Functionally Relevant Histone-DNA Interactions Extend Beyond the Classically Defined Nucleosome Core Region*

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We demonstrate that core histones can affect the accessibility of a DNA element positioned outside of the classically defined nucleosome core region. The distance between a well positioned nucleosome and the binding site for the 5 S-specific transcription factor TFIIIA was systematically varied and the relative binding affinity for TFIIIA determined. We found that core histone-DNA interactions attenuate the affinity of TFIIIA for its cognate DNA element by a factor of 50–100-fold even when the critical binding region lies well outside of the classically defined nucleosome core region. These results have implications for the validity of parallels drawn between the accessibility of general nucleosomes to DNA sequences in chromatin and the activity of actual sequence-specific DNA binding factors.

In eukaryotes, the primary repeating subunit of chromatol is the nucleosome (1, 2). Each nucleosome contains about 200 bp of DNA, an octamer of core histone proteins, (H2A/H2B/H3/H4)4, and a single linker histone. An obvious function of chromatol structure is to bring about the ordered compaction of DNA within the nucleus. In addition, in vitro and in vivo experiments have shown that nucleosomes also play a functional role in both activating and repressing gene activity (2–4). However, in general, the presence of histone-DNA interactions greatly reduces the DNA binding activity of sequence-specific factors that mediate gene transcription (5, 6). Thus, the presence of a nucleosome may effectively reduce or eliminate the occupancy of crucial DNA elements at the concentrations of trans-acting factors found in vivo.

A major question is how transcription occurs within the context of nucleosomes. Recent work has uncovered several strategies employed in eukaryotic nuclei for alleviating nucleosome repression (2, 4). Active expression of certain genes is dependent upon energy-intensive active disruption of histone-DNA interactions outside of the classically defined nucleosome core region. The disintegration of DNA within the nucleus. In addition, the nucleosome core particle DNA is defined by protection from nucleosome core region. However, the full extent of the repressive influence of a single nucleosome core has not been accurately determined. The reach of restrictive histone-DNA interactions is generally considered to be equivalent to ~146 base pairs, the amount of DNA within the nucleosome core region. However, the nucleosome core particle DNA is defined by protection from histone-DNA interactions within a single nucleosome core on the binding of the transcription factor, TFIIIA. We show that the entire cognate element for TFIIIA must be moved well beyond the edge of the nucleosome core region to recover high affinity binding by this factor. The results suggest that histone-DNA interactions 15–20 base pairs beyond the edge of the nucleosome core region have significant consequences that may be relevant for the activation of 5 S RNA genes in vivo.

EXPERIMENTAL PROCEDURES

Construction of Modified 5 S DNAs—The HpaII-XbaI fragment flanked by asymmetric BstXI linkers from pJHXI-BstXI, which contains the Xenopus borealis somatic 5 S RNA gene (22), was prepared and modified by standard three- and four-primer polymerase chain reaction methods. The primers were designed to modify the 5 S DNA fragment to obtain inserts of ~35 base pairs in increments of 5 bp between positions +50 and +51 (with respect to the transcription start nucleotide of 5 S RNA gene, +1). Inserts were designed as tandem repeats of the sequence nucleotide of 5 S RNA gene, +1). Inserts were designed as tandem repeats of the sequence between positions +5 to +55 appended to sequences +51 to +132; the +50 construct has uninterrupted 5 S RNA gene from +102 to +60 appended to sequences +51 to +132, and so on. A fragment containing a 20-base pair insert was not obtained because of the inability to be propagated in bacterial cells. The modified fragments were then restricted with BstXI and cloned into pBSIIa(+)(Stratagene). The HpaII-XbaI fragments were radiolabeled at the 5′ end of either strand with T4 polynucleotide kinase and [γ-32P]ATP by standard methods.

Nucleosome Core Reconstitutions—Nucleosome cores were reconstituted onto radiolabeled DNA fragments either by exchange with stripped chromatin or by dialysis from high salt with purified chicken erythrocyte histones (22). Under no circumstances did we detect differences in results due to the reconstitution methodology employed. In the histone exchange method, a large molar excess (~50-fold) of stripped chromatin was mixed with radiolabeled DNA, and NaCl concentration was adjusted to 1 M. The mix was then incubated for 1 h at room temperature.
temperature. The salt concentration was then diluted to 0.8, 0.6, 0.2, 0.1, and 0.05 M NaCl by adding 10 mM Tris-Cl, pH 8.0. For the salt dialysis method, nonspecific carrier DNA (10 μg), radiolabeled DNA (0.25 μg), and purified core histones (mass ratio histone/DNA = 0.8) were mixed in a total volume of 200 μl in 2.0 M NaCl. The sample was dialyzed for at least 1.5 h against several buffers containing 10 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 1 mM EDTA, and decreasing NaCl concentrations of 1.2, 1.0, 0.8, and 0.6 M. A final dialysis was performed for 12 h against 10 mM Tris-HCl, pH 8.0. After reconstitution, only naked DNA, (H3/H4)2 tetramer-DNA complexes and nucleosomes were detectable by nucleoprotein gel electrophoresis.

Micrococcal Nuclease Mapping—Hpa II-Xba I DNA fragments were internally labeled at the Bst I site as described (22). Nucleosomes or (H3/H4)2 tetramer-DNA complexes were reconstituted as described and then digested for 5 min at room temperature with 0.75, 0.4, 0.2, and 0.1 unit of micrococcal nuclease (Worthington). Cus2+ concentration was adjusted to 0.5 mM concomitantly with addition of micrococcal nuclease. Digestion was terminated with addition of EDTA and SDS. The DNA was recovered by ethanol precipitation and resolved by electrophoresis in non-denaturing 6% polyacrylamide gels. DNA fragments of 147 bp were gel-extracted and restricted with EcoRV to map the termini of the micrococcal nuclease digestion products (24). The digestion products were then resolved by electrophoresis in denaturing gels (25).

Hydroxyl Radical Footprinting—DNA fragments were end-labeled either at the Hpa II site or at the Xba I site. Nucleosome cores reconstituted onto Hpa II-Xba I-labeled fragments were treated with hydroxyl radicals as described, and reactions were quenched with the addition of glycerol to a final concentration of 5% (22). Samples were then directly loaded onto preparative 0.7% agarose gels and electrophoresed for 1.5–2 h at 120 V. Radiolabeled bands were identified by autoradiography of the wet gel; DNA was recovered from the gel and analyzed by denaturing 6% polyacrylamide gels. DNA fragments of 147 bp were gel-extracted and restricted with EcoRV to map the termini of the micrococcal nuclease digestion products (24). The digestion products were then resolved by electrophoresis in denaturing gels (25).

**RESULTS**

We have used hydroxyl radical footprinting to reveal the precise location of histone-DNA interactions within a well-positioned nucleosome assembled on a DNA fragment containing a 5 S RNA gene from *X. borealis* (Fig. 1) (27). Interestingly, the assembly of this DNA fragment into a nucleosome prevents binding of the 5 S-specific transcription factor TFIIIA to the internal promoter of the 5 S gene (18). As previously reported, histone-DNA interactions can be detected well beyond the edge of the nucleosome core region located between positions −70 and +79 as defined by micrococcal nuclease digestion (18, 27). Importantly, these contacts extend through the entire internal promoter and include the crucial +80 to +92 region necessary and sufficient for TFIIIA binding (Fig. 1) (28, 29). To assess more accurately the significance of these histone-DNA interactions with regard to TFIIIA binding, we designed constructs to gradually move the TFIIIA-binding site away from the positioned nucleosome.

To obtain the constructs needed for this study, it was first necessary to locate the DNA element that drives sequence-dependent positioning of the histone octamer. Sequence-dependent conformational variations in B-DNA structure such as variation in minor groove width are reflected in the hydroxyl radical cleavage patterns of naked DNA (30, 31). The hydroxyl radical cleavage pattern of naked Xenopus 5 S DNA contains regions of strikingly periodic and uniform modulation (Fig. 1), reminiscent of the cleavage pattern obtained with stably curved DNA sequences (31, 32). Such DNA structures have been shown to efficiently direct nucleosome positioning (31, 33). Importantly, quantitative densitometry clearly shows that these periodic cleavage modulations directly align with the much larger modulations within the hydroxyl radical cleavage pattern of 5 S DNA assembled into a nucleosome (Fig. 2). Thus, a discrete region within the 5 S DNA has a conformation that yields a hydroxyl radical cleavage pattern similar in nature, but not in magnitude, to the severely curved DNA found within the nucleosome. In addition, the position of this element corresponds to the approximate intranucleosomal location of the TFIIIA-binding site (28, 29). To assess more accurately the significance of these histone-DNA interactions with regard to TFIIIA binding, we designed constructs to gradually move the TFIIIA-binding site away from the positioned nucleosome.

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nucleosome positioning element within the 5 S DNA fragment (Fig. 2).

The putative nucleosome positioning element we identified is located approximately between positions −30 to +50 within the 5 S DNA sequence. This element abuts the TFIIIA-binding site within the internal promoter region located approximately from +50 to +95 (Fig. 2) (28). Thus, we designed constructions of the 5 S DNA fragment to gradually separate the TFIIIA and nucleosome-binding sites by placing insertions between nucleotides +50/+51 (Fig. 3). Modified 5 S DNAs were obtained by standard polymerase chain reaction methods. The length of the inserts was increased in increments of 5 bp to a maximum of 35 bp, except that a plasmid bearing an insert of +20 bp could not be propagated in bacterial cells (Fig. 3). Inserts were designed as tandem repeats of the original sequence in order to provide as little disruption of the 5 S sequence as possible (see “Experimental Procedures”).

We first analyzed if the insertions had any effect on the formation or positioning of nucleosomes reconstituted in vitro. Reconstitutions were carried out such that about 50% of the labeled nucleoprotein complexes were (H3/H4)2 tetramer-DNA complexes, and the other 50% were nucleosomes (Fig. 4). The presence of both complexes was desired in order to provide an internal nucleoprotein control for TFIIIA binding (see below). The identity of each nucleoprotein complex was confirmed by co-migration of purified reconstituted tetramer or octamer in agarose gels (data not shown; see Ref. 22). We next determined the position of the nucleosomes on our 5 S DNA constructs by mapping the limits of the 146-base pair nucleosome core DNA produced during micrococcal nuclease digestion. Micrococcal nuclease digestion was carried out as described under “Experimental Procedures,” and the DNA products were resolved on non-denaturing polyacrylamide gels (Fig. 5). In agreement with the nucleoprotein gel, all constructs were efficiently reconstituted into canonical nucleosomes as evidenced by the production of the 146-bp nucleosome core particle DNA during micrococcal digestion.

The precise position of the nucleosome was determined by recovering the 146-bp DNA from the gel, cleavage with EcoRV at position +31 within 5 S DNA, and analyzing the products on a sequencing gel (Fig. 6). Note that because the DNA is labeled internally at position −23, only one of the two fragments produced by EcoRV digestion of the nucleosome core DNA will be observed (see “Experimental Procedures”). The digestion products indicate that all constructs have nucleosome positioning identical to the unmodified wt fragment. Specifically, two major bands are observed of −100 and 130 nucleotides in length (Fig. 6A). The −100-nucleotide band corresponds to the major nucleosome position mapped previously on the wt 5 S DNA fragment (23, 24). The 130-bp band was unexpected based on earlier mapping studies of octamer-5 S DNA complexes (18, 24). Since an octamer located at this position would be expected to bind TFIIIA even on the wt 5 S DNA construct (see below), we next determined whether this band might be due to the
that this 146-base pair product is an intermediate formed during micrococcal digestion of tetramer-DNA complexes in which trimming has occurred only on the downstream end of the complex. In this case, the predicted position of the downstream edge of the tetramer would be exactly coincident with the position previously mapped in footprinting experiments (24). Nonetheless, the data indicate that the presence of the inserts does not alter the position of the nucleosome with respect to the nucleosome positioning element among the 5 S DNA fragments (Fig. 6B).

We next carried out hydroxyl radical footprinting of the different nucleosome constructs to confirm the mapping results and to obtain a more accurate assessment of the location of histone-DNA interactions (27). Complexes were prepared with DNAs containing a radioactive end label on either strand, cleaved with hydroxyl radicals, and the nucleosomes resolved on preparative nucleoprotein gels. The DNA products were purified and analyzed on sequencing gels (Fig. 7). As evident when the DNA was radioactively labeled at the downstream end of the 5 S fragment, the insertions had the effect of moving the nucleosome footprint progressively further away, by discrete 5-bp increments, from the TFIIIA-binding site (Fig. 7). Conversely, when the label was incorporated at the upstream end of the 5 S DNA fragments, all nucleosome footprints occurred at the same position with respect to the end of the fragment containing the nucleosome positioning element and were qualitatively identical to that observed on the wt fragment (results not shown; see Fig. 1).

The above results indicate that the nucleosome is present at the same location with respect to the nucleosome positioning element in every construct, and thus histone-DNA interactions are gradually retracted from the TFIIIA-binding site in a 3'-5' direction. To determine the effect on 5 S promoter accessibility, we performed nucleoprotein gel shift assays. Complexes reconstituted on each of the 5 S constructs were incubated with increasing amounts of purified TFIIIA in binding buffer, and the resulting products were resolved in agarose gels. As the TFIIIA concentration increased, the intensity of the free DNA band for each construct was reduced, and a new band corresponding to TFIIIA-DNA complex appeared (Fig. 8). Importantly, quantitative titrations with the naked DNA fragments alone showed that the TFIIIA binding affinity was identical for all 5 S constructs (results not shown). Thus, the mid-point in the titration for TFIIIA binding to the naked DNAs in the nucleoprotein experiments occurred when the free concentration of active TFIIIA was $\sim 1 \times 10^{-9}$ M and was used as an internal reference (36, 37). By using the naked DNAs as a reference, an approximate relative dissociation constant for TFIIIA binding to all nucleoprotein complexes was determined by quantitative densitometry with corrections for residual den-
FIG. 7. Hydroxyl radical footprinting of nucleosomes reconstituted with modified 5 S DNA fragments. Nucleosomes were reconstituted with each of the 5 S DNA fragments labeled at the XbaI site, downstream of the 5 S RNA gene (see Fig. 3). The complexes were subjected to hydroxyl radical cleavage, octamer-DNA complexes isolated by nucleoprotein gel electrophoresis, and the cleavage patterns analyzed on sequencing gels as described. Lanes 1–6 show Maxam-Gilbert guanine base-specific reactions done with the +5, +10, +15, +25, +30, and +35 5 S fragments, as markers. Lanes 7–13 show the hydroxyl radical footprints of nucleosomes assembled with the wt, +5, +10, +15, +25, +30, and +35 fragments, respectively. Schematic indicates the location of the 5 S RNA gene coding sequence (black oval); the binding site for TFIIIA (hatched box), the region of DNA in contact with core histone proteins as determined by the footprinting analysis; and the nucleosomal dyad (black oval). The positions of the inserts and the radioactive end label are also indicated. Note that the position of the histone octamer (oval) is shown only for the wt 5 S DNA.

DISCUSSION

We have tested if histone-DNA interactions outside of the classically defined nucleosome core region can significantly modulate the binding of a eukaryotic transcription factor, TFIIIA. This protein has a modular structure, with nine individually folded zinc finger domains (2). These nine fingers are combined into three distinct structural domains containing fingers 1–3, 4–6, and 7–9 (39, 40). Previous work with truncated forms of TFIIIA revealed that 90% of the binding free energy is contributed by interaction of the first three fingers of this protein with 5 S sequences between +80 and +92 in the 5 S gene (28, 29). Thus, a polypeptide containing fingers 1–3 binds DNA with an affinity only 3–4-fold less than the intact protein. About half of the remaining stability is contributed by finger 5 and half by fingers 7–9 (41). Thus, blockage of the binding of fingers 4–9 would cause a loss in affinity of only about 3-fold.

In the case of (H3/H4)2 tetramer-wt 5 S DNA complex, we...
The hypothesized modes of TFIIIA binding to the set of differentially translationally positioned tetramer complexes and nucleosomes are shown. For tetramer complexes (Tet) assembled with the wt and +5 fragments, only fingers 1–3 of TFIIIA can bind to the DNA, whereas fingers 1–6 are bound to DNA in the +10 and +15-tetramer complexes. All fingers of TFIIIA are presumed bound in tetramer complexes assembled with the +25, +30, and +35 5 S DNA fragments. Nucleosomes assembled with the wt, +5, +10, +15, and +25 5 S DNA fragments (Oct) are refractory to TFIIIA binding, whereas fingers 1–3 are bound within the +30 and +35-nucleosome complexes. The translational positions of the tetramers and nucleosomes corresponding to each of the 5 S DNA fragments are indicated. The nine zinc fingers of TFIIIA (small ovals) and the location of fingers 1 and 9 in the model are indicated.

The data indicate that core histone-DNA interactions can have significant effects on accessibility of linker DNA. Furthermore, these results suggest that the region of linker DNA between two neighboring nucleosomes, which is as accessible as naked DNA, is effectively much smaller than previously thought for some transcription factors. Thus strategies in which cis-acting elements are places between nucleosomes may require much more precise positioning than was previously evident or may require concurrent nucleosome disruption activities or histone modifications, such as are thought to be relevant for intra-core DNA binding activities.

Previous experiments have shown that nucleosome positioning on the 5 S DNA fragment at least partly recapitulates the nucleosome positioning observed in vivo (43). Experiments with reconstituted templates showed that the presence of a nucleosome on the X. borealis somatic 5 S rRNA gene efficiently inhibits transcription in Xenopus oocyte extracts, whereas the template could be transcribed in presence of incomplete nucleosome core composed by H3/H4 tetramer (44, 45). Transcription in these experiments is correlated with the apparent ability of TFIIIA to bind to the reconstituted templates (18). The results presented here suggest that nucleosome sliding to expose the TFIIIA-binding site within a linker region between nucleosomes might not be sufficient to allow activation of 5 S genes in vivo since little unrestricted linker DNA is expected to exist between nucleosomes spaced at intervals of approximately 200 base pairs (45). Thus, other factors, such as histone acetylation may play an important role in activation of these genes, concomitant with displacement of histone H1 (19, 46, 47).
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