The H3-H4 N-Terminal Tail Domains Are the Primary Mediators of Transcription Factor IIIA Access to 5S DNA within a Nucleosome

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Reconstitution of a DNA fragment containing a *Xenopus borealis* somatic type 5S rRNA gene into a nucleosome greatly restricts the binding of transcription factor IIIA (TFIIIA) to its cognate DNA sequence within the internal promoter of the gene. Removal of all core histone tail domains by limited trypsin proteolysis or acetylation of the core histone tails significantly relieves this inhibition and allows TFIIIA to exhibit high-affinity binding to nucleosomal DNA. Since only a single tail or a subset of tails may be primarily responsible for this effect, we determined whether removal of the individual tail domains of the H2A-H2B dimer or the H3-H4 tetramer affects TFIIIA binding to its cognate DNA site within the 5S nucleosome in vitro. The results show that the tail domains of H3 and H4, but not those of H2A and/or H2B, directly modulate the ability of TFIIIA to bind nucleosomal DNA. In vitro transcription assays carried out with nucleosomal templates lacking individual tail domains show that transcription efficiency parallels the binding of TFIIIA. In addition, we show that the stoichiometry of core histones within the 5S DNA-core histone-TFIIIA triple complex is not changed upon TFIIIA association. Thus, TFIIIA binding occurs by displacement of H2A-H2B-DNA contacts but without complete loss of the dimer from the nucleoprotein complex. These data, coupled with previous reports (M. Vettese-Dadey, P. A. Grant, T. R. Hebbes, C. Crane-Robinson, C. D. Allis, and J. L. Workman, *EMBO J.* 15:2508–2518, 1996; L. Howe, T. A. Ranalli, C. D. Allis, and J. Ausio, *J. Biol. Chem.* 273:20693–20696, 1998), suggest that the H3/H4 tails are the primary arbiters of transcription factor access to intranucleosomal DNA.

The eukaryotic cell condenses its genetic material and controls access to DNA regulatory elements contained within by the hierarchical arrangement of DNA and proteins in the chromatin complex. Several aspects of chromatin are thought to play direct roles in modulating gene expression. These include nucleosome positioning, the effect of linker histones and core histone tail domains, and the formation of higher-order structures such as the 30-nm fiber (59, 61). It has been shown that in most cases the assembly of regulatory sequences with core histone proteins into a nucleosome can severely restrict *trans*-acting factor binding to nucleosomal DNA in vitro (11). Polach and Widom demonstrated that linked equilibria exist in which histones and *trans*-acting factors compete for binding to DNA elements within nucleosomes (39, 40). Competition by histone proteins is critically important for the proper regulation of transcription in several systems, such as maintenance of the stably repressed state of the yeast PHO5 gene in the absence of inducer in vivo (17, 48). Likewise, the ability of positioned nucleosomes to restrict access of critical replication factors has been demonstrated in vivo (46). The binding of H1 may also contribute to this regulation by restricting the translational mobility of the nucleosome to critical locations containing cognate DNA sequences involved in transactivation (38, 45, 52, 56).

The four core histones, H2A, H2B, H3, and H4, have amino-terminal tail domains defined by sensitivity to limited trypsin proteolysis (5). In addition, H2A also has a C-terminal tail. The tail domains can undergo a number of posttranslational modifications which correlate with differential chromatin activities and structures (20, 61). Acetylation of the tails has been

linked to transcriptional activation and results in the neutralization of the positive charge on the ϵ -amino group of lysines within the tails which is likely to result in a decrease in the affinity of the tails for DNA binding (7, 8, 28). Thus, proteolytic removal of the histone tails has been viewed as approximating the effect of acetylation of lysines in the tail domains. However, the exact mechanism by which acetylation facilitates transcription of chromatin templates is unknown. Previous work has shown that either proteolytic removal of the tails or acetylation of these domains results in an increase in the accessibility of selected transcription factors to nucleosomal DNA in vitro (15, 30, 33, 57, 58) and a marked reduction in the stability of the fully compacted 30-nm fiber (see below) (1, 13, 14, 55). In addition, biophysical evidence indicates that removal of the tails decreases fiber-fiber association (44). Thus, the tails influence chromatin structure and DNA accessibility at the level of individual nucleosomes, as well as higher-order structures (19).

Transcription factor IIIA (TFIIIA) is a 40-kDa protein comprised of nine zinc finger domains which binds specifically to the internal promoter of the 5S rRNA gene (60). TFIIIA binding is the primary step in activation of 5S transcription (29), and modulation of TFIIIA binding has been shown to be involved in regulation of 5S gene expression in vivo (2, 6). TFIIIA binds in a linearly extended fashion specifically to the 50-bp internal promoter within the 5S gene to generate a nucleoprotein complex recognized by the polymerase III factors TFIIC and TFIIB (16, 60). Reconstitution of the *Xenopus borealis* somatic gene with core histone proteins yields a population of complexes in which >80% of the nucleosomes adopt unique translational and rotational positions with regard to the DNA sequence (23, 24, 56). Histone-DNA contacts within the 5S nucleosome occlude the 5S internal promoter and greatly reduce the apparent affinity of TFIIIA for its cognate site (25, 51, 53, 62). Hydroxyl radical footprinting has

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FIG. 1. Histone-DNA interactions within the 5S nucleosome or H3-H4 tetramer-5S DNA complex. Histone-DNA contacts within the nucleosome (solid oval) or the H3-H4 tetramer (dotted oval) assembled with a DNA fragment containing an *X. borealis* somatic-type 5S RNA gene (arrow) are depicted (24, 25). The location of the internal control region (gray box) is indicated.

shown that core histone-5S DNA contacts within the 5S nucleosome extend from ~ -90 to $\sim +90$ within the 5S sequence, throughout the internal promoter, including a region (located at positions +80 through +90) most critical for TFI_{II}A binding (Fig. 1) (25, 43). Interestingly, TFI_{II}A binds with high affinity to 5S DNA templates assembled with only H3-H4 tetramers and these complexes exhibit much greater transcriptional activity than nucleosomal templates (9, 21, 25, 53, 54).

Removal of all of the core histone tails by limited trypsin proteolysis greatly reduces the competition by the histones for binding of the internal promoter and allows high-affinity binding by TFI_{II}A to the nucleosome (30). Surprisingly, footprinting analysis showed that the extent of histone-DNA contacts within the trypsinized 5S nucleosome is identical to that of the wild-type (WT) nucleosome and that the nucleosome position on the 5S template is not dramatically affected by removal of the core histone tails (22). Recent observations indicate that transcriptionally silent 5S genes are associated with unacetylated H4, whereas transcriptionally active genes are associated with the acetylated form of H4 (27). In addition, acetylation of H4 has been strongly correlated with transcription factor access to nucleosomal DNA in vitro (57). Given that the histone tails are likely to have multiple and independent functions within chromatin (20), it is possible that an individual core histone tail or a subset of the tail domains plays a predominant role in mediating the access of DNA-binding *trans*-acting factors such as TFI_{II}A to intranucleosomal DNA.

In this study, we utilized the well-characterized *Xenopus* 5S mononucleosome model system to evaluate the role of the different core histone tails in modulating TFI_{II}A binding to nucleosomal DNA. We found that removal of the H3-H4 tails is sufficient to restore the accessibility of nucleosomal DNA to TFI_{II}A and increase the transcriptional permissibility of 5S nucleosomal templates in vitro. Interestingly, removal of the H2A/H2B dimer tails, either together or individually, had no effect on the ability of TFI_{II}A to bind nucleosomal DNA. These results support a model in which acetylation of the H3 and H4 tail domains is the primary determinant of access of DNA-binding factors to DNA within the nucleosome.

MATERIALS AND METHODS

DNA fragments. A 215-bp fragment DNA template which contains a somatic-type 5S rRNA gene isolated from the frog *X. borealis* was used for nucleosome reconstitution and was obtained from the plasmid XP-10 by digestion with *Eco*RI and *Dde*I (Fig. 1) (24). The TFI_{II}A binding site is located within the internal promoter (+45 to +95), and reconstitutions with this template yield nucleosomes positioned such that the dyad axis is approximately 75 bp from the *Eco*RI site, near the transcription start site at +1 (Fig. 1) (24, 42). The fragment was radiolabeled at the 5' *Eco*RI site with [γ -³²P]ATP and T4 polynucleotide kinase

by standard methods (62). The dinucleosome template for the transcription experiments was prepared as previously described (56). The 424-bp *Xba*I-*Xho*I fragment from pX5S197-2 containing one tandem repeat of two 5S RNA genes was radiolabeled at both 5' ends as described above.

Mutant H2A/H2B dimer preparation. Coding sequences for *X. laevis* WT H2A and H2B genes were subcloned into either pET3a or pET3d (Novagen) (31). PCR strategies were used to obtain coding sequences for the desired tailless mutant forms (-nH2A, H2Ac-, -nH2Ac- and -nH2B), which were inserted into pET3a. *Escherichia coli* BL21(DE3) cells (Novagen) were transformed with the constructs, and expression was induced after the cells reached log-phase growth in the presence of 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for an additional 3 h at 37°C. Cells were then spun down, resuspended in 10 mM Tris-HCl (pH 8.0)-2 mM EDTA-0.1% Triton X-100-0.25 mM phenylmethylsulfonyl fluoride-lysozyme at 100 μ g/ml, and incubated at room temperature for 30 min. The lysate was brought to 0.6 M NaCl-10 mM Tris-HCl (pH 8.0)-2 mM EDTA, and the suspension was incubated on ice and then sonicated (2-min intervals for 10 min with a 30% duty cycle and a power setting of 50% using a Branson 250 sonicator). The preparation was centrifuged at 13,000 $\times$ g for 30 min, and the supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Supernatants containing approximately stoichiometric quantities of expressed H2A and H2B were combined, the NaCl concentration was reduced to <0.5 M by dilution, and the preparation was incubated with BioRex 70 resin (Bio-Rad) as previously described (31). The H2A-H2B dimers were then eluted with 1 M NaCl-Tris-EDTA and stored at -80°C in the elution buffer. WT H2A-H2B dimers and H3-H4 tetramers were also prepared from chicken erythrocyte chromatin (62).

Tailless H3-H4 tetramer preparation. Soluble linker histone-lacking chromatin (1.65 mg/ml) was prepared from chicken erythrocyte nuclei (62). Trypsinization of the soluble chromatin was accomplished using 1.0 ml of a 50% slurry of insoluble agarose-trypsin beads (Sigma) prepared as follows. The beads were washed once with H₂O and then twice with 10 ml of 10 mM Tris (pH 8.0). Supernatant was removed after each wash by centrifugation at 6,000 rpm (4,000 $\times$ g) for 2 min. An 8.0-ml volume of soluble chromatin was mixed with the beads, and the mixture was placed on a tube rotator for 16 h at room temperature and then gently spun to remove the trypsin-beads. Trypsin inhibitor was added to a final concentration of 50 ng/ml to ensure against residual trypsin activity, and the level of digestion was checked by SDS-PAGE (18% gel) (see Fig. 2A). The trypsinized chromatin was digested with micrococcal nuclease to generate core particles (146 bp) and then loaded onto a 5 to 20% sucrose gradient containing 4 M urea, 0.34 M NaCl, and 20 mM Tris HCl (pH 8.0) as previously described (3). Trypsinized tetramer-core DNA complexes were separated from the H2A-H2B dimers by ultracentrifugation at 20°C for 24 h at 38,000 rpm in an SW41 rotor. Fractions containing the trypsinized tetramer-DNA complexes were identified by SDS-PAGE and combined, and the DNA was removed by making the sample 0.4 M with respect to HCl. The sample was incubated on ice for 45 min and centrifuged to pellet the DNA, and the supernatant containing the trypsinized H3-H4 histones was neutralized with 10 M NaOH. Purified, trypsinized H3 and H4 were stored at -80°C until needed. Completely recombinant octamers lacking H3, H4, or both tails were the kind gift of Karl Nightingale, Department of Molecular Biology, University of Cambridge, Cambridge, United Kingdom.

Nucleosome reconstitution. Nucleosomes were reconstituted with labeled 5S DNA using either salt gradient dialysis or histone exchange (62). Mononucleosomes for stoichiometric analysis were reconstituted with unlabeled 5S DNA without competitor calf thymus DNA. Dinucleosomes for transcription experiments were reconstituted with a mixture of labeled and unlabeled 5S dimer template and linearized pBSII DNA as a nonspecific competitor DNA. The dinucleosomes were purified by 5 to 20% sucrose gradient as previously described, and fractions were analyzed on 0.7% agarose nucleoprotein gels (62). Tetramer-DNA complexes were reconstituted in the same manner without H2A/H2B. The stoichiometry of all complexes was further verified by sucrose gradient sedimentation.

TFI_{II}A binding assays. TFI_{II}A was prepared as previously described by Smith et al. (47). Alternatively, TFI_{II}A was overexpressed in bacterial cells and purified as previously described (10). TFI_{II}A prepared by either method behaved the same way in nucleosome binding assays (62). TFI_{II}A binding reactions were carried out with 10 μ l of the reconstituted histone-DNA complexes (~ 250 ng of total DNA and ~ 25 fmol of total 5S DNA) in binding buffer (50 mM NH₄Cl, 10 mM HEPES [pH 7.4], 5 mM MgCl₂, 20 μ M ZnCl₂, 5 mM dithiothreitol, 0.02% Nonidet P-40, 5% [vol/vol] glycerol, 20 mg of bovine serum albumin per ml, 100 ng of calf thymus DNA) in a final volume of 13 μ l. Complexes were incubated with 0.25 to 128 ng of purified TFI_{II}A as indicated in the figure legends. Samples were incubated for 1 h at 23°C, loaded directly onto a 0.7% agarose gel with 0.5 $\times$ TB buffer (45 mM Tris borate [pH 8.3]), and electrophoresed for ~ 2 h at 20 mA (25). EDTA was omitted from all buffers. Approximate affinities were estimated from the amount of TFI_{II}A required to shift 50% of the reconstituted material compared to the naked-DNA control in each titration.

Histone stoichiometry analysis. Antibodies directed against histones H2A and H2B were obtained by immunopurification from an antiserum generated against all histones (50). Briefly, purified H2A and H2B were deposited onto nitrocellulose and the membrane was saturated with 5% dried milk and then incubated with the panhistone antiserum for 1 h. The membrane was then extensively

FIG. 2. Histones H2A-H2B and the core histone tail domains inhibit the interaction of TFIIIA with 5S DNA assembled into a nucleosome. Nucleosomes and H3-H4 tetramer complexes were reconstituted with the *EcoRI-DdeI* 5S DNA fragment and analyzed on 0.7% agarose nucleoprotein gels. (A) SDS-PAGE containing Bethesda Research Laboratories low-molecular-weight markers (lane 1), soluble chromatin (lane 2), and stripped, trypsinized chromatin (lane 3). The asterisk indicates bovine pancreatic trypsin inhibitor. (B) Relative mobilities of the naked 5S DNA fragment (lane 1), the tetramer-5S DNA complex (lane 2), and the 5S nucleosomes reconstituted with WT core histones (lane 3). Oct, octamer; Tet, tetramer. (C, D, and E) Removal of the core histone tails or the H2A-H2B dimer facilitates TFIIIA binding to nucleosomal DNA. Increasing amounts of TFIIIA were incubated with nucleosomes containing either WT (C) or trypsinized (D) core histones or the (H3-H4)₂ tetramer-5S DNA complex (E). In each panel, lane 1 contains naked 5S DNA, lane 2 contains reconstituted complexes incubated without TFIIIA, and lanes 3 to 11 contain complexes incubated with 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 ng of TFIIIA, respectively. Bands corresponding to naked 5S DNA (DNA); the 5S DNA-TFIIIA complex (A/DNA); tetramer, the WT octamer, or trypsinized octamer complexes (Tet, Oct, and Tryp, respectively); the tetramer-5S DNA-TFIIIA triple complex (A/Tet); and the trypsinized octamer-5S DNA-TFIIIA triple complex (A/Oct) are indicated.

washed with phosphate-buffered saline-Tween, and antibodies binding to H2A-H2B proteins were stripped with 100 mM glycine, pH 2.5. The solution was then buffered with Tris to pH 7.0. In order to increase the specificity of the antibodies for the dimer fraction, any residual reactivity against H3 or H4 was exhausted by immunodepleting the antibody fraction against H3 and H4 deposited onto nitrocellulose. The reactivity of the purified antibodies was confirmed by Western blotting (see Fig. 6A) (49).

Bulk preparations of reconstituted trypsinized mononucleosomes for stoichiometry analysis were prepared as described above. The amount of TFIIIA required to form the triple complex was determined by careful stepwise titration as evaluated on 0.7% agarose nucleoprotein gels. The gels were electrophoresed at 20 mA for 2 h, and then the DNA-containing complexes were identified by soaking the gels in 0.5× TB buffer with ethidium bromide, followed by UV illumination. Bands corresponding to unbound trypsinized nucleosomes and the triple complex containing 5S DNA-trypsinized core histones-TFIIIA were excised (30). Gel slices were placed into Spin-X tubes, placed at -80°C for 30 min, and then spun at 13,000 rpm in a Brinkman tabletop microcentrifuge for 30 min to elute the protein and DNA. The DNA concentration in the resultant eluate was quantitated using a Hoefer DyNA Quant 200, and samples were normalized with respect to DNA content. Immunoblot analysis was carried out using the specific anti-H2A-H2B antibodies and secondary antibodies coupled to horseradish peroxidase and ECL (Amersham). The blots were analyzed by densitometry.

Hydroxyl radical footprinting. Reconstituted mononucleosomes were treated with hydroxyl radicals, and reactions were quenched by the addition of glycerol to a final concentration of 5% as previously described (23). Samples were loaded onto preparative 0.7% agarose gels and electrophoresed for ~ 2 h at 20 mA. The bands were identified by autoradiography of the wet gel; DNA was recovered and separated by electrophoresis on denaturing 6% polyacrylamide gels. The gels were dried, and footprints were analyzed by autoradiography (62).

Transcriptional analysis. Transcription experiments were carried out with the 5S dinucleosome as previously described (56). The dinucleosome template was reconstituted with the different full-length (WT) and tailless histones as described above, complexes were resolved in sucrose gradients, and the dinucleosome fractions were used for the transcription assays. A 20- μl volume of templates (0.5 ng/ μl) was incubated in 20 μl of *Xenopus* egg extract supplemented with 1 μl of nucleoside triphosphates (10 mM each ATP, GTP, and CTP and 1 mM UTP), 0.6 μg of TFIIIA, 1 μl of RNasin, and 2 μl of [α - ^{32}P]UTP for 1 h at room temperature. The reactions were stopped by addition of 100 μl of 5 mM EDTA-1% SDS-10 mM Tris-HCl (pH 8.0), nucleic acids were purified by phenol-chloroform extraction and ethanol precipitation, and transcription products were analyzed by denaturing gel electrophoresis and autoradiography.

RESULTS

H2A-H2B dimers and the core histone tail domains inhibit binding of TFIIIA to 5S DNA in a nucleosome. We have reported that the assembly of a *Xenopus* 5S RNA gene into a nucleosome greatly restricts access of the TFIIIA to the gene promoter (25, 30). However, recent reports suggest that TFIIIA binds 5S nucleosomal DNA dependent upon translational position (26, 37, 42). Thus, our initial efforts focused on recapitulating previous in vitro studies of TFIIIA binding to nucleosomes reconstituted with a 215-bp DNA fragment containing an *X. borealis* somatic 5S rRNA gene (Fig. 1). Nucleosomes were reconstituted using either a stepwise salt dialysis method or a histone exchange procedure. The resulting reconstitutions were analyzed by separation in nucleoprotein gels (Fig. 2B), and the identity of the putative nucleosome band was confirmed by sucrose gradient sedimentation (data not shown). TFIIIA binding to the nucleosome was assessed by a nucleoprotein gel shift assay (Fig. 2C to E). Previous work has demonstrated that any TFIIIA binding is likely to result in a dramatic shift in the position of the complex on the gel (25). These studies showed that reconstitution of a nucleosome onto this DNA fragment containing WT core histones resulted in loss of detectable high-affinity binding by TFIIIA (Fig. 2C), consistent with previous results (25, 30, 51). In contrast, TFIIIA binding to the naked DNA was clearly detectable. We estimate that the apparent association constant for TFIIIA binding is reduced by at least a factor of 10^3 for DNA within the nucleosome compared to the naked 5S DNA. This result was not dependent on the method of reconstitution (results not shown).

Also consistent with previous reports (25, 30, 51, 54, 62), we

found that removal of all of the histone tails by limited trypsin proteolysis (Fig. 2D) or removal of the H2A-H2B dimers (Fig. 2E) restores high-affinity binding by TFIIIA. Titration of TFIIIA with the 5S DNA fragment reconstituted with a trypsinized histone octamer resulted in diminution of the nucleosome band and the emergence of a new complex previously identified as a nucleosome-TFIIIA triple complex (Fig. 2D) (25). The results also show that the affinity of TFIIIA for naked DNA is higher than that for the trypsinized 5S nucleosome complex; 50% of the naked 5S DNA was bound at an approximately fivefold lower concentration of TFIIIA than were nucleosomes containing trypsinized histones. Likewise, reconstitution of the 5S DNA fragment with only the (H3/H4)₂ tetramer generated a complex which clearly exhibited high-affinity binding with TFIIIA (Fig. 2E). Throughout the titration of TFIIIA, we observed a diminution of the bands corresponding to naked DNA and the tetramer complex and the appearance of new bands corresponding to the 5S DNA-TFIIIA and tetramer-5S DNA-TFIIIA complexes.

The H3-H4 tails alone mediate TFIIIA high-affinity binding to the 5S nucleosome. Based on the above observations, we hypothesized that the N- and/or C-terminal tail domains of H2A-H2B were responsible for the inhibition of TFIIIA binding to its site within the 5S nucleosome. To test our hypothesis, we used directed mutagenesis techniques and bacterial overexpression to generate H2A and H2B lacking tail domains (Fig. 3A). Dimers lacking either a single tail, a subset of tails, or all of the three tail domains were reconstituted into mononucleosomes along with WT H3-H4 tetramers and the 215-bp 5S DNA fragment. The final amount of each dimer required for octamer complex formation was determined empirically by careful stepwise titration with previously established levels of DNA and WT tetramer (H3/H4). The electrophoretic mobility of each reconstituted nucleosome was examined, and the identity of the putative nucleosome band was confirmed by sucrose gradient sedimentation (results not shown) and hydroxyl radical footprinting (see Fig. 7). The reconstituted nucleosomes were then tested for the ability to be bound by TFIIIA. Surprisingly, we did not detect TFIIIA binding to nucleosomes lacking either H2A or H2B tails (Fig. 3B and C). Furthermore, nucleosomes in which the N- and C-terminal tails of H2A were deleted singularly or in combination with the H2B tail were also refractory to high-affinity binding by TFIIIA (data not shown). Finally, nucleosomes lacking all three dimer tail domains did not exhibit detectable high-affinity binding by TFIIIA (Fig. 3D).

We then determined if removal of the H3-H4 tetramer tails affected TFIIIA binding. We prepared purified tailless H3-H4 tetramer from trypsin-digested bulk nucleosome core particles by the sucrose-urea gradient method of Ausio et al. (3) (Fig. 4A). A careful titration was performed with the trypsinized tetramer to determine the optimal amount required for tailless H3-H4 tetramer-5S DNA complex formation. The WT H2A-H2B dimer was then titrated to obtain a homogeneous preparation of octamer-DNA complex which was confirmed by sucrose gradient sedimentation. In clear contrast to the experiments with tailless dimers, TFIIIA binding to the nucleosomes lacking only the H3/H4 tail domains was apparent (Fig. 4A). This was evidenced by the diminution of the octamer band and the appearance of a new shifted complex representing the TFIIIA-nucleosome complex. Next, we examined whether the deletion of specific H2A-H2B dimer tails could increase the apparent binding affinity of TFIIIA in combination with the tailless H3-H4 tetramer. Surprisingly, no further increase in TFIIIA binding was detected when dimers lacking either both tails of H2A (Fig. 4B) or the N-terminal tail of H2B (Fig. 4C)

FIG. 3. Removal of the H2A-H2B tail domains does not increase access of TFIIIA to nucleosomal DNA. (A) Overexpression of mutant H2A-H2B dimer proteins. Lanes: 1, Bethesda Research Laboratories low-molecular-weight marker; 2, wtH2A-wtH2B; 3, -nH2A-wtH2B; 4, H2Ac-wtH2B; 5, wtH2A/-nH2B; 6, wtH3-wtH4. (B, C, and D) Nucleosomes were reconstituted with the *EcoRI-DdeI* 5S DNA fragment, WT H3 and H4 and nH2Ac-wtH2B (B), wtH2A-nH2B (C), or the completely tailless nH2Ac-nH2B dimer (D). In each panel, lane 1 contains the naked 5S DNA fragment; lane 2 contains nucleosomes incubated without TFIIIA, and lanes 3 to 10 contain nucleosomes incubated with 64, 32, 16, 8, 4, 2, 1, and 0.5 ng of TFIIIA, respectively. Complexes are as indicated in the legend to Fig. 2. Designations to the right of each panel indicate the tail domains not present within each nucleosome preparation.

were reconstituted with the tailless H3-H4 tetramer into a nucleosome. However, we were able to detect a moderate additional increase in affinity when nucleosomes were reconstituted with trypsinized tetramer and the completely tailless H2A-H2B dimer (data not shown). We conclude from these results that removal of the H3-H4 tail domains is necessary and sufficient to generate a nucleosome that is bound by TFIIIA with high affinity.

We then asked if removal of either the H3 or H4 tails is sufficient to allow TFIIIA access to nucleosomal DNA. 5S nucleosomes were reconstituted with fully recombinant his-

FIG. 4. Removal of the H3-H4 N-terminal tail domains facilitates high-affinity binding of TFIIIA to nucleosomal DNA. (A, B, and C) TFIIIA binding to 5S nucleosomes lacking H3-H4 tail domains. Nucleosomes were reconstituted with the *EcoRI-DdeI* 5S DNA fragment, trypsinized (tailless) H3/H4, and wtH2A-wtH2B (A), nH2Ac-wtH2B (B), or wtH2A-nH2B (C). In each panel, lane 1 contains the naked 5S DNA fragment, lane 2 contains nucleosomes incubated without TFIIIA, and lanes 3 to 11 contain nucleosomes incubated with 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 ng of TFIIIA, respectively. Positions of complexes are indicated as in the legend to Fig. 2.

tones lacking only the H3 tails, the H4 tails, or both sets of tails (Fig. 5A). The ability of TFIIIA to bind was assessed by gel shift as described above. We found that removal of the H3 tails facilitated marginal binding by TFIIIA (Fig. 5B), while nucleosomes lacking only the H4 tails exhibited only barely detectable binding by TFIIIA (Fig. 5C). Importantly, removal of both tails (Fig. 5D) recapitulated the results obtained with trypsinized H3-H4 (Fig. 4A). We estimated that the binding affinity of TFIIIA for the nucleosome lacking H3-H4 tails was reduced ~50-fold in comparison to the affinity for naked DNA (Fig. 5D).

Core histone stoichiometry does not change upon TFIIIA binding. There are several non-mutually exclusive mechanisms by which removal of the histone tails might increase the accessibility of nucleosomal DNA. Tail removal might decrease the stability of DNA wrapping, effectively increasing the conformational equilibrium constant for DNA site exposure within the nucleosome (39). In addition, removal of the tails might facilitate nucleosome mobility or displacement of H2A-H2B dimers, thereby facilitating TFIIIA binding (25). In order to help distinguish between these possibilities, we determined the effect of TFIIIA binding on the stoichiometry of the core histones within the nucleosome by immunoblot analysis of the shifted triple complex which contains DNA, trypsinized core histones, and TFIIIA. Immunoaffinity-purified antibodies specific to the H2A-H2B dimer were prepared, and the specificity

FIG. 5. Both the H3 and H4 N-terminal tail domains modulate binding of TFIIIA to nucleosomal DNA. (A) Relative mobility of nucleosomes reconstituted with the *EcoRI-DdeI* 5S DNA fragment and lacking the H3 or H4 tail domains. Nucleosomes were reconstituted with recombinant full-length core histones (lane 1) or core histones lacking only the H3 tails (lane 2), the H4 tails (lane 3), or both the H3 and H4 tails (lane 4). Also shown are the naked 5S DNA fragment (lane 5) and the TFIIIA-5S DNA complex (lane 6). (B, C, and D) TFIIIA binding to 5S nucleosomes lacking the H3 tail domains (B), the H4 tail domains (C), both the H3 and H4 tails (D) and nucleosomes containing full-length core histones (E). Positions of complexes are indicated in the legend to Fig. 2. In each panel, lane 1 contains naked 5S DNA, lane 2 contains nucleosomes incubated without TFIIIA, and lanes 3 to 12 contain nucleosomes incubated with 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 ng of TFIIIA, respectively.

FIG. 6. TFIIIA binding does not alter core histone stoichiometry within the 5S nucleosome. Nucleosomes containing trypsinized histones and the 5S DNA fragment were prepared as described in Materials and Methods and incubated with TFIIIA. TFIIIA-bound and unbound complexes were isolated by electrophoresis on a 0.7% nucleoprotein gel, and DNA and proteins were eluted from the gel (see Materials and Methods). (A) Immunoblot of SDS-PAGE containing WT H2A/H2B dimers only (lane 1), all four WT core histones (lane 2), and all four trypsinized core histones (lane 3). (B) Densitometric analysis of the TFIIIA-bound versus the unbound tailless octamer-5S DNA complex. Equivalent amounts of bound or unbound nucleosomes (normalized with respect to DNA content) were spotted onto the filter and probed with the H2A-H2B-specific antibodies as described in Materials and Methods. The relative amount of signal is plotted as a function of the total DNA content of the sample.

of the antibodies for both WT and tailless H2A-H2B was confirmed by Western blotting (Fig. 6A). Nucleosomes were reconstituted with trypsinized histones and incubated in the presence or absence of TFIIIA as previously described. Bands corresponding to the TFIIIA-nucleosome complex and unbound nucleosomes were isolated from preparative nucleoprotein gels, and the protein-DNA complexes were blotted onto nitrocellulose. The DNA contents of the samples were quantified by fluorimetry and used to normalized the nucleosome concentrations in the samples. Immunoblots showed that the ratios of DNA to H2A-H2B dimer within unbound nucleosomes was identical to the amount of dimer within the nucleosomes bound by TFIIIA (Fig. 6B). Note that the loss of one or both dimers would result in a 50 or 100% decrease in the amount of H2A-H2B, which is well within the estimated precision of our assay ($\pm 5\%$). We noted that binding reactions contain 10 μg of unassembled naked competitor DNA per ml, which should act as a sink for any free H2A-H2B dimers. Therefore, we concluded that the binding of TFIIIA to the tailless nucleosome does not involve the displacement of an H2A-H2B dimer.

FIG. 7. Removal of core histone tail domains does not alter the extent of histone-DNA contact within the nucleosome. (A) Hydroxyl radical footprints of nucleosomes reconstituted with the end-labeled 5S DNA fragment and various WT and tailless core histones. Nucleosomes were probed with hydroxyl radicals, and the cleavage products were analyzed by sequencing gel electrophoresis and autoradiography as described in Materials and Methods. Lane 1 contains products of the Maxam-Gilbert G-specific sequencing reaction with the 5S DNA fragment included as a marker. Shown are the cleavage patterns of the naked DNA fragment (lane 2), nucleosomes reconstituted with full-length WT histones or trypsinized histones (lanes 3 and 4, respectively), nucleosomes reconstituted with nH2Ac-nH2B and WT H3-H4 (lane 5), and nucleosomes reconstituted with WT H2A-H2B and tailless H3-H4 (lane 6). (B) Same as in panel A but subjected to electrophoresis for a longer time to resolve longer DNA fragments. The region of 5S DNA contacted by histone proteins (oval), the position of the 5S gene (arrow), the internal control region (striped box), and the approximate location of the nucleosomal dyad are indicated.

Removal of core histone tail domains does not alter nucleosome position or the extent of histone-DNA contacts. Next, we wished to determine if nucleosomes lacking various tail domains assembled onto 5S DNA in the same position and exhibited the same extent of histone-DNA contacts as previously determined for the WT and trypsinized core histones (22). Histone-DNA contacts with the various nucleosomes reconsti-

FIG. 8. Removal of the N-terminal tails of H3 and H4 increases the transcriptional competence of 5S nucleosomal templates. In vitro transcription of reconstituted 5S dinucleosomes was carried out in a *Xenopus* egg extract, and transcripts were analyzed on a 6% denaturing polyacrylamide gel as described in Materials and Methods. Shown are products of transcription carried out with the naked DNA template (lane 1) and templates reconstituted with WT core histones (lane 2), WT H2A-H2B-trypsinized H3-H4 (lane 3), and completely trypsinized core histones (lane 4). Levels of 5S RNA transcription, as determined by PhosphorImager analysis and normalized with respect to the radiolabeled template DNA band, are listed below the lanes. The transcriptional activity of the naked DNA template was taken as 100%.

tuted with the *EcoRI-DdeI* 5S DNA were mapped by hydroxyl radical footprinting (Fig. 7). As expected (22), identical patterns of protection were found for nucleosomes containing WT or trypsinized histones (Fig. 7, lanes 3 and 4). Moreover, the hydroxyl radical cleavage patterns for nucleosomes reconstituted with the tailless dimer-WT tetramer octamer (Fig. 7, lane 5) or the WT dimer-tailless tetramer octamer (Fig. 7, lane 6) were identical to that of the WT octamer (Fig. 7, lane 3). Importantly, upon removal of histone tail domains, no loss of tail-dependent histone-DNA contacts was detected in the internal promoter region, a region contacted primarily by the H2A-H2B dimer (22, 25). Therefore, high-affinity TFIIIA binding observed with the WT dimer-trypsinized tetramer nucleosome does not appear to be attributable to variance in the tight positioning of the nucleosome on 5S DNA or a loss of tail-dependent histone-DNA contacts near the periphery of the unbound nucleosome.

Removal of H3-H4 tails increases the transcriptional activity of 5S nucleosomal templates in vitro. We next tested if the effect of removal of the H3-H4 N-terminal tails on TFIIIA binding correlates with more efficient transcription of 5S nucleosomal templates. Nucleosomes were reconstituted onto a template containing a tandem repeat of the *X. borealis* somatic 5S DNA since short DNA templates transcribe in extracts in-

efficiently (56). Reconstituted dinucleosomes were isolated on sucrose gradients as previously described, and transcription was performed with a fixed amount of template in *Xenopus* egg extracts (Fig. 8). Consistent with previous results (56), we found that reconstitution of the 5S template with full-length (WT) histones severely inhibits transcription, to about 30 to 40% of that obtained with the same amount of naked DNA template (Fig. 8, cf. lanes 1 and 2). Note that the presence of ancillary proteins, including the remaining polymerase III transcription factors, is likely to stimulate factor binding in the extracts, thereby allowing transcription to occur on templates assembled with unmodified histones (56). Interestingly, the results show that the dinucleosomal template containing WT H2A-H2B and trypsinized H3-H4 was reproducibly more efficiently transcribed than the template reconstituted with the completely full-length histones (Fig. 8, lane 3). The fully trypsinized template supported the highest level of transcription of the three reconstituted nucleosomes we assayed (Fig. 8, lane 4).

DISCUSSION

The major conclusion of this work is that the H3-H4 tetramer tail domains are the major arbiters of TFIIIA access to 5S DNA assembled within a nucleosome. These results provide direct evidence for the hypothesis that individual tails or subsets of tails perform separate functions in chromatin (19, 20). Interestingly, we found that H3-H4 tail removal did not cause a loss of histone-DNA contacts within the unbound nucleosome or lead to a change in the stoichiometry of the core histones upon TFIIIA binding. Further, we showed that the ability of TFIIIA to bind is paralleled by a change in the efficiency of transcription of the nucleosomal templates. In agreement with previous studies, these results demonstrate a direct correlation between TFIIIA binding activity and the transcriptional competence of chromatin templates containing the *X. borealis* somatic 5S RNA gene (6, 9, 21, 45, 53).

Our results are consistent with previous observations regarding TFIIIA binding to *X. borealis* somatic-type 5S DNA assembled into a nucleosome. TFIIIA did not exhibit specific high-affinity binding to 5S nucleosomes containing WT core histones (Fig. 2C, 5E), whereas TFIIIA did bind with high affinity to both the H3-H4 WT tetramer-5S DNA complex (Fig. 2E) and a nucleosome containing trypsinized core histones (Fig. 2D), as previously reported (25, 30, 54, 56, 62). These results suggested that the H2A-H2B dimer tails were the main source of restriction to TFIIIA binding. However, we found that removal of the H3-H4 tails, but not that of any or all of the H2A-H2B tail domains, was sufficient to relieve repression of TFIIIA binding (Fig. 3 to 5). Interestingly, TFIIIA binding to nucleosomes lacking only the H3 or H4 tails can be detected, with removal of the H3 tail leading to greater enhancement of TFIIIA binding (Fig. 5). This result suggests that the H3-H4 tails mediate a structural transition within the nucleosome which results in greater accessibility of the internal control region to TFIIIA. Interestingly, this region is contacted primarily by the H2A-H2B dimers within the 5S nucleosome (25, 41).

Several previous reports also support a differential and dominant role for the H3-H4 tail domains in nucleosome structure. Bradbury and coworkers have reported that acetylation of the H3-H4 tails alone can mediate the reduction in the number of DNA supercoils constrained per nucleosome observed in minichromosomes reconstituted with acetylated core histones (35, 36). Interestingly, it is likely that this effect of H3-H4 acetylation is manifest through a reduction of DNA writhe near the edge of the nucleosome and does not include any apparent loss of histone-DNA contacts at the edge of the

nucleosome (4, 35). Moreover, Workman and coworkers have reported a tight correlation between the affinity with which several transcription factors bind nucleosomal DNA and the level of H4 acetylation (57). The core histone tails have been shown to modulate the access of several other transacting factors to nucleosomal DNA. Inhibition of binding of both GAL4-AH and the retinoic acid receptor to their cognate sites within nucleosomal DNA was relieved by removal of the core histone tail domains using limited trypsin proteolysis (33, 58). We note that the different affinities of TFIID for nucleosomes lacking all tails versus those lacking only the H3-H4 tails indicate that the H2A-H2B tails also contribute to intracore DNA accessibility (see reference 4). Thus, these observations, together with the present results, indicate a predominant role for the H3-H4 tails in mediating intracore DNA accessibility.

Previous studies have presented evidence for TFIID binding to a similar *X. borealis* somatic 5S rRNA gene fragment reconstituted into a nucleosome with WT core histones (26, 37, 42). The binding of TFIID observed in these studies has been attributed to heterogeneous positioning of the nucleosome along the 5S DNA fragment. However, our results show that all of the nucleosomes assembled onto our 5S DNA fragment significantly impeded TFIID binding (see above). It should be noted that our DNA fragment was prepared in a different manner and is slightly shorter than those used in the aforementioned studies (26, 37). Several lines of evidence, including those obtained by hydroxyl radical footprinting, nucleosome mapping by micrococcal and restriction nuclease digestions, and site-directed histone cross-linking studies, indicate that ~80% of the nucleosomes reconstituted with our 5S DNA template adopt a single or closely related set of translational positions (23, 24, 31, 32, 51, 56). More important, any heterogeneity leading to exposure of the 5S internal promoter would clearly be evident since a fraction of the nucleosome population would be bound by TFIID (51). One possibility is that some reconstitution procedures yield populations of nucleosomes with a distribution of translational positions different than that obtained in the present study. Multiple translational positions may have been "trapped out" during the reconstitution, since it has been demonstrated that nucleosomes prepared by some salt dialysis techniques may exhibit relative populations of translational positions not representative of the thermodynamically defined equilibrium distribution of translational positions at low salt concentrations (12, 18). In these cases, heating the sample to 37°C in low-salt buffers allows equilibration between translational positions and redistribution of nucleosomes on the DNA fragment (12). We note that heating our preparations of 5S nucleosomes did not alter translational positions, as judged by PAGE, and had no effect on the ability of TFIID to bind (results not shown). Further, observed differences in TFIID binding could be due to small sequence variations in the 5S DNA templates used. Minor sequence variations could have a significant effect on nucleosome positioning, which could result in increased translational heterogeneity. In addition, the binding of TFIID itself is very DNA sequence sensitive. Consider that the fourfold preference of TFIID for somatic versus oocyte-type 5S genes is attributable to base pair changes at residues +53 and +55 within the internal promoter region (43). These possibilities are now being addressed.

Previously reported results suggest that TFIID directly competes with an H2A/H2B dimer for DNA binding (25, 51, 53, 54). Positioning of the nucleosome with respect to the 5S RNA gene places the 50-bp TFIID binding site (internal promoter region), +45 through +95, near the edge of the histone octamer-DNA interactions (Fig. 1) (25, 41). A region within

the internal promoter necessary and sufficient for high-affinity TFIID binding is exposed in the (H3-H4)₂ tetramer-5S DNA complex. DNA-protein contacts for this complex extend only halfway into the internal promoter region, to position +65, thereby allowing TFIID access to the critical nucleotides located at +80 through +90 within the internal promoter (25). Correspondingly, TFIID binds the tetramer-5S DNA complex with a binding constant nearly equivalent to that observed for binding of naked 5S DNA (25; this study). Thus, competition between an H2A/H2B dimer and TFIID for binding to this critical 10-bp stretch of DNA near the edge of the nucleosome appears to be a major determinant of TFIID binding.

It is formally possible that removal of the H3-H4 tails relieves simple steric hindrance to TFIID binding. In this model, these tails would have to adopt different positions within the tetramer-DNA complex compared to the nucleosome. However, the fact that both the H3 and H4 tails contribute to TFIID access to DNA suggests a more complex mechanism. A dynamic model has been proposed in which the nucleosome undergoes a conformational transition to states allowing access to internal sites (39, 40). Our data suggest that modifications to the H3-H4 tetramer tail domains have a direct consequence for the conformational equilibrium constant for exposure of sites near the edge of the nucleosome. Interestingly, hydroxyl radical footprinting indicates that the apparent extent of histone-DNA contacts within the total population of unbound nucleosomes is unaffected by tail removal (Fig. 7). Note that the tail domains themselves do not contribute to the nucleosomal cleavage pattern produced by hydroxyl radical (22). Thus, removal of the H3 or H4 tails is likely to result in a change in the conformational dynamics undergone by the nucleosome rather than an overt change in the main conformation or configuration within the ensemble of nucleosomes. As a direct consequence of this alteration, steric constraints at the edge positions of the nucleosome would be decreased, allowing competition for binding to the internal promoter to shift in favor of TFIID instead of the H2A-H2B dimer (39). Interestingly, our data show that TFIID binding does not dislodge the H2A-H2B dimer; rather, there is an accommodation of the nucleosome structure to facilitate this binding event. DNA may be displaced from the surface of the H2A-H2B dimer without release of the dimer, since the H2B N-terminal tail would serve to tether the dimer within the complex (34). Moreover, it is possible that only a subset of the nine zinc fingers with TFIID are bound within the triple complex and/or TFIID binding displaces histone-DNA interactions. These possibilities are currently under investigation.

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