to the recognition of DNA: one side of the double helix is occluded, since it faces the core histones; the adjacent superhelix of DNA further prevents access (Klug and Lutter, 1981); and DNA is distorted into an 80 bp circle (Richmond et al., 1984) and overwound from 10.5 bp to 10.0 bp per turn over much of its length (Hayes et al., 1991). Furthermore, the positively charged core histone tails that lie on the outside of the nucleosomal DNA might be expected to compete with transcription factors for binding to the negatively charged phosphodiester backbone. It is therefore surprising that certain transcription factors can still recognize DNA when it is wrapped around the core histones (Perlmann and Wrange, 1988; Piña et al., 1990; Archer et al., 1991; Taylor et al., 1991; Perlmann, 1992).

Transcription factor TFIIIA has a well-defined interaction with 5S RNA genes (Smith et al., 1984; Fairall et al., 1986; Vrana et al., 1988; Hayes and Tullius, 1992), and these same genes have also been used to generate model chromatin structures (Rhodes, 1985; Gottesfeld, 1987; Hayes et al., 1990, 1991). TFIIIA has been found to associate specifically with histone-bound 5S DNA (Rhodes, 1985; Hayes and Wolffe, 1992). This interaction provides an excellent opportunity to determine whether positioning or modification of the histone proteins might affect the accessibility of a transcription factor to its recognition element within a nucleosome.

Two modifications to the nucleosome have been proposed to influence the association of sequence-specific DNA-binding proteins with chromatin. Depletion of histones H2A/H2B from the nucleosome facilitates the binding of RNA polymerase II (Baer and Rhodes, 1983) and TFIIIA (Hayes and Wolffe, 1992). Likewise, acetylation of the core histone tails potentially destabilizes the nucleosome and might facilitate access of regulatory molecules (Oliva et al., 1990; Walker et al., 1990). In both cases, overall histone–DNA contacts are presumed to be weakened through removal either of the histones themselves or of the positively charged tails from contact with DNA. We have examined the effect of these nucleosome modifications on TFIIIA access to the 5S RNA gene. We find that the tetramer of histones, $(H3/H4)_2$, is capable of preventing TFIIIA binding to 5S DNA, depending on the position of histone–DNA contacts. In contrast, acetylation of the core histones allows TFIIIA to bind to the histone–DNA complex without apparent impediment.

Results

Histone Acetylation Facilitates TFIIIA Binding to the *Xenopus borealis* 5S RNA Gene in a Nucleosome

We wished to determine whether acetylation of the core histone tails might influence the accessibility of nucleosomal DNA to transcription factors. This analysis is simplified for a 5S RNA gene, since both acetylated and nonacetylated core histones recognize the same nucleosome positioning elements (Marvin et al., 1990). A tetramer of histones $(H3/H4)_2$ and trypsinized histone octamers $(H2A/H2B/H3/H4)_2$ also recognize the same nucleosome positioning elements (Dong et al., 1990; Dong and van Holde, 1991; Hayes et al., 1991). Model nucleosomes assembled on the *X. borealis* somatic 5S RNA gene have well-defined histone–DNA contacts that include the binding site for TFIIIA (Rhodes, 1985; Hayes et al., 1990, 1991).

A simple method for detecting transcription factor interaction with histone–bound DNA is to resolve reconstituted nucleosomes on nondenaturing agarose gels in the presence or absence of the factor. Free DNA that remains in the reconstitution reaction serves as an internal control for factor interaction. A single histone octamer, $(H2A/H2B/H3/H4)_2$, bound to the *X. borealis* 5S RNA gene prevents TFIIIA binding to the gene, whereas under the same conditions, naked DNA is completely bound (Figure 1, Octamer). In contrast, a single histone tetramer $(H3/H4)_2$ associated with the same gene allows TFIIIA to bind (Figure

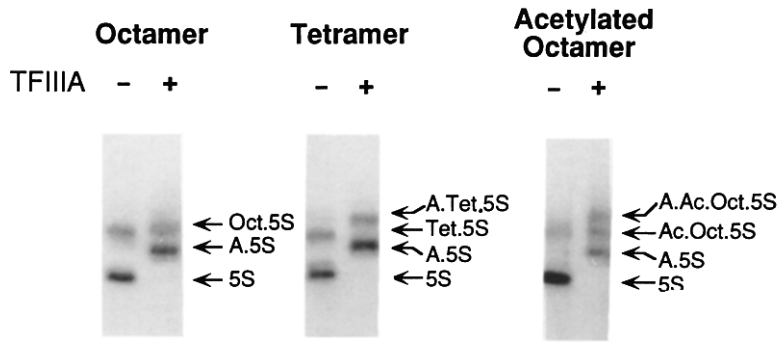

Figure 1. TFIIIA Binds to the X. borealis 5S RNA Gene Associated with an Acetylated Octamer or an Unmodified Tetramer, but Not to the Gene Associated with an Unmodified Octamer

An autoradiograph of mobility shifted complexes is shown. The presence (+) or absence (−) of TFIIIA (10-fold molar excess) is indicated. All lanes are from the same gel. Histones were prepared from HeLa cells. The octamer and acetylated octamer were reconstituted onto 5S DNA by exchange from long chromatin; the tetramer was assembled onto 5S DNA through salt–urea dialysis (see Experimental Procedures).

1, Tetramer; Hayes and Wolffe, 1992). Acetylation of the core histones used to assemble the single histone octamer onto the 5S RNA gene facilitates the association of TFIIIA with the gene (Figure 1, Acetylated Octamer). The histones used in these experiments are shown in Figure 2. Two gel systems are used, that of Laemmli (1970) to resolve histones independent of posttranslational modification (SDS; Figure 2, lanes 1, 2, 5, and 6), and that of Zwiedler (1978), which has a Triton acid–urea buffer that resolves histones dependent on acetylation (TAU; lanes 3 and 4). Histone octamers and tetramers (Figure 2, lanes 1 and 2), unmodified and acetylated histones (lanes 3 and 4), and intact octamers and trypsinized histones (lanes 5 and 6) are clearly resolved. The unmodified histones resolved in lane 3 of Figure 2 contain low levels of histone acetylation, best visualized for histone H4; these levels are much lower than those for histones isolated from butyrate-treated HeLa cells (see Experimental Procedures).

We examined the organization of the nucleoprotein complexes resolved in the nondenaturing gels (Figure 1) by DNAase I footprinting. In these experiments, it was important to form the complexes in solution, to digest with DNAase I, and then to resolve the complexes in the nondenaturing gel before deproteinization and resolution on a denaturing gel (Hayes and Wolffe, 1992). This eliminates the possibility that mixtures of binary complexes (e.g., histones plus DNA or TFIIIA plus DNA) could give the appearance of a tertiary complex (e.g., histones plus TFIIIA plus DNA).

There are several similarities and differences in the organization of DNA associated with different types of histone. Our results indicate that the DNA within nucleosomes assembled with acetylated histones has a similar organization to those assembled with normal histone octamers (Figure 3, compare lanes 6 and 10). The acetylated particles are more readily digested by DNAase I in comparison with the normal particles, but both show identical rotational positioning of the DNA on the histone octamer (cleavage sites are indicated by horizontal bars between lanes 11 and 12; Whitlock and Simpson, 1977; Marvin et al., 1990). Previous work has shown that histone tetramer $(H3/H4)_2$ is able both to recognize the same nucleosome positioning element as the complete octamer and to organize the central 120 bp of DNA identically to the octamer (Hayes et al., 1991; Dong and van Holde, 1991). Although DNA has a similar rotational position on the histone tetramer, the relative cleavage efficiency by DNAase I clearly differs on the particle surface (compare lanes 13 and 16 of Figure 3). This is particularly noticeable at the dyad axis of the reconstituted particle, where a major DNAase I cleavage site is found in octamer-associated DNA (horizontal arrow) that is absent when the tetramer is bound to DNA. These differences serve as a useful distinction between DNA associated with a tetramer of histones and DNA associated with an octamer of histones.

Isolation of the putative tertiary complexes between TFIIIA, histones, and 5S DNA, followed by resolution of the DNAase I cleavage pattern, reveals both strong his-

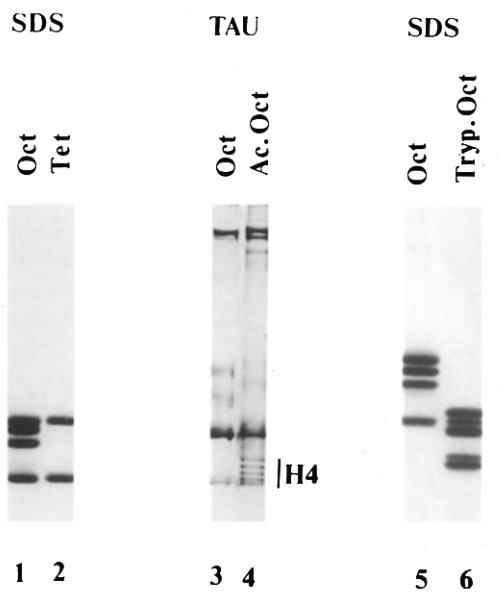

Figure 2. Core Histones on Gels Containing SDS or Triton Acid–Urea

Core histones are shown resolved on gels containing SDS (SDS, lanes 1, 2, 5, and 6) or Triton acid–urea (TAU, lanes 3 and 4). Lane 1 shows an octamer of histones, while lane 2 shows a tetramer of histones. Histones with low or high levels of acetylation are shown in lanes 3 and 4. Histone H4, which has clearly resolved acetylation states, is indicated. Each band from the bottom to the top of the indicated region represents the acetylation of an additional lysine in the N-terminal tail. Lane 6 shows a core histone octamer that has been cleaved with trypsin; an intact histone octamer is shown in lane 5. Samples in lanes 1, 2, 5, and 6 were silver stained (Wray et al., 1981), while samples in lanes 3 and 4 were stained with Coomassie blue.

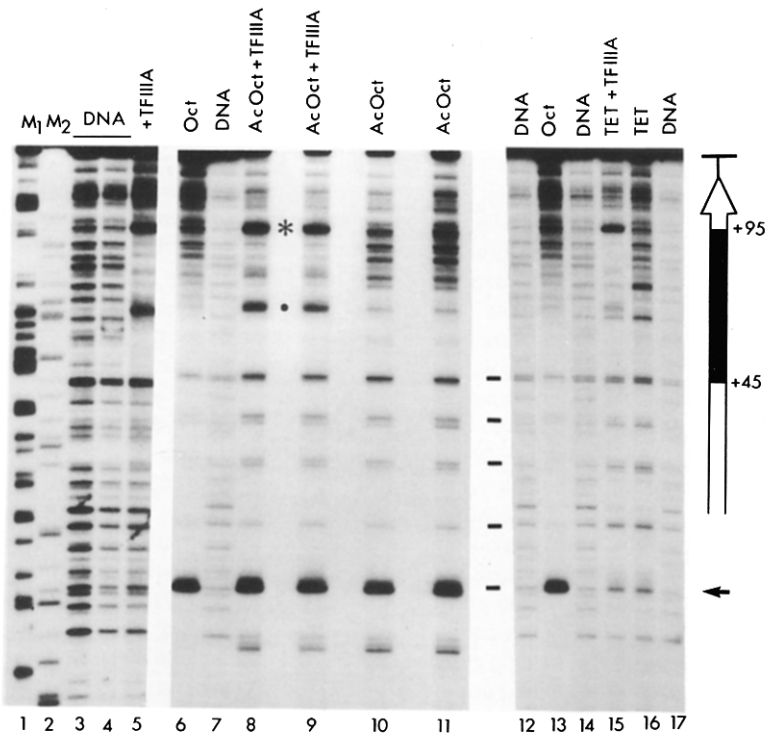

Figure 3. The Tertiary Complex of Acetylated Histones–TFIIIA–X. borealis 5S DNA

DNA from gel-isolated DNAase I-digested complexes is shown resolved on a denaturing acrylamide gel. Lanes 1 and 2 are markers. Lanes 3, 4, 7, 12, 14, and 17 are digestion patterns of naked DNA; lane 5 is of a TFIIIA–DNA complex; lanes 6 and 13 are of an unmodified octamer–DNA complex; lanes 8 and 9 are of the tertiary complex of TFIIIA–acetylated octamer–DNA; lanes 10 and 11 are of the acetylated octamer–DNA complex; lane 15 is of the tertiary complex of TFIIIA–tetramer–DNA; and lane 16 is of the tetramer–DNA complex. The 5S RNA gene is indicated by the open arrow, and the TFIIIA-binding site is indicated by by the closed box. The horizontal arrow indicates the pseudodyad axis of symmetry in the nucleosome core. The asterisk between lanes 8 and 9 indicates the position of DNAase I hypersensitivity at +95, and the dot indicates the position at +62/+63. Cleavage sites shared between the octamer and tetramer due to the rotational positioning of DNA surface are indicated by horizontal bars between lanes 11 and 12. Histone–DNA complexes were assembled as described in Figure 1.

tone and TFIIIA binding within the same complex (Figure 3, lanes 8, 9, and 15). The presence of both histones and TFIIIA was confirmed by isolation of the nucleoprotein complex, followed by radioiodination of the proteins (D. P. and A. P. W., data not shown). Comparison with a TFIIIA footprint on naked DNA (Figure 3, lane 5) indicates similar DNAase I hypersensitive sites at the 3′ end of the TFIIIA-binding site within all of the tertiary complexes (asterisk between lanes 8 and 9 at +95, relative to the start site of 5S RNA gene transcription +1). The 3′ end of the TFIIIA-binding site is also protected in both tertiary complexes, TFIIIA–histone tetramer–5S DNA and TFIIIA-acetylated histone octamer–5S DNA. These results confirm the specific association of TFIIIA with its binding site in these histone–DNA complexes. No additional protection from DNAase I cleavage characteristic of nonspecific TFIIIA association is apparent in the tertiary complexes (Winsor et al., 1988). Interestingly, the hypersensitive site in the middle of the TFIIIA-binding site (indicated by the dot between lanes 8 and 9 of Figure 3 at +62/+63) is only readily apparent in the complex of TFIIIA with the acetylated octamer. This suggests that the association of TFIIIA with the acetylated octamer–5S DNA complex differs from that with the tetramer–5S DNA complex. We conclude that acetylation of the core histones facilitates the association of TFIIIA with a nucleosome including the X. borealis 5S RNA gene.

A Tetramer of Histones H3/H4 Can Prevent TFIIIA Binding to the X. laevis 5S RNA Gene in the Nucleosome

Two explanations exist for the differential accessibility of TFIIIA to the X. borealis 5S RNA gene associated with a tetramer compared with an octamer of histone. It is possible that histones H2A/H2B make contacts with key regulatory elements in the TFIIIA-binding site (+81 to +91; Hayes and Tullius, 1992) that remain free when only histones H3/H4 are present (Hayes and Wolffe, 1992). A second explanation is that a histone tetramer–5S DNA complex is an intrinsically unstable structure in comparison with the histone octamer–5S DNA complex. If the first explanation is correct, movement of the tetramer to include contacts with the key TFIIIA regulatory elements should prevent access of TFIIIA to the gene. If the second explanation is correct, TFIIIA binding to the tetramer–5S DNA complex will occur independently of the position of histone–DNA contacts along the gene.

Histone octamers associate with somatic 5S RNA genes of X. laevis differently than with those of X. borealis (Rhodes, 1985; Gottesfeld, 1987). This is probably due to sequence differences between X. laevis and X. borealis located 20–30 bp to either side the dyad axis of the X. borealis nucleosome (FitzGerald and Simpson, 1985; Hayes et al., 1990). Both 5S RNA genes are incorporated into positioned nucleosomes (see Figures 3 and 6); however, the translational positions of the two nucleosomes differ (Rhodes, 1985; Gottesfeld, 1987). Hydroxyl radical footprinting (Hayes et al., 1991) and micrococcal nuclease digestion (Dong and van Holde, 1991) indicate that both the tetramer and the octamer are in contact with the whole TFIIIA-binding site of the X. laevis 5S RNA gene (data not shown; Figure 4). We find that a histone tetramer assembled on the X. laevis 5S RNA gene will inhibit TFIIIA binding under conditions in which naked 5S DNA is completely bound by TFIIIA (Figure 5A, lanes 3–5). As a control, TFIIIA association with a tetramer–X. borealis 5S RNA gene com-

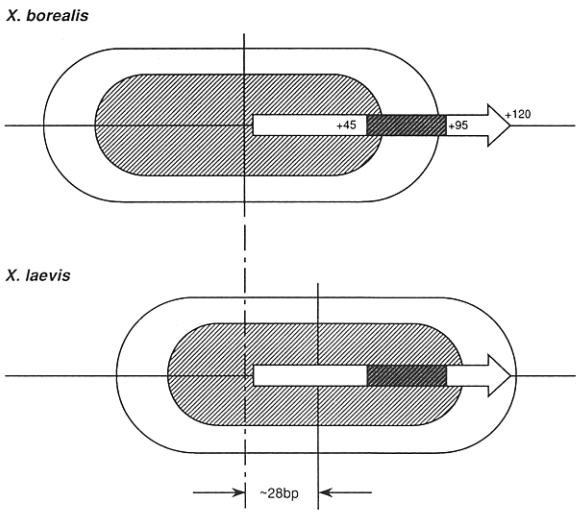

Figure 4. Histone–DNA Contacts Differ between Nucleosomes Including the X. laevis and X. borealis 5S RNA Gene

Model to show the position of histone tetramer (hatched ellipsoid) and histone octamer (open ellipsoid) on X. borealis and X. laevis 5S RNA genes (open arrow). The TFIIIA-binding site is indicated by the hatched box. Vertical lines indicate the approximate positions of the dyad axes of the nucleosomes. In the X. laevis nucleosome, the dyad axis is approximately 28 bp closer to the 3′ end of the 5S RNA gene than it is in the X. borealis nucleosome.

plex readily occurs (Figure 5A, lanes 8 and 9). Several minor bands around the main nucleosomal reconstitute (Figures 5A and 5B, lanes 8) are a consequence of variations in translational position of the histone tetramer or octamer relative to the 5S DNA fragment (see Meersseman et al., 1992). We have found all of the various bands to contain the expected complement of core histones (D. P., data not shown). All of the histone–DNA complexes behave identically in the TFIIIA binding assay (Figure 5A, lanes 8 and 9; Figure 5B, lanes 9 and 10). We conclude that differences in the position of the histone tetramer relative to the TFIIIA–binding site can determine the association of TFIIIA. The histone tetramer alone is capable of preventing transcription factor access to key regulatory elements.

Histone Acetylation Relieves the Inhibition of TFIIIA Binding Due to Both Tetramer and Dimer Contacts with the 5S RNA Gene

Having established that a subnucleosomal particle, the tetramer $(H3/H4)_2$, can prevent TFIIIA binding to the X. laevis 5S RNA gene, we examined the effect of histone acetylation on TFIIIA binding to a nucleosome including the X. laevis 5S RNA gene. Previous work has indicated that nucleosome assembly on the X. laevis 5S RNA gene prevented TFIIIA binding (Gottesfeld, 1987). However, nucleosomes assembled with acetylated histones on X. laevis DNA allow TFIIIA binding (Figure 5B, lanes 3–5). As a control, nucleosomes assembled with acetylated histones on the X. borealis gene also allowed efficient TFIIIA binding (Figure 5B, lanes 8–10).

We examined the organization of the nucleoprotein complexes resolved in the nondenaturing gels (Figure 5B) by DNAase I footprinting. As seen with the X. borealis 5S RNA gene, the histone tetramer provides a footprint that is distinct from that of the acetylated octamer (Figure 6, compare lane 9 with lanes 11 and 12; similar results were obtained with both DNA strands, but only data from the noncoding strand are shown). 5S DNA has the same rotational organization when wrapped around histones in both complexes, but the relative cleavage frequencies of DNAase I along the DNA backbone differ. The tertiary complex between TFIIIA and the acetylated octamer–5S DNA complex reveals both strong histone and TFIIIA binding (Figure 6, lane 10). Comparison with a TFIIIA footprint on naked DNA (Figure 6, lanes 5 and 6) indicates that similar DNAase I hypersensitive sites appear in the tertiary complex (asterisk at +95 and dot +62/+63 between lanes 6 and 7).

The histone tetramer prevents TFIIIA binding to the X. laevis 5S RNA gene, while TFIIIA can bind when an acetylated octamer is bound to the same DNA. Acetylation of the core histones also relieves the inhibition of TFIIIA binding directed by H2A/H2B interactions with the X. borealis 5S gene (Figures 1, 3, and 5; Hayes and Wolffe, 1992). We conclude that acetylation of the core histones can facilitate TFIIIA binding to regions in contact with histones H3/H4 and to those in contact with histones H2A/H2B.

The Level of Histone Acetylation That Facilitates TFIIIA Access to DNA in the 5S Nucleosome

Bradbury and colleagues have shown that a distribution of 2–3 acetyl groups per molecule of histone H4 approaches the maximal level of acetylation of core histones achieved in vivo (Perry and Chalkley, 1981; Norton et al., 1989). Moreover, this level of acetylation does not lead to major alterations in the internal organization of the nucleosome (Marvin et al., 1990), in agreement with earlier biophysical results (Ausio and van Holde, 1986). However, not every core histone is uniformly acetylated (see Turner et al., 1992). Hence, it is only possible to discuss the average distribution of acetylated histones. We examined the average level of histone H4 acetylation in the donor chromatin required to allow nucleosome cores reconstituted onto a 5S RNA gene to bind TFIIIA. Histone H4 acetylation was examined both by electrophoresis on Triton acid–urea gels (Zweidler, 1978) and by immunoblotting using antibodies specific to the acetylated amino terminus of histone H4 (Lin et al., 1989). We find that histones that are acetylated, with an average of 2–3 acetyl groups per molecule of histone H4 (see Figure 2, lane 4), allow more than half of the 5S RNA gene nucleosomes to be bound by TFIIIA (data not shown). Quantitation of binding affinity using limiting concentrations of TFIIIA reveals that the protein associates with the acetylated 5S gene nucleosomes with approximately equal affinity to naked 5S DNA ($K_D = 10^{-9}$ M; see Del Rio and Setzer, 1991; Liao et al., 1992). Examination of the time course of TFIIIA association to naked DNA and histone–DNA complexes indicates that equilibrium binding is achieved within 2–3 min in both cases (data not shown). We have not yet determined how many acetylated

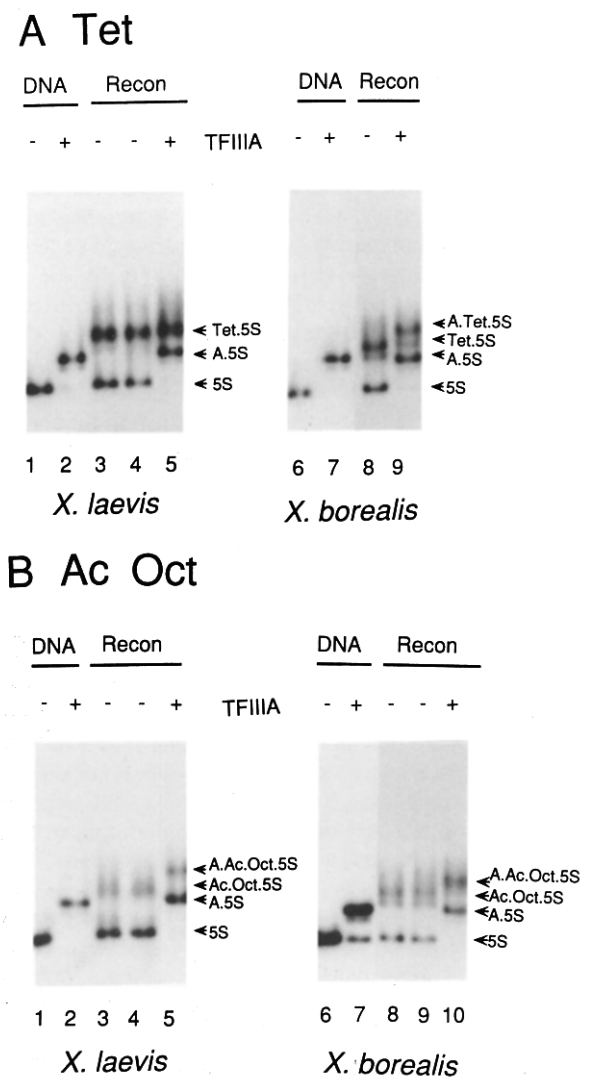

Figure 5. TFIIIA Binding Is Inhibited by a Tetramer on the X. laevis 5S Gene, but Is Facilitated by Histone Acetylation

(A) Single tetramers associated with X. laevis 5S DNA (lanes 3–5) or X. borealis 5S DNA (lanes 8 and 9) were mixed with TFIIIA as indicated (10-fold molar excess). Free DNA (lanes 1 and 2 and lanes 6 and 7) was used as a control, with or without TFIIIA. All lanes are from the same gel.

(B) Single acetylated octamers associated with X. laevis 5S DNA (lanes 3–5) or X. borealis 5S DNA (lanes 8–10) were mixed with TFIIIA as indicated (10-fold molar excess). DNA labeled at the noncoding or coding strands was used. Free DNA (lanes 1 and 2 and lanes 6 and 7) was used as a control, with or without TFIIIA. All lanes are from the same gel. Histone–DNA complexes were assembled as described in Figure 1. The donor chromatin was included (without histone transfer) in the naked DNA control reactions.

lysines in histone H4 reflect the overall histone acetylation levels in the nucleosome that allow TFIIIA access. However, we have determined that the acetylation levels of H4 in the donor chromatin are an accurate reflection of those in the reconstitutes (data not shown).

We conclude that levels of acetylation of the core histones that cause no major conformational change in the nucleosome (Ausio and van Holde, 1986; Marvin et al., 1990) are sufficient to allow TFIIIA to access the 5S RNA gene in a nucleosome.

Histone Acetylation Does Not Influence Either the Extent of Histone-Bound DNA or the Helical Repeat of DNA in the Nucleosome

Careful studies by Bradbury and colleagues have shown that with increasing levels of acetylation (from less than 1 up to 2–3 acetyl groups per molecule of H4), the linking number change per 5S nucleosome decreases from −1.0 (Simpson et al., 1985) to −0.82 (Norton et al., 1989). These results suggest that histone acetylation has the ability to release negative supercoils previously constrained by nucleosomes into a closed chromatin loop, thereby functioning as a eukaryotic gyrase (Norton et al., 1989). The cause of this change in linking number is unknown, but possibilities include a conformational change in the nucleosome altering the path of DNA in the particle, a change in the helical repeat of DNA in the nucleosome, or a change in the writhe of DNA between nucleosomes (see White et al., 1989). Either of the first two possibilities might contribute to the increased accessibility of TFIIIA to 5S DNA in a single nucleosome containing acetylated core histones.

Bradbury and colleagues investigated these possible changes to the organization of DNA in chromatin (Norton et al., 1990; Marvin et al., 1990), concluding that acetylation of histones H3/H4 alone was enough to cause the linking number change. Hydroxyl radical footprinting is a sensitive technique for defining both histone–DNA contacts and changes in DNA helical repeat within the nucleosome. Application of this technique to complexes of histones and 5S DNA has revealed that each octamer of histones might contact up to 180 bp of DNA (Hayes et al., 1990, 1991) and each tetramer up to 120 bp (Hayes et al., 1991; Hayes and Wolffe, 1992; see also Dong and van Holde, 1991). We therefore examined the hydroxyl radical footprint of single nucleosomes reconstituted on the X. borealis 5S RNA gene with acetylated or normal histones. This gene reconstituted with either an acetylated or normal octamer gives identical footprints (data not shown). A key region of the 5S RNA gene for contact with TFIIIA (+81 to +91) is not released from the nucleosome as a consequence of acetylation, but is released as a consequence of the loss of H2A/H2B (Hayes and Wolffe, 1992). We conclude that the helical repeat of DNA in the nucleosome is identical whether or not the core histones are acetylated and that this equivalence of organization extends over approximately 180 bp. Therefore, either the path of DNA in the particle or the writhe of DNA between nucleosomes must change on acetylation of the histones.

Proteolytic Removal of the Core Histone Tails Allows TFIIIA to Bind to the 5S RNA Gene in the Nucleosome

Treatment of nucleosome core particles with trypsin will remove approximately half of the N-terminal tail of the core histones, including all but one of the lysine residues acetylated in vivo (reviewed by Böhm and Crane-Robinson,

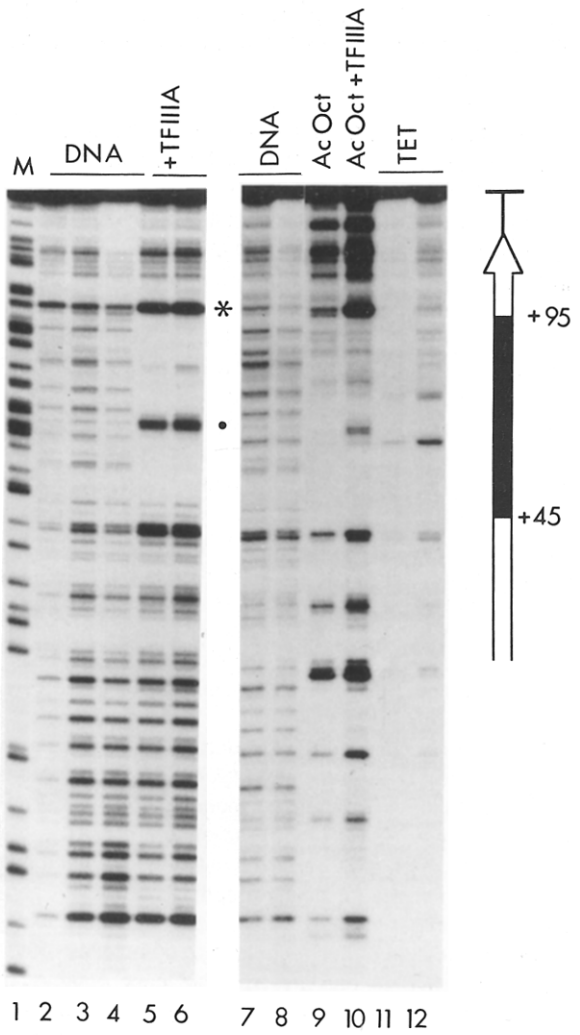

Figure 6. The DNAase I Footprint of a Tertiary Complex of TFIIIA–Acetylated Octamer–X. laevis 5S DNA

DNA from gel isolated DNAase I-digested complexes is shown resolved on a denaturing acrylamide gel. Lane 1 shows markers; lanes 2, 3, 4, 7, and 8 are digestion patterns of naked DNA; lanes 5 and 6 are of the complex of TFIIIA with DNA; lane 9 is of the acetylated octamer–5S DNA complex; lane 10 is of the TFIIIA–acetylated octamer–5S DNA complex; and lanes 11 and 12 are of the tetramer–5S DNA complex. The 5S RNA gene is indicated by the open arrow and the TFIIIA-binding site by the closed box. The asterisk between lanes 6 and 7 indicates the DNAase I hypersensitive site in the TFIIIA footprint at +95, and the dot indicates the site at +62/+63. Histone–DNA complexes were assembled as described in Figure 1.

1984). Only lysine 27 of histone H3 is not removed by trypsin and is potentially acetylated (Marvin et al., 1990). Trypsin digestion causes the N-terminal peptides to dissociate from DNA (Grigoryev and Krashennikov, 1982; Goldblatt and Bustin, 1975). Thus, it has been suggested that proteolytic removal of the tails is equivalent to their apparent release from DNA contacts as a consequence of acetylation (Cary et al., 1982). If the histone tails prevent TFIIIA binding to the 5S RNA gene in a nucleosome as a consequence of their own interaction with DNA, then either acetylation or proteolysis might be expected to allow TFIIIA to bind.

Removal of the histone tails using trypsin (Figure 2, lane 6) does not affect the positioning of the trypsinized octamer on the X. borealis 5S RNA gene (Dong et al., 1990; Hayes et al., 1991), nor does it influence the extent of histone–DNA contacts or the helical repeat of DNA compared with the normal octamer–5S DNA complex (Hayes et al., 1991). Addition of TFIIIA to the trypsinized octamer–5S DNA complex leads to tertiary complex formation (Figure 7A, lanes 3 and 4). It is important to show that a complete octamer of trypsinized histone is transferred to DNA in this experiment; hence, we examined the histones in the reconstituted complexes before TFIIIA addition. We find that a complete octamer of trypsinized histones is present (Figure 7B); the presence of intact histone octamers was also examined as a control (compare Figure 7B with Figure 2, lanes 5 and 6). This excludes the possibility that the nucleoprotein complexes assembled in this experiment were deficient in histones H2A/H2B. DNAase I digestion and isolation of the nucleoprotein complexes reveal a specific TFIIIA footprint existing with that of the trypsinized histones (Figure 8, lane 8). Earlier failures to obtain TFIIIA binding to trypsinized octamer–5S DNA complexes in this experiment (Hayes and Wolffe, 1992) probably resulted from digestion of TFIIIA by residual trypsin. The current protocol included controls that monitored the integrity of the TFIIIA protein. Careful monitoring of the activity of individual batches of trypsin and trypsin inhibitor is necessary to avoid residual proteolytic activity. The TFIIIA–trypsinized octamer–5S DNA complex differs from that of the TFIIIA–histone tetramer–5S DNA complex (Figure 7A, lanes 1 and 2; Figure 8, lane 4; also see Figure 3). For example, the DNAase I hypersensitive site at the dyad axis of the histone–DNA complex is less marked with the TFIIIA–tetramer–5S DNA complex than with the TFIIIA–trypsinized octamer–5S DNA complex (Figure 3, horizontal arrow). Differences also exist in the TFIIIA-binding site (between +45 and +95). We conclude that removal of the core histone tails is sufficient to allow TFIIIA to bind to 5S DNA in a nucleosome. Histone acetylation potentiates TFIIIA association with DNA in the nucleosome, probably through mechanisms relying on the release of the histone tails from contact with DNA.

Discussion

The interaction of TFIIIA with a 5S RNA gene in a nucleosome depends on the acetylation of the core histone tails. Acetylation relieves the inhibition of TFIIIA binding seen when the 5S RNA gene is already associated with unmodified histones present either as a tetramer, $(H3/H4)_2$, or as an octamer, $(H2A/H2B/H3/H4)_2$. We conclude that acetylation of the core histones might be expected to exert a major influence on the accessibility of chromatin to regulatory molecules.

Formation of Tertiary Complexes of TFIIIA, Histones, and 5S DNA

Substantial information exists concerning the interaction of the zinc finger protein TFIIIA with the 5S RNA gene (Smith et al., 1984; Fairall et al., 1986; Vrana et al., 1988; Hayes and Tullius, 1992). The nine zinc fingers of TFIIIA

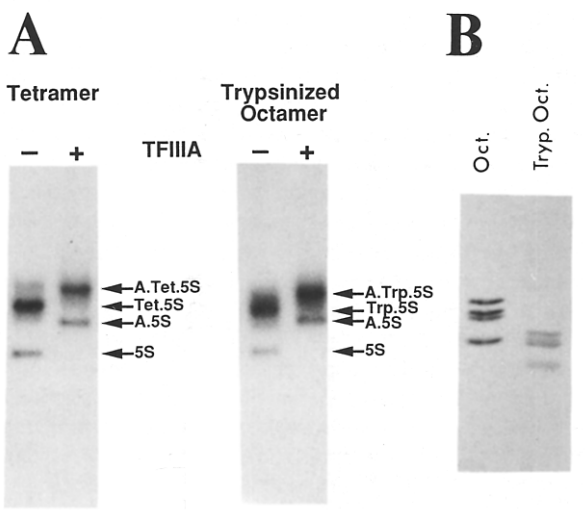

Figure 7. Tryptic Removal of the Core Histone Tails Facilitates TFIIIA Binding to the X. borealis 5s RNA Gene in a Nucleosome

(A) An autoradiograph of mobility shifted complexes is shown. The presence (+) or absence (–) of TFIIIA (10-fold molar excess) is indicated. All lanes are from the same gel. Histones were prepared from chicken erythrocytes, the tetramer being reconstituted onto 5S DNA by exchange from trypsinized nucleosome core particles (see Experimental Procedures).

(B) The histones present in the trypsinized octamer–5S DNA preparations after the exchange reaction (Tryp. Oct.) and in contral octamer–5S DNA preparation (Oct.) are shown after silver staining.

are proposed to interact in a linear array with over 40 bp of DNA, including major groove contacts at either end of the complex. Such an extensive interaction would appear incompatible with the simultaneous wrapping of the same DNA helix around the histone core. However, stable TFIIIA binding can be established by making use of specific contacts only at the 3′ end of the binding site (Sakonju and Brown, 1982; Smith et al., 1984; Vrana et al., 1988). Here, TFIIIA has strong contacts with DNA at +69 and between +81 and +91 on the noncoding strand (Hayes and Tullius, 1992). These sites are more accessible on the X. borealis somatic 5S RNA gene associated with a tetramer than on a a complete octamer of core histones (Hayes et al., 1991; Hayes and Wolffe, 1992). This differential accessibility can explain why TFIIIA can interact with the tetramer–X. borealis 5S DNA complex, but not with the octamer–X. borealis 5S DNA complex (Figure 1). However, movement of the tetramer–5S DNA contacts to include those at the 3′ end of the X. laevis 5S RNA gene prevents TFIIIA from forming a tertiary complex (Figure 5A). Thus, a histone tetramer is capable of preventing transcription factor access to DNA.

Acetylated histone octamers assembled on both the X. borealis and X. laevis 5S RNA gene have contacts with the entire TFIIIA binding site (Figures 3, 4, and 6; Hayes et al., 1990). The extent of interaction is identical to that of unmodified histone octamers, yet TFIIIA still binds to the genes associated with acetylated histones. Therefore, the nature of histone–DNA contacts with the acetylated octamer, rather than the length of DNA helix associated with histones, must change. DNAase I footprinting shows that TFIIIA binding to the complex of the acetylated octamer with 5S DNA occurs at the 3′ end of the binding site between the DNAase I hypersensitive sites at +62/+63 and at +95. These encompass the key recognition elements for stable protein binding to naked DNA (Hayes and Tullius, 1992). This is true for the complexes formed by TFIIIA with the acetylated histone octamers associated with the X. borealis and X. laevis gene and for that with the unmodified tetramer associated with the X. borealis gene. The footprint of the acetylated octamer does not change as a con-

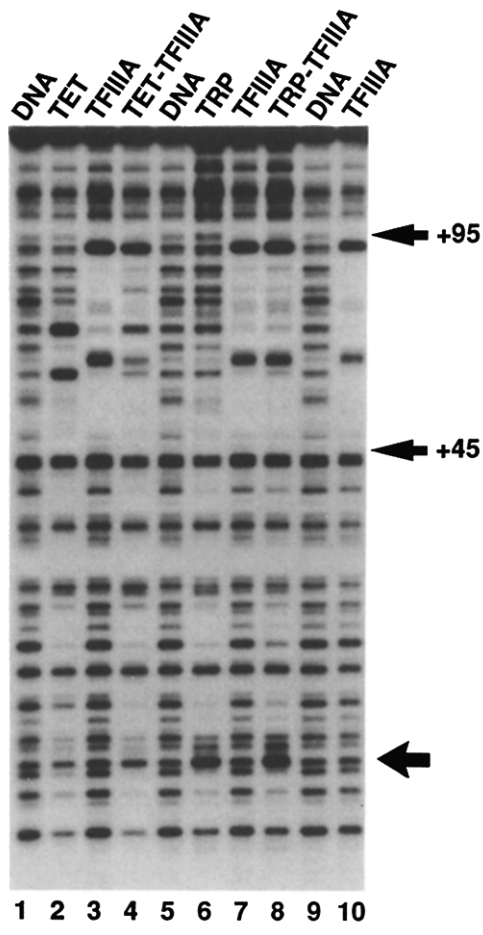

Figure 8. The DNAase I Footprint of a Tertiary Complex of TFIIIA–Trypsinized Octamer–X. borealis 5S DNA

Tetramers and trypsinized octamers assembled onto X. borealis 5S DNA were digested with DNAase I in the presence or absence of TFIIIA, and nucleoprotein complexes were isolated by native gel electrophoresis. DNA associated with each band on the native gel was purified and analyzed by sequencing gel electrophoresis. Lanes 1–4 and lanes 5–8 contain digestion products from experiments with the tetramer and the trypsinized octamer, respectively. Shown are the digestion products obtained from the free DNA bands in lanes 1 and 5; the tetramer and trypsinized octamer binary complexes in lanes 2 and 6, respectively; the TFIIIA–5S DNA complexes in lanes 3 and 7, respectively; and the TFIIIA–tetramer–5S DNA and TFIIIA–trypsinized octamer–5S DNA tertiary complexes in lanes 4 and 8, respectively. For comparison, the digestion patterns of free and TFIIIA-bound DNA produced by conventional methods are shown in lanes 9 and 10, respectively. Arrows indicate the approximate limits of the TFIIIA-binding site at positions +45 and +95 in the X. borealis 5S gene. The lowest horizontal arrow indicates the dyad axis of the histone–DNA complex. Histone–DNA complexes are assembled as described in Figure 7.

sequence of TFIIIA binding, and for both the X. borealis and X. laevis gene it is clearly distinguishable from that of the histone tetramer. This result, together with the clear differences in mobility on nondenaturing gels, strongly suggests that no histone displacement from the complex occurs as a result of TFIIIA binding.

Earlier work had suggested that the interaction of TFIIIA with a histone-associated 5S RNA gene was dependent on the type of 5S RNA gene (Rhodes, 1985; Gottesfeld, 1987) and the stoichiometry of the histones (Tremethick et al., 1990; Almouzni et al., 1991; Clark and Wolffe, 1991; Hayes and Wolffe, 1992). Our results, however, systematically vary histone stoichiometry, histone–DNA contacts, and histone acetylation and show that the binding of TFIIIA to model nucleosome templates depends on all three variables.

Histone Acetylation and the Association of Transcription Factors with Nucleosomal DNA

Our results address three possible models to explain how histone acetylation facilitates the association of TFIIIA with its binding site in a nucleosome (Figure 9). These can be divided into two classes: those requiring alterations in organization of the nucleosome and those postulating a direct interaction of the tails with the transcription factor. The histone tails can associate with DNA in the core particle (Cary et al., 1978) through a large number of electrostatic interactions between basic lysine residues in the tails and the phosphodiester backbone (Walker, 1984). These interactions can be greatly reduced by acetylation of the histone tails (Cary et al., 1982). Thus, it is possible that acetylation of the histone tails might prevent these polypeptide chains from occluding the interaction of a transcription factor with DNA. This concept is supported by tryptic removal of the tails, including all but one of the lysine residues that might potentially be acetylated, which facilitates TFIIIA binding (Figures 7 and 8). Thus, we suggest that acetylation of the histone tails is relieving an inhibitory effect directed by interaction of these protein domains with DNA.

Acetylation of the histone tails has been suggested to unfold the nucleosome (reviewed by Oliva et al., 1990). Under our experimental conditions (Figure 2), changes in nucleosome conformation are subtle. van Holde and colleagues have detected no major changes in the integrity of "tailless" or hyperacetylated particles (Ausio and van Holde, 1986; Ausio et al., 1989; but see Oliva et al., 1990). Likewise, Bradbury and colleagues detect no increase in the accessibility of the H3 sulphydryl groups within the nucleosome upon acetylation (Marvin et al., 1990); however, a 20% reduction in linking number change per nucleosome is detected (Norton et al., 1989). Three possible explanations for this change exist: the helical repeat of DNA on the histone octamer could change, the path of the DNA in the nucleosome could be altered, or the path of DNA between nucleosomes could vary. We have determined that the helical repeat of 5S DNA in the nucleosome does not change. Thus, the path of DNA in chromatin must be influenced by acetylation (see White et al., 1989). Two major structures blocking uniform transcription factor access to DNA are the globular domains of the histones around which DNA is wrapped and the adjacent superhelix of DNA. Either of these surfaces might be altered following acetylation of the tails. The driving force for such an alteration could be electrostatic repulsion derived from the two adjacent DNA helices. In fact, dissociation of the histone tails from DNA directed by an increase in salt concentration does correlate with an "opening up" of nucleosome structure (Daban and Cantor, 1982; Walker, 1984). DNA recognition elements might be exposed as a result of such conformational changes.

A more direct role of the histone tails in mediating the interaction of transcription factors with DNA follows from genetic and structural experiments in yeast. Grunstein and colleagues have investigated the role of the NH_2-terminus of histone H4 in gene repression and activation. Mutations in this domain, including potentially acetylated lysines, can prevent activation of certain genes (Durrin et al., 1991) and the repression of others (Kayne et al., 1988; Johnson et al., 1990). Histone acetyltransferases are required to mediate repressive effects at the silent mating loci (Whiteway and Szostak, 1985). Potential interactions between the histone H4 tails and the $\alpha 2$ repressor might influence nucleosome positions (Roth et al., 1992). Although it is possible that comparable interactions between the histone tails and TFIIIA might directly facilitate its interaction with nucleosomal DNA, the fact that removal of the histone tails also allows TFIIIA to bind renders this possibility unlikely (Figures 7 and 8).

Biological Significance of Increased Transcription Factor Access to Acetylated Nucleosomes

Histone acetylation is strongly correlated with three events in vivo. Allfrey suggested that acetylated histones were preferentially associated with transcriptionally active chromatin (Allfrey et al., 1964), and this observation has been substantiated by numerous studies (for example, Walker et al., 1990; Lin et al., 1989). Histone acetylation has been suggested to precede transcriptional activation (Chen and Allfrey, 1987; Chen et al., 1990). Our results suggest that acetylation of histones within nucleosomes might facilitate transcription complex formation on the gene that is to be actively transcribed. TFIIIA is only the first of several transcription factors to bind to DNA in the assembly of a transcription complex on a 5S RNA gene (Wolffe and Brown, 1988), thus, the role of histone acetylation in the access of the complete transcription complex to a 5S RNA gene remains to be resolved (see Roberge et al., 1991). A second biological role also related to histone displacement is the acetylation of histones prior to their displacement by protamines (Sung and Dixon, 1970; Christensen et al., 1986). We suggest that the same mechanism that allows TFIIIA to bind facilitates protamine binding. Acetylation of histones is also strongly correlated with histone deposition on newly replicated DNA (Waterborg and Matthews, 1984; Perry and Annunziato, 1989). Histones are progressively deacetylated as chromatin structure matures. Thus, the acetylation of histones within nascent nucleosomes might serve to facilitate transcription factor access to newly replicated DNA, allowing genes to be programmed for the tran-

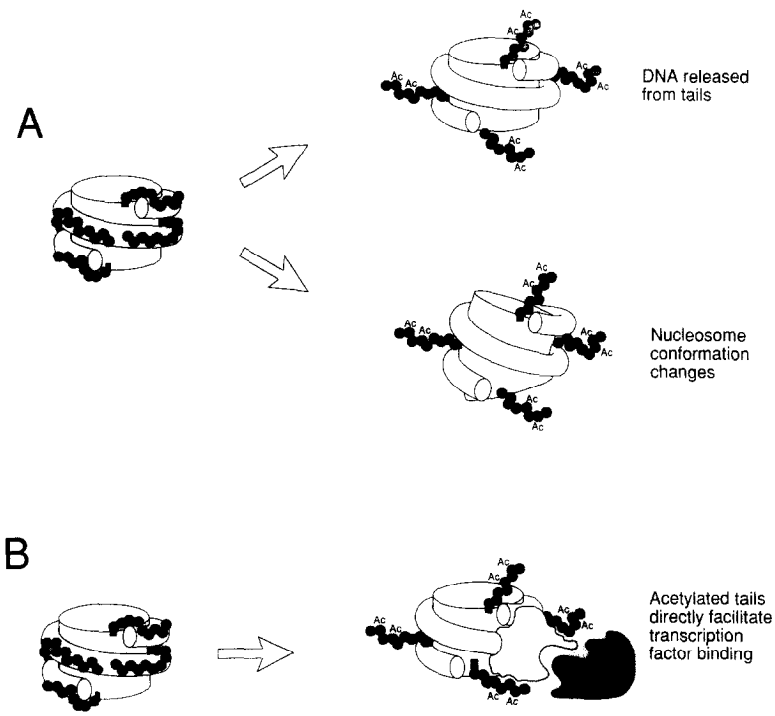

Figure 9. Models for the Influence of Histone Acetylation on Transcription Factor Binding

(A) Those that involve a change in nucleosome organization.

(B) Those that involve contacts between the acetylated histone tails and transcription factors.

scription factors available. The subsequent deacetylation of histones would serve to establish a chromatin state that would prevent subsequent gene activation. These three possible biological roles remain to be further explored in vivo through the analysis of protein–DNA interactions on specific genes.

Experimental Procedures

DNA Fragments

Radiolabeled DNA fragments contained a single copy of either the X. laevis or X. borealis somatic 5S RNA gene. A 186 bp HindIII–DdeI fragment derived from pXP-1 (Wolffe and Brown, 1987) containing the X. laevis gene and a 214 bp EcoRI–DdeI fragment derived from pXP-10 (Wolffe et al., 1986) containing the X. borealis gene were used for nucleosome reconstitutions after radiolabeling at the noncoding strand with polynucleotide kinase or at the coding strand with Klenow DNA polymerase. pXP-1 was labeled at the HindIII site, and pXP-10 was labeled at the EcoRI site. After radiolabeling, the second restriction reaction with DdeI was carried out, and the uniquely labeled fragments were electrophoretically purified in a nondenaturing 8% polyacrylamide gel, followed by band isolation and electroelution.

Purification of Chromatin and Histones

HeLa cells were grown either on flasks with a plating density of approximately 5 × 10^6/150 cm^2 or in suspension with Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. For hyperacetylated chromatin preparations, media containing 8 mM sodium butyrate from a freshly prepared stock solution made by alkali treatment of n-butyric acid (Sigma) were supplied, and the cells were grown for 24 hr at 37°C. Thereafter, cells were harvested and treated to prepare nuclei following the procedure of Ausio and van Holde, with slight modifications (Ausio and van Holde, 1986). Extensive sonication, producing chromatin lengths of 10–30 nucleosomes, was employed instead of traditional micrococcal nuclease digestion of nuclei. All buffers contained 10 mM sodium butyrate to minimize residual histone deacetylase activity in the chromatin preparations. Purified chromatin was treated with Dowex 50W × 2 in 0.65 M NaCl, 0.05 M potassium phosphate (pH 6.8) to remove linker histone H1 (Thoma et al., 1979; Bolund and Johns, 1973). Histones were resolved on SDS–18% polyacrylamide and Triton acid–urea–15% polyacrylamide gels (Zweidler, 1978). Triton acid–urea gels were modified from the published protocol to optimize resolution (19:1 15% acrylamide:bisacrylamide, 6.3 M urea, 7 mM Triton X-100, 5% acetic acid). Gels were prerun for 2–3 hr with a top buffer layer of 6.3 M urea, 7 mM Triton X-100 containing 0.5 M 2-aminoethanethiol-HCl (Aldrich) as a scavenger. Electrophoresis was carried out in 5% acetic acid with an applied constant current of 3 mA/cm in a buffer-cooled electrophoresis tank for approximately 5 hr. Analytic gels were subsequently fixed and stained with Coomassie blue. Proteins from Triton acid–urea gels were electrotransferred onto nitrocellulose and stained with ponceau S (Sigma) to evaluate transfer efficiency. Western blotting and immunochemical staining with polyclonal antibodies (a gift of David Allis) confirmed the acetylation of histone H4 (Delcuve and Davie, 1992; Lin et al., 1989).

Histones from adult chicken erythrocytes and HeLa cells were prepared as described by Simon and Felsenfeld (1979) using hydroxylapatite chromatography and salt gradients to elute histone groups H2A+H2B and H3+H4 in a stepwise manner. In brief, sheared or micrococcal nuclease–digested chromatin isolated from Triton X-100–washed nuclei was adsorbed onto hydroxylapatite at low ionic strength (<0.05 M NaCl, 10 mM Tris–HCl [pH 8.0], 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride). Elution of linker histones H1 and H5 was achieved with 2 column volumes of 0.65 M NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride. Histones H2A and H2B were then eluted with 0.93 M NaCl; histones H3 and H4 were eluted with 2 M NaCl in the same buffer. Histones were quantitated spectrophotometrically at 230 nm. This method fractionated core histones efficiently and with extremely high purity, as determined by SDS–polyacrylamide gel electrophoresis. Aliquots were rapidly frozen on dry ice and stored at −70°C.

To generate trypsinized nucleosomal structures, nucleosome core particles (0.35 mg/ml DNA) in 35 mM NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol were prepared (Ausio et al., 1989), and portions were treated with trypsin from bovine pancreas (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone; Sigma) at a concentration of 6 μg/ml for 7 min at 25°C to remove the core histone tails. The reaction was stopped by adding trypsin inhibitor from soybean (Boehringer Mannheim) to a concentration of 60 μg/ml and cooled on ice. Histones were analyzed in SDS–18% polyacrylamide

gels and stained with Coomassie blue. These trypsinized cores were used immediately for histone exchange reconstitutions (see below) and were stable for at least 2 weeks (data not shown). We found that residual trypsin in the exchange reconstitution would not necessarily further digest the histones in the trypsinized cores, but would digest TFIIIA added to the reaction. This digestion would inhibit TFIIIA binding. To control for the presence of intact TFIIIA in the binding reaction, the integrity of the protein should be examined in the presence of the trypsinized reconstitutes. It was found necessary to titrate individual batches of trypsin (Sigma) with individual batches of trypsin inhibitor (Boehringer Mannheim) to establish conditions under which digestion of TFIIIA in the reaction did not occur.

Nucleosome Reconstitution

Nucleosomes were assembled on radiolabeled DNA either by exchange with short chromatin (10–30 nucleosomes on average) or core particles or by dialysis from high salt–urea with purified histones (Tatchell and van Holde, 1977; Camerini-Otero et al., 1976). In no circumstances did we detect differences in results due to the reconstitution methodology employed or the source of unmodified histones. In the histone exchange method, approximately 5-fold template mass excess of normal or hyperacetylated donor chromatin was mixed with radiolabeled DNA in silicanized tubes followed by slow adjustment of NaCl concentration (to 1 M) by dropwise addition and vortexing. Tubes were placed on ice between handling steps. Samples were then transferred to a dialysis bag (with a molecular size limit of 6–8 kd) and dialyzed against 1.0 M NaCl, 5 M urea, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, and 10 mM 2-mercaptoethanol for at least 6 hr at 4°C. Following this initial dialysis, samples went through successive changes of 90 min each in the same buffer, but with NaCl concentrations of 0.8 M and 0.6 M. Samples were then dialyzed in the 0.6 M NaCl buffer lacking urea for 4 hr, followed by a final dialysis against 10 mM Tris–HCl (pH 8.0) and 1 mM 2-mercaptoethanol for 12 hr at 4°C. A similar methodology was employed using the core particles (with or without trypsin treatment).

In the salt–urea gradient method using purified core histones, radiolabeled DNA (<0.05 μg), unlabeled nonspecific carrier DNA (from either calf thymus or λ phage digested with restriction enzymes HaeIII or HhaI to generate approximate sizes ranging from 0.1 to 0.5 kb in length), and histones were mixed at various protein:DNA ratios ranging from 0.4 to 0.8 in a total volume of 100–200 μl of high salt buffer (2 M NaCl, 10 mM Tris–HCl [pH 8.0], 10 mM 2-mercaptoethanol, and 1 mM EDTA). Unlabeled carrier DNA was necessary to determine nucleic acid concentration accurately for correct histone:DNA stoichiometry. Samples were dialyzed against the 2 M NaCl buffer containing 5 M urea for 12 hr at 4°C and then into successive changes of 90 min each in the same buffer, but with NaCl concentrations of 1.2 M, 1.0 M, 0.8 M, and 0.6 M. Two further changes were carried out with 0.6 M NaCl buffer lacking urea for a total of 4 hr at 4°C. Thereafter, samples were exhaustively dialyzed against 10 mM Tris–HCl (pH 8.0) and 1 mM 2-mercaptoethanol for at least 12 hr.

The two methods described resulted in the assembly of single nucleosomes on the labeled DNA templates as monitored by nucleoprotein gel electrophoresis (0.8% agarose in 45 mM Tris–borate [pH 8.3] and 1 mM EDTA) and SDS–polyacrylamide gel electrophoresis of band-isolated histones (data not shown). Reconstitutions of histone octamer–DNA complexes were achieved with either method; histone tetramer–DNA complexes were achieved with the salt–urea gradient method. The uncomplexed DNA in the reconstitution samples provided an important internal control for the binding of TFIIIA.

Purification of TFIIIA

7S storage particles and TFIIIA were purified as described by Smith et al. (1984). In brief, immature ovary homogenate was fractionated on glycerol gradients, bound to diethylaminoethyl cellulose, and eluted on a salt gradient. The 7S particle fractions were adjusted to 0.1 M KCl in 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM $ZnCl_2$, 20% glycerol (buffer A). RNAase A was added to the mixture (50 μg of enzyme per mg of protein), which was incubated for 5 min, and then the volume was brought up 2-fold with buffer A containing 10 M urea (to a final urea concentration 5 M). The mixture was then loaded onto a 2 ml (bed volume) BioRex-70 column, and TFIIIA was eluted with increasing concentration of KCl (the protein eluting at a final concentration of 1 M). No other proteins were detectable by SDS–polyacrylamide gel electrophoresis and silver staining.

TFIIIA Binding Reactions and Gel Mobility Retardation Assay

Approximately 10 fmol of labeled DNA was used for quantitative gel retardation experiments in a total reaction volume of 10–20 μl of binding buffer (20 mM HEPES [pH 7.5], 70 mM NH_4Cl, 7 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM dithiothreitol, 0.02% Nonidet P-40/5% [v/v] glycerol, 20 μg/ml bovine serum albumin) (Hayes and Wolffe, 1992). TFIIIA was added to approximately 10-fold mass excess, unless stated otherwise. Reactions were equilibrated at room temperature for 15–20 min. Samples were loaded directly into 0.8% agarose gels in 0.5 × TB buffer (45 mM Tris–borate [pH 8.3]) with applied voltage of 4 V/cm for 3 hr at room temperature. EDTA was omitted in all binding and electrophoresis buffers to avoid denaturing TFIIIA. The Mg^{2+} concentration in the binding buffer was varied from 7 mM to 1 mM with no difference in TFIIIA association with reconstitutes.

DNAase I and Hydroxyl Radical Footprinting

Reconstitutes with or without TFIIIA (10-fold excess, unless otherwise stated) were treated with either DNAase I or hydroxyl radicals prior to resolving nucleoprotein complexes on preparative 0.8% agarose gels (Hayes and Wolffe, 1992). The approximate concentration of DNAase I (Boehringer Mannheim) necessary to generate cutting was determined empirically to be 0.2–0.4 mg of enzyme per mg of nucleic acid under our experimental conditions. DNAase I reactions were carried out on ice for 1 min, and the entire reaction volume was transferred directly into a preparative gel with an applied voltage of 5 V/cm with 45 mM Tris–borate (pH 8.3). The reaction mixture contained 5% glycerol, and all visual tracking dyes such as bromophenol blue or xylene cyanol were omitted. The hydroxyl radical reactions were carried out as described by Hayes et al. (1990) with the following modification: final concentrations of Fe(II)EDTA and H_2O_2 were 100 μM and 0.012%, respectively. Free radical reactions were quenched with the addition of glycerol to a concentration of 5%, and the entire reaction volume was transferred to a gel, as described above. After electrophoresis, wet gels were placed against film to locate bands, and those of interest were excised for electroelution. Recovered DNA samples were further treated with proteinase K digestion in the presence of 0.2% SDS and phenol–chloroform extraction, ethanol precipitated, and then resolved on a denaturing 7 M urea–6% polyacrylamide gel in 90 mM Tris–borate–EDTA (pH 8.3) buffer. Specific DNA markers were produced by Maxam and Gilbert cleavage at G residues.

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