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Nucleosome Binding by the Polymerase I Transactivator Upstream Binding Factor Displaces Linker Histone H1

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Upstream binding factor (UBF) is a vertebrate RNA polymerase I transcription factor that can bend and wrap DNA. To investigate UBF's likely role as an architectural protein of rRNA genes organized in chromatin, we tested UBF's ability to bind rRNA gene enhancers assembled into nucleosome cores (DNA plus core histones) and nucleosomes (DNA plus core histones plus histone H1). UBF bound with low affinity to nucleosome cores formed with enhancer DNA probes of 162 bp. However, on nucleosome cores which contained ~60 bp of additional linker DNA, UBF bound with high affinity similar to its binding to naked DNA, forming a ternary DNA-core histone-UBF complex. UBF could be stripped from ternary complexes with competitor DNA to liberate nucleosome cores, rather than free DNA, suggesting that UBF binding to nucleosome cores does not displace the core histones H2A, H2B, H3, and H4. DNase I, micrococcal nuclease, and exonuclease III footprinting suggests that UBF and histone H1 interact with DNA on both sides flanking the histone octamer. Footprinting shows that UBF outcompetes histone H1 for binding to a nucleosome core and will displace, if not dissociate, H1 from its binding site on a preassembled nucleosome. These data suggest that UBF may act to prevent or reverse the assembly of transcriptionally inactive chromatin structures catalyzed by linker histone binding.

In the nucleus, genes do not exist as naked DNA but are complexed with histones and a variety of nonhistone chromosomal proteins to form chromatin (14, 15). Nucleosomes are a fundamental unit of chromatin, organizing DNA into bead-like structures ~10 nm in diameter (42). Each nucleosome includes ~146 bp of DNA wrapped ~1.75 times around a histone octamer that is composed of two molecules each of the core histones H2A, H2B, H3, and H4 (23), ~50 to 70 bp of additional linker DNA (73), and one molecule of linker histone, such as histone H1 or a related variant (41, 61). The types and abundance of linker histones expressed in the cell are developmentally regulated and are likely to play a role in regulating genomic expression patterns (12, 22, 74). A number of studies have shown that chromatin composed of core histones allows transcription and that core histones can be displaced by a transcribing polymerase (24, 31, 62, 63). However, linker histone binding decreases the transcriptional potential of chromatin templates in vitro due to stabilization of core histone-DNA interactions (21) and constraints on nucleosome mobility (29, 47, 67). At a structural level, binding of linker histones is also associated with further packaging of 10-nm-diameter chromatin fibers into 30-nm and higher-order chromatin structures thought to contain transcriptionally inert DNA and inactive genes (16, 18, 56, 64, 72, 74). Thus, proteins which interfere with linker histone binding might be expected to preserve the chromatin in a transcriptionally competent state by preventing or reversing higher-order chromatin packaging (15, 43, 44).

Little is known about the regulation of rRNA genes organized in chromatin. In eukaryotes, rRNA genes are arranged in tandem arrays with copy numbers typically in the hundreds to thousands (36, 46, 58). Available evidence based on electron microscopy and psoralen accessibility suggests that not all rRNA genes are active at one time (7, 10, 11, 32). Inactive genes appear to be packed in regular nucleosomal arrays based on their micrococcal nuclease accessibility, whereas the chromatin of active genes appears to be less organized (7). Active rRNA gene chromatin in vertebrates is likely to be influenced by upstream binding factor (UBF), an RNA polymerase I transcription factor localized to the nucleolus, where rRNA transcription and ribosome assembly take place (for a review, see reference 59). UBF shows sequence similarity with high-mobility-group (HMG) proteins (20), an extensive family of abundant nonhistone chromosomal proteins thought to be preferentially associated with active chromatin (17, 28, 43, 51). Like other HMG proteins, UBF apparently interacts with the minor groove of duplex DNA (8), bends and wraps linear DNA fragments (1, 53), and binds structured nucleic acids such as DNA kinked by cisplatin (66) or cruciforms or four-way junctions (8, 19, 26). UBF binds DNA as a dimer (33) and is abundant. Initial estimates suggested ~75,000 molecules of UBF per cell based on estimates of the yield of footprinting activity upon purification to apparent homogeneity, but more-recent estimates based on quantitative Western blotting suggest 10^5 to 10^6 molecules per cultured *Xenopus laevis* cell (34, 59). Assuming 2×10^5 UBF molecules per cell, an approximate stoichiometry of ~100 UBF dimers for each of the ~10^3 rRNA genes in a diploid *Xenopus* or mammalian somatic cell is calculated. Consistent with its high copy number, UBF has multiple binding sites within the intergenic spacers that separate adjacent rRNA genes. These sites include the gene promoter and upstream promoter-proximal repetitive elements known to function as enhancers in *Xenopus* and mouse (5, 27, 35, 49). In *X. laevis*, these occur in blocks of 8 to 12 alternating 60- and 81-bp elements (each 81-bp enhancer includes a complete 60-bp element) (3, 57) to which UBF binds cooperatively with a stoichiometry of one UBF dimer per two enhancers (54). The typical *X. laevis* rRNA gene intergenic spacer contains two to five such blocks of enhancers, possibly accounting

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for 10 to 25 UBF dimers. An additional one or two UBF dimers are thought to bind the gene promoter and each of the two to five duplicated spacer promoters (30, 48), raising the estimate to 15 to 35 UBF dimers bound. Recently, repetitive sequences at the distal 5′ end of the *X. laevis* intergenic spacer (region 0/1) were shown to bind UBF and stimulate transcription (37). Sequences downstream of the transcription start site have also been shown to be footprinted by UBF (30). Therefore, it seems likely that UBF binds throughout the intergenic spacer, perhaps requiring ~50 UBF dimers. UBF is present in high enough copy number to not only saturate these binding sites but also bind throughout much of the coding sequences. These considerations suggest that UBF may play an important structural role in organizing the chromatin structure of rRNA genes.

To test whether UBF binding to rRNA gene sequences is compatible with conventional histone-organized chromatin, we examined UBF's ability to interact with enhancer sequences assembled into nucleosome cores (DNA plus core histone octamer, no linker histone) containing 162 to 222 bp of DNA and nucleosomes (222 bp of DNA plus core histone octamer plus linker histone). We show that UBF binds efficiently to nucleosome cores in a linker DNA-dependent manner to form a ternary complex. UBF outcompetes histone H1 for binding to nucleosome cores and appears to displace, if not dissociate, H1 from nucleosomes. These results suggest that UBF might help maintain rRNA genes in a transcriptionally competent state by inhibiting or reversing their packaging into higher-order chromatin structures dependent on linker histone binding.

MATERIALS AND METHODS

UBF purification. UBF was purified from whole-cell and nuclear extracts of *X. laevis* kidney cell line XLK-2 by sequential chromatography on DEAE, Biorex, and Mono-Q as described previously (48, 54). Fast protein liquid chromatography was used for all chromatographic steps. Peak fractions from the final Mono-Q column dialyzed into UBF storage buffer (50% glycerol, 25 mM HEPES [pH 7.9], 200 mM KCl, 1 mM dithiothreitol [DTT], 0.1 mM EDTA) were used in all DNA and nucleosome binding studies. Typical UBF concentrations in storage buffer were 5 to 10 ng/μl.

DNA probes. The probes used in this study have been described in detail previously (50, 54). Enhancer-bearing plasmid DNA was linearized, 5′ end labeled using gamma-labeled [^{32}P]ATP and T4 polynucleotide kinase, and then cut with a second restriction enzyme to liberate the probe fragment labeled on only one end. Labeled probes were purified by electrophoresis through a nondenaturing 5% polyacrylamide gel followed by elution into 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. Nucleosome cores were reconstituted onto labeled ribosomal DNA (rDNA) enhancer probes by the octamer transfer method (60) as described by Cote et al. (9). Approximately 100 to 150 ng of gel-purified probe DNA was mixed with 4 to 6 μg of HeLa H1-depleted oligonucleosomes in 1 M NaCl in a 20-μl volume. Following incubation at 37°C for 30 min, reconstitution onto labeled probe DNA was achieved by serial dilution to 0.3 M NaCl by adding sequentially 3.6, 7, 9.4, and 26 μl of 50 mM HEPES (pH 7.5)–1 mM EDTA–5 mM DTT–0.5 mM phenylmethylsulfonyl fluoride (PMSF) buffer with 30-min incubations at 30°C between each dilution. A final dilution to 0.1 M NaCl was achieved by the addition of 134 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–0.1% Nonidet P-40–5 mM DTT–0.5 mM PMSF–20% glycerol–100 μg of bovine serum albumin per ml. Assembly of probe DNA into mononucleosome cores was nearly 100% on the basis of analysis of reconstitution reaction products by native polyacrylamide gel electrophoresis. Reconstituted nucleosome cores were purified on 8 to 28% sucrose gradients subjected to centrifugation at 38,000 rpm for 11 h at 3°C in a Beckman SW50L rotor. Gradient fractions were characterized by scintillation counting, native gel electrophoresis, and DNase I digestion to identify peak nucleosome core fractions and assess their quality by the size and periodicity of the DNase I footprints. Peak fractions were used in all experiments.

Gel mobility shift and DNase I and exonuclease III footprinting. Gel mobility shift and DNase I footprinting assays were performed as described previously (48, 54), with minor modifications. Typical gel mobility shift binding reaction mixtures included ~0.2 to 0.5 ng of end-labeled naked DNA or reconstituted nucleosome core probe DNA, 5 to 20 ng of purified UBF or histone H1, 10 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10% glycerol, and 3 to 5% sucrose in a final volume of 20 μl. A 100-ng amount of plasmid DNA was included as a competitor in some reaction mixtures (see figure legends). The binding reaction mixtures were incubated for 30 to 40 min at room temperature and then loaded onto a native 4.5% acrylamide (30:1 acrylamide: bisacrylamide)–50 mM Tris–50 mM borate–0.5 mM EDTA gel and run in the same buffer at 200 V for 2.5 to 3 h at 4°C. The gels were generally transferred to filter paper, dried under a vacuum, and exposed to X-ray film overnight.

For DNase I footprinting (2), the binding reaction mixtures were essentially identical to those described above except that the volume was initially 50 μl. Following the 30- to 40-min incubation period, 50 μl of a 10 mM $MgCl_2$–5 mM $CaCl_2$ solution was added, and then 5 to 10 ng of DNase I (Worthington) was added. A 90-μl volume of stop buffer (20 mM EDTA [pH 8.0], 1% sodium dodecyl sulfate [SDS], 0.2 M NaCl, and 250 μg of yeast tRNA per ml) was added after 60 s of DNase I digestion. Following phenol-chloroform (24:1, vol/vol) extraction, DNA was precipitated with 2 volumes of cold (−10°C) absolute ethanol and recovered by centrifugation at 14,000 × *g* for 15 min at room temperature. The pellets were resuspended in formamide loading buffer, and DNase digestion products were resolved by electrophoresis on an 8 M urea–8% polyacrylamide gel. The gels were transferred to filter paper, vacuum dried, and exposed to X-ray film overnight. The micrococcal nuclease footprinting protocol was essentially identical except that only $CaCl_2$ was added to the binding reaction mixtures, to a final concentration of 3 mM, and digestion was performed with 0.5 to 5.0 U of micrococcal nuclease (Worthington) for 70 s at 25°C.

Footprints of gel-shifted complexes were obtained by combining the two methods (4). Binding reactions and DNase I cleavage were done as described above except that the reactions were scaled up four- to fivefold. DNase digestion was stopped by addition of 1/9 volume of 100 mM EDTA–50 mM EGTA, and samples were loaded immediately on a running 4.5% native polyacrylamide gel. Resolved complexes were electroblotted onto DE-81 paper as for Western blotting (see below). The filter was exposed to X-ray film, and DNA-protein complexes were excised and eluted in 500 μl of buffer (50 mM Tris-HCl [pH 8.0], 1.0 M NaCl, 1 mM EDTA, 0.1% SDS, 10 μg of tRNA per ml) for 1 h at 68°C. After phenol-chloroform extraction and ethanol precipitation, DNA products were subjected to electrophoresis on an 8 M urea–8% polyacrylamide gel and visualized by autoradiography as for the standard footprinting protocol.

Duplicate DNA and protein blots of gel mobility-shifted protein-DNA complexes. Duplicate DNA and protein blots of gel mobility-shifted protein-DNA complexes were obtained essentially as described by Vettese-Dadey et al. (71). The mobility shift gel was soaked for 5 min in transfer buffer (12 mM Tris, 95 mM glycine, 20% methanol, 0.05% SDS), placed on a sheet of 3MM filter paper, and overlaid with a nitrocellulose filter followed by a sheet of 3MM filter paper, a DE-81 filter (Whatman), and again 3MM filter paper. All filters were cut to the size of the gel and presoaked in transfer buffer. Transfer of protein and DNA was accomplished by using a semidry blotting apparatus (Sartorius) at 50 mA for 3 to 4 h or at 25 mA overnight. The nitrocellulose membrane with retained proteins was subjected to conventional Western blotting (65) using as the primary antibody a 1:1,000 dilution of a rabbit polyclonal serum raised against the amino-terminal 328 amino acids of UBF overexpressed in *Escherichia coli*. A horseradish peroxidase-conjugated second antibody was used for chemiluminescent detection of UBF-antibody complexes on X-ray film according to the protocol of the supplier (Amersham). Radioactive probe DNA captured on the DE-81 filter was visualized by direct exposure of the filter to X-ray film.

RESULTS

Assembly of nucleosome cores on *X. laevis* enhancer rDNA. To test UBF's ability to interact with nucleosome cores assembled on DNA fragments of defined lengths, we used tandem *X. laevis* rRNA gene enhancers that have been well characterized with respect to UBF binding (50, 53, 54). These included tandem 81- and 60-bp enhancers in their natural arrangement or tandem 60-bp enhancers (60-2 probes) within repeats of 73 bp due to addition of linker sequences needed for multimerization (50). Homopolymeric 60-n (where n = 2, 4, 8, or 10) repeats are equivalent to natural 81/60 repeats for both enhancer function and UBF binding (50, 54). For the experiments whose results are presented here, the 60-2 enhancer construct was used with various amounts of flanking plasmid polylinker DNA to alter the final size of the probe.

Histone octamers from H1-depleted HeLa nucleosomes were transferred onto labeled enhancer DNA probes by stepwise salt dilution, and reconstituted mononucleosome cores were further purified by sucrose gradient centrifugation. Nucleosome cores displayed reduced electrophoretic mobility compared to that of free DNA (Fig. 1A, compare lanes 1 and 2). DNase I digestion of free DNA and DNA of nucleosome cores revealed the expected footprint on the nucleosome core (Fig. 1B, compare lanes 1 and 2). Protected and nuclease-hypersensitive sites alternate to form a ladder with a period-

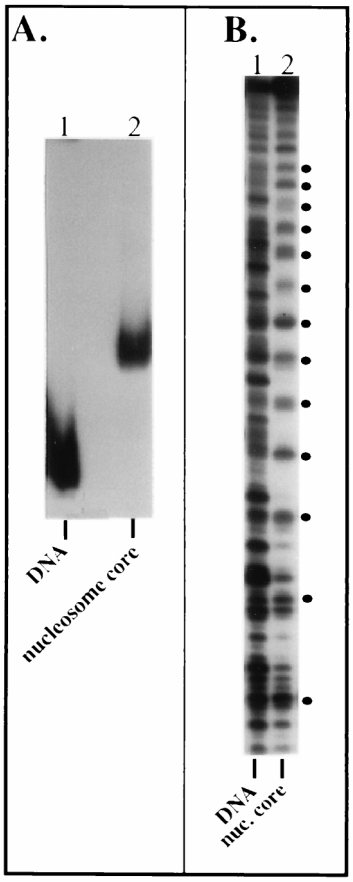


FIG. 1. Assembly of nucleosome cores on tandem rRNA gene enhancers. (A) Free probe DNA (lane 1) and DNA assembled into mononucleosome cores (lane 2) were resolved by electrophoresis on a native 4.5% polyacrylamide gel and visualized by autoradiography. The probe consists of two *X. laevis* rRNA gene enhancers linked head-to-tail and flanked by ~75 bp of plasmid polylinker DNA. (B) Limited DNase I digestion profiles of free (lane 1) and nucleosome core (lane 2) DNAs. Digestion products were resolved on an 8% denaturing polyacrylamide gel and visualized by autoradiography. Nucleosome core assembly results in altered digestion over an ~150-bp region of the probe, causing a ladder of hypersensitive sites at ~10-bp intervals (dots) alternating with sequences protected from digestion. The strong phasing and extent of the footprint suggest that the histone octamer is positioned nonrandomly. Though the DNase footprint is subtle at either end of the region protected by the histone octamer, exonuclease III and micrococcal nuclease footprinting support the location of the histone octamer as depicted by the dots (see also Fig. 6).

icity of 10 to 11 bp due to the rotational phasing of the DNA helix on the surface of the histone octamer (Fig. 1B, lane 2). This repeating pattern of DNase I cutting was not observed with naked DNA (Fig. 1B, lane 1). The regular periodicity of the nucleosome digestion profile suggests that the histone octamer is nonrandomly located and occupies either a single translational position (position relative to the ends of the fragment) on the probe or several translational positions that are in phase with one another. The same DNase I cleavage pattern was obtained with nucleosome cores stored at 4°C for several days, suggesting their stable assembly.

Interaction of UBF with enhancer DNA assembled into nucleosome cores. UBF binds to naked enhancer DNA or to nucleosome cores assembled on the same probe to severely reduce their electrophoretic mobility through native polyacrylamide gels (Fig. 2A, lanes 2 and 4, respectively). The UBF-DNA complex has a greatly reduced mobility compared to that of a histone octamer assembled on the same probe to form a nucleosome core (Fig. 2A, compare lanes 2 and 3). The greater mobility of the mononucleosome core probably reflects two things. First, the histone octamer has a lower mass (~110 kDa) than a UBF dimer (~170 kDa). Second, the nucleosome core is probably more compact than a UBF-DNA complex, given that the DNA is wrapped almost twice around the core histone octamer but a DNA fragment of similar length is wrapped only once by a UBF dimer (1, 53). The second effect (wrapping density) probably exerts the greater influence on mobility. Interestingly, UBF incubated with the mononucleosome core probe resulted in the appearance of a UBF-dependent complex with a gel mobility slightly higher than that of a complex of UBF and free DNA (Fig. 2A, compare lanes 2 and 4). Full-length recombinant UBF expressed in a baculovirus system (the generous gift of Brian McStay, Dundee, Scotland) yielded the same result (data not shown), arguing against the

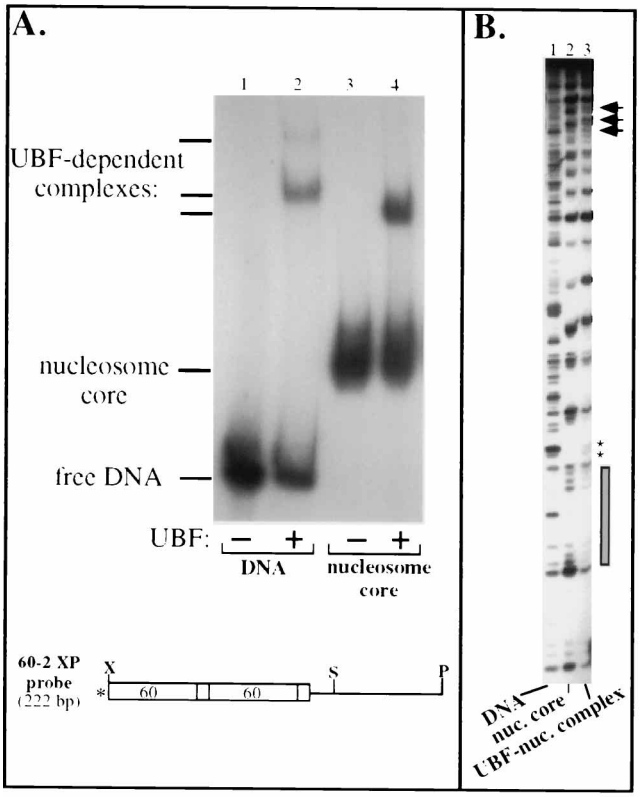


FIG. 2. (A) Interaction of UBF with free DNA or nucleosome cores results in protein-DNA complexes with similar gel mobilities. Approximately 10 ng of purified UBF was incubated with the 222-bp 60-2 XP enhancer probe in its free (lane 2) or nucleosome core (lane 4) form, and the resulting complexes were resolved by native polyacrylamide gel electrophoresis. Note that the UBF-dependent complex formed with nucleosome cores (lane 4) has a slightly higher mobility than the major (lower) complex formed between UBF and free DNA (lane 2). Lanes 1 and 3 are controls showing the mobility of free DNA or nucleosome cores in the absence of UBF. The lowest-mobility UBF-DNA complex at the top of lane 2 results from weak binding of a second UBF dimer. The 60-2 XP probe used in all experiments is diagrammed at the bottom. The repeating unit of the probe is 73 bp, consisting of a complete 60-bp enhancer and linker sequences (gray boxes). (B) Combined gel shift and footprinting shows that UBF-dependent nucleosome core complexes have DNase I footprints similar to those of nucleosome cores alone. Lane 1, DNase I digestion pattern of naked DNA not complexed with histones or UBF; lane 2, digestion profile of the nucleosome core DNA in the absence of UBF; lane 3, footprint of a UBF-dependent nucleosome core complex eluted from a mobility shift gel (DNase treatment occurred prior to running of the mobility shift gel). UBF-induced hypersensitive sites (asterisks and arrows) and the UBF-footprinted region (bar) are indicated.

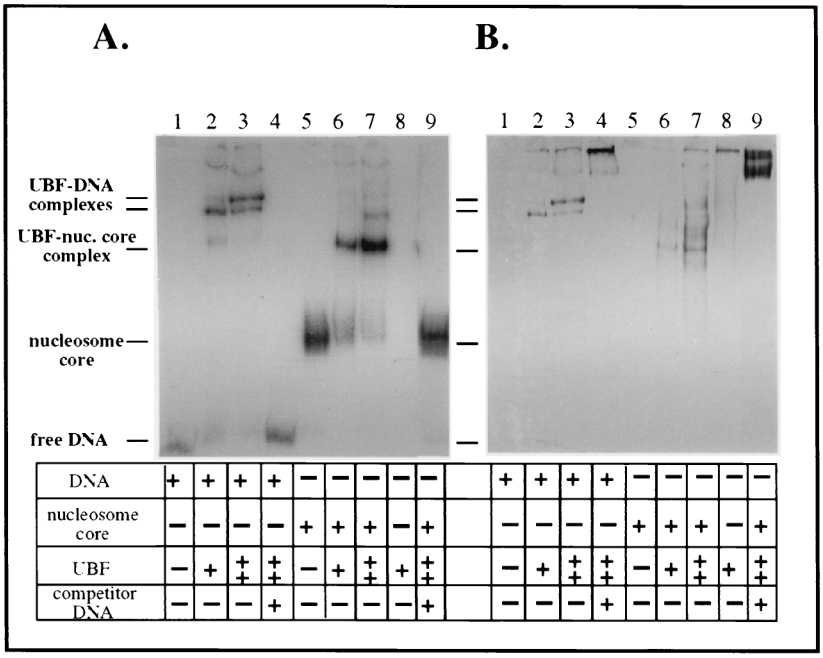


FIG. 3. UBF forms a ternary complex with the nucleosome core without displacing the histone octamer. DNA and protein from a single mobility shift gel were transferred to DEAE (DE-81) and nitrocellulose filters to produce the images shown in panels A and B, respectively. The DNA blot on the DE-81 filter was visualized by autoradiography. The protein blot on nitrocellulose was incubated with polyclonal antiserum raised against recombinant UBF and visualized by using a secondary antibody and enhanced chemiluminescent detection. A total of 10 (+) or 20 (++) ng of purified UBF was incubated with free 60-2 XP probe DNA (lanes 2 to 4) or 60-2 XP probe DNA assembled into a nucleosome core (lanes 6 to 9) for a total of 40 min prior to loading of the gel. In lane 4, 100 ng of competitor plasmid DNA was included in the binding reaction mixture. In lane 9, 100 ng of competitor DNA was added for the final 20 min, after UBF and the nucleosome core had interacted for 20 min. Lanes 1, 5, and 8, controls revealing the relative migration of free probe, nucleosome cores, and UBF on the native gel. nuc., nucleosome.

possibility that contaminating proteins in the UBF preparation affected binding.

The UBF-dependent complex formed on the nucleosome core was examined by a combined gel shift and footprinting assay. In this technique, binding reaction mixtures were treated with DNase I prior to resolution of the protein-DNA complexes on a native gel. The nicked DNA within shifted complexes was then purified and subjected to electrophoresis on a denaturing gel. As shown in Fig. 2B, comparison of the naked DNA (lane 1) and nucleosome core DNA (lane 2) reveals the characteristic ladder of hypersensitive sites and protections in the latter. Interestingly, the footprint of the UBF-shifted nucleosome core complex was very similar to that of the nucleosome core alone (Fig. 2B, compare lanes 2 and 3). However, subtle differences were apparent, including several new hypersensitive sites (Fig. 2B, asterisks and arrows; the latter region is better seen when the opposite strand is labeled, as is shown below [see Fig. 5B]) and weak protection of sequences proximal to the labeled end of the 60-2 XP probe. These results suggest that UBF does not dramatically alter the structure of a nucleosome core but does have a measurable effect.

The fact that UBF did not drastically alter the footprint of the UBF-dependent nucleosome core complex suggested that UBF did not dislodge or dissociate the core histones from the nucleosome. Thus, the decreased mobility of the UBF-dependent complex relative to that of the nucleosome core suggested that the former might be a UBF-histone octamer-DNA ternary complex (Fig. 2A, lane 4). A plausible alternative was that UBF interacted transiently with the nucleosome core to alter its footprint and gel mobility. To address these possibilities, we used the gel mobility shift assay to resolve UBF-dependent complexes and combined DNA competition and Western blotting of these complexes to deduce their compositions (Fig. 3). The logic was that if UBF displaced the histones from a nucleosome core probe, stripping the UBF from the probe by competition with an excess of unlabeled competitor DNA would release free DNA. In contrast, if the UBF-dependent supershift of the nucleosome core was due to ternary-complex formation without disruption of core histone binding, removing UBF from the complex would release the nucleosome core, which would migrate at its characteristic position in the gel. To follow UBF among the shifted complexes in this experiment, the complexes in the native polyacrylamide gel were transferred electrophoretically to back-to-back nitrocellulose and DEAE filters. Proteins were bound to the nitrocellulose, but double-stranded DNA passed through the nitrocellulose and was retained on the DEAE filter. The DEAE filter was exposed to X-ray film to obtain an autoradiographic image of the DNA blot (Fig. 3A). The nitrocellulose filter, in turn, was probed with a polyclonal antibody against *Xenopus* UBF and was developed like a conventional Western blot by using chemiluminescent detection on X-ray film (Fig. 3B).

DNA incubated with UBF resulted in one or two (at higher UBF concentrations) UBF-dependent complexes, as deduced from the shift in the mobility of the labeled DNA probe (Fig. 3A, lanes 2 and 3) and the immunological detection of UBF on the replica Western blot at precisely the same location as the shifted DNA (Fig. 3B, lanes 2 and 3). Inclusion in the binding reaction mixtures of 100 ng of unlabeled enhancer-bearing plasmid DNA as a competitor prevented UBF from binding the labeled probe appreciably, so that all probe DNA ran at the position of free DNA (Fig. 3A, lane 4) and all UBF detected in the Western blot was found at the top of the gel, presumably associated with the large (~3-kb) plasmid DNA (Fig. 3B, lane 4). DNA assembled into a nucleosome core migrated more slowly than free probe DNA (Fig. 3A, lane 5), and no cross-

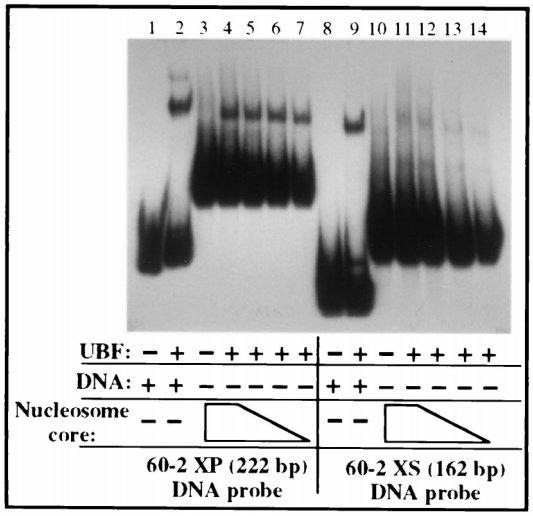


FIG. 4. UBF requires linker DNA for nucleosome core binding. Enhancer probes 60-2 XP (lanes 1 to 7) and 60-2 XS (lanes 8 to 14) were end labeled to the same specific activity and tested for their ability to interact with UBF (~10 ng) as free DNA (lanes 2 and 9) or nucleosome cores (lanes 3 to 7 and 11 to 14) by gel mobility shift assay. In lanes 4 to 7 and 12 to 14, decreasing amounts of nucleosome cores (fourfold range) were present in the UBF binding reaction mixtures. Lanes 1 and 8, controls showing the relative migration of the linear DNA probes; lanes 3 and 10, controls showing the migration of nucleosome cores in the absence of UBF. Note that UBF binds with similar affinities to the free probes and the nucleosome core formed by using the longer (222-bp) probe (lanes 2, 9, and 4 to 7).

reaction between the anti-UBF antiserum and the histones of the nucleosome core was observed (Fig. 3B, lane 5). Addition of UBF to nucleosome cores again resulted in mobility-shifted complexes that had UBF associated with them (Fig. 3, lanes 6 and 7). Addition of competitor DNA to UBF-nucleosome core binding reaction mixtures after UBF had bound virtually abolished the appearance of shifted probe complexes (Fig. 3A, lane 9), resulting in probe DNA migrating at the position of the nucleosome core rather than at the position of free DNA. Likewise, all UBF was detected at the top of the gel, presumably associated with the competitor DNA (Fig. 3B, lane 9). A final control shows that UBF loaded on the gel in the absence of DNA forms a smear but does not migrate to a discrete position (Fig. 3B, lane 8). Collectively, the data of Fig. 2 and 3 suggest that UBF interacts with nucleosome cores to form a ternary complex. UBF can be stripped from such ternary complexes with competitor DNA, resulting in the release of the nucleosome core rather than free probe DNA (Fig. 3, lanes 9). The possibility that UBF causes disruption of core histones that can rapidly reassemble on the probe when UBF is removed is unlikely in the presence of excess competitor DNA.

Interaction of UBF with a nucleosome core depends on linker DNA. We addressed whether UBF interacts with the histones and/or DNA of the core particle or if additional linker DNA is required. To do this, we tested 60-2 probes of various lengths, ranging from 146 to 222 bp, assembled with core histone octamers. Figure 4 shows the results for two such probes, 60-2 XS and 60-2 XP (the relative locations of the *Sma*I and *Pvu*II sites are shown in Fig. 2A). These DNA fragments differ by ~60 bp due to inclusion of additional plasmid polylinker sequences in 60-2 XP.

Certain DNA binding characteristics of UBF should be mentioned for consideration of the data of Fig. 4. UBF is a highly sequence-tolerant DNA-binding protein for which no consensus binding site can be defined (8); therefore, the translational position of a discrete binding site relative to the surface of the histone octamer is not critical. Though it does not require specific sequences, UBF displays a profound length requirement for DNA binding (54). A UBF dimer binds maximally to linear DNA probes of ~140 bp, will not bind linear fragments shorter than ~60 bp, and binds only weakly to linear probes of 60 to 90 bp (54). However, UBF can bind to four-way junctions in which the duplex arms are only ~15 bp long (8, 19, 26), suggesting that prebent DNA circumvents the length requirement observed with linear duplex DNA molecules. We suspect that the minimal length of linear duplex DNA that can be bent and wrapped by UBF explains the length requirement (53).

UBF binds with similar affinities to free 60-2 XP and 60-2 XS probe DNAs, as expected, given that both are longer than 140 bp (Fig. 4, lanes 2 and 9, respectively). The highest faint shifted complex visible in lane 2 (and seen also in Fig. 2 and 3) results from the possibility of weak binding of a second UBF dimer to linear probes longer than 200 bp (strong binding to two UBF dimers requires 250 to 280 bp of DNA) (54). Upon assembly of the 60-2 probes with core histones, UBF still binds efficiently to nucleosome cores assembled on the 222-bp probe (Fig. 4, lanes 4 to 7), forming a single ternary complex, but binding to the cores assembled with 162 bp of DNA was barely detectable (lanes 11 to 14). Note that the amount of UBF was held constant in Fig. 4, while nucleosome core amounts varied over a fourfold range in lanes 4 to 7 and in lanes 11 to 14. Because the amount of UBF-dependent ternary complex was largely independent of the nucleosome core concentration, this suggests that UBF was limiting in these reactions. Therefore, comparison of ternary-complex formation on the two probes should reflect the relative affinities of UBF for the two types of chromatin structures. The data suggest an ~20-fold preference for nucleosome cores with an extra 60 bp of linker DNA. Furthermore, comparison of UBF binding to the linear or nucleosome core versions of the 222-bp probe suggests that UBF's affinity for nucleosome cores is similar to that for naked DNA (Fig. 4, compare lanes 2 and 7, in which similar amounts of DNA are present in the reaction mixtures). The slightly better UBF binding to the linear form of the probe is likely to reflect the fact that binding in this case is partially cooperative due to the possibility of loading two UBF dimers (54), accounting for the two shifts visible in Fig. 4, lane 2, whereas the single UBF-shifted complex visible with the nucleosome core probe suggests that UBF binding in this case is probably noncooperative.

One possible explanation for strong binding of UBF to the 222-bp, but not the 162-bp, nucleosome core probe is that UBF binds to DNA extending out from the region in close contact with the histone octamer. If it is assumed that the octamer interacts with ~146 bp of DNA at one extreme end of the probe, a maximum of ~76 bp of free linear duplex DNA would be available on the other end. UBF can bind to duplex DNA of this length, but only weakly, as mentioned above (54). Furthermore, the footprinting data of Fig. 1 and 2 suggest that the histone octamer is positioned approximately in the center of the probe, rather than at one end. Therefore, ~30 to 40 bp of duplex DNA is expected to protrude from each side of the nucleosome core when the 222-bp probe is used. Though UBF cannot bind linear DNA molecules that are this short, recall that UBF will bind four-way junctions in which the duplex arms are ~15 bp long (8). These artificial cruciforms are thought to have arms spaced so that they mimic the juxtaposition of DNA entering and exiting a nucleosome core, and, in fact, linker histone H1 binds efficiently to four-way junction DNA in vitro (70, 74). On the basis of these considerations, the strong linker-dependent binding of UBF in Fig. 4 suggested that UBF might

bind to two short segments of linker DNA on either side of the histone octamer. Footprinting data (below) support this hypothesis.

UBF displaces or dissociates linker histone H1 from nucleosome cores. The linker requirement for UBF binding to nucleosome cores suggested that UBF might compete with linker histone binding. Purified histone H1 was included in the nucleosome reconstitution reaction mixture or was added directly to nucleosome cores under low-salt conditions identical to those used for UBF-nucleosome core interaction experiments (69). The two methods yielded equivalent mononucleosomes that displayed slightly decreased gel mobilities relative to that of nucleosome cores (Fig. 5A, compare lanes 3 to 6 with lane 2), as expected on the basis of other published reports (68, 69). Whereas nucleosome cores left ~35 bp of DNA accessible to exonuclease III on both ends (again indicating that the histone octamer was positioned almost exactly in the middle of the probe), reconstitution of histone H1 caused protection to be extended ~15 bp on both ends of the probe (data not shown), consistent with the expected properties of a properly assembled nucleosome (39).

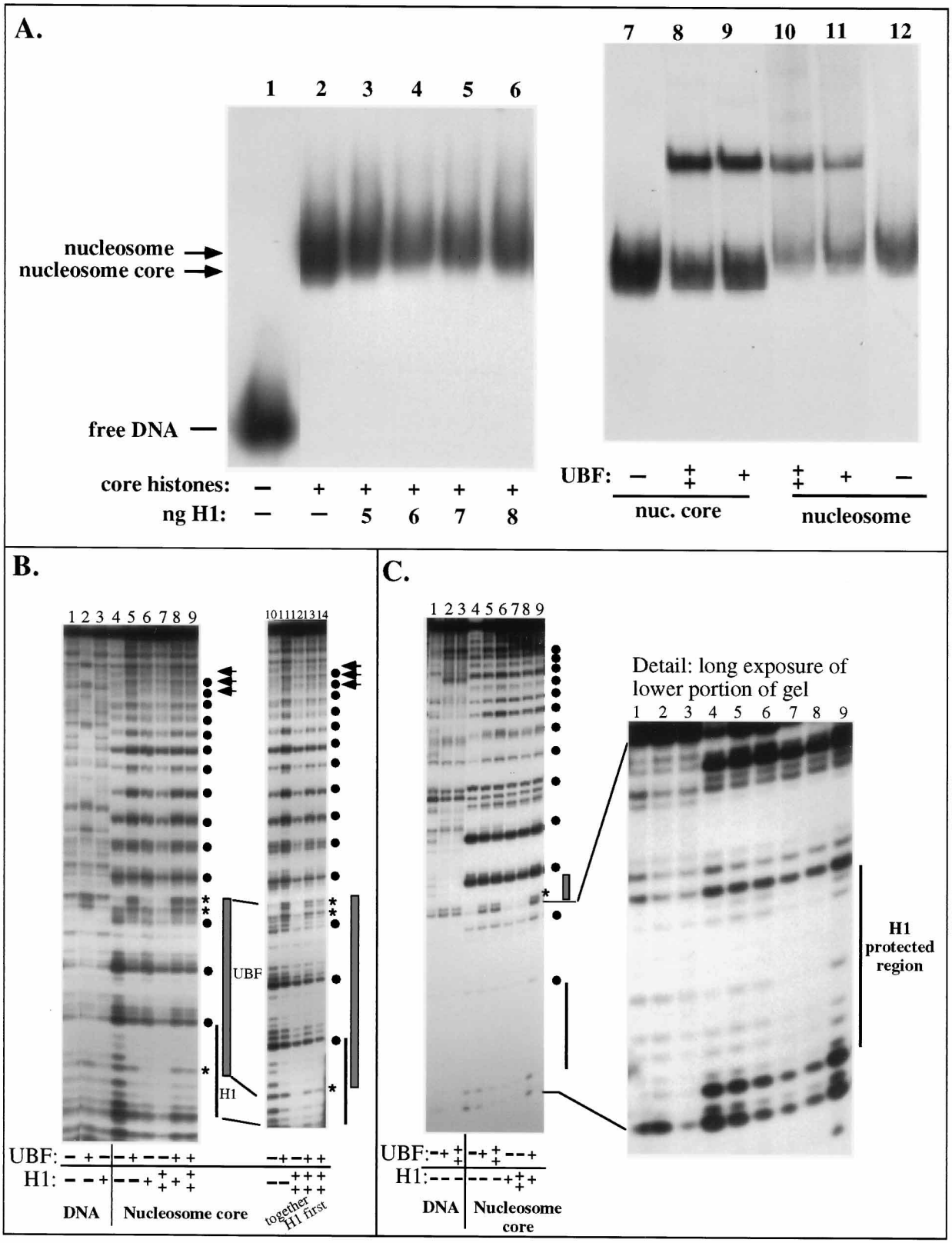
UBF interacted with reconstituted nucleosomes to cause a shifted complex whose mobility was the same as that of a UBF-nucleosome core complex (Fig. 5A, compare lanes 10 and 11 with lanes 8 and 9). This suggested that UBF might dissociate and replace histone H1 upon binding to a nucleosome. Unfortunately, we were unable to demonstrate the fate of H1 conclusively using the gel mobility shift assay and Western blotting approach, as in Fig. 3, due to the failure of anti-H1 antibodies to cross-react with native (nondenatured) H1 in this assay (73a). An alternative approach we tried was to strip UBF from the nucleosome-UBF complexes to see if complexes with the mobility of nucleosome cores (without H1), rather than nucleosomes (with H1), would be recovered. However, the competitor DNA also stripped H1 efficiently from nucleosomes in the presence or absence of UBF, so that nucleosome cores were recovered in either case (data not shown). Thus, another approach was needed.

Histone H1 binding to nucleosome cores and UBF-H1 competition were both visualized by DNase I footprinting (Fig. 5B and C). As shown in Fig. 5B, lane 4, core histones interact with central sequences of the 60-2 XP probe, causing the characteristic 10-bp periodic ladder of hypersensitive sites and protected regions (compare to the naked-DNA digestion pattern in lane 1). Addition of UBF altered the DNase digestion pattern on the flanks of the region interacting with the histone octamer (Fig. 5B, lane 5), causing the appearance of hypersensitive sites as well as footprints proximal to the labeled end of the probe. These alterations matched those observed previously with the same probe in the combined gel shift-footprinting assay (Fig. 2B). Note that the UBF-dependent hypersensitive sites (Fig. 5B, asterisks) occur at the same positions on free and nucleosome core probes (compare lane 2 to lanes 5, 8, and 9). Histone H1 altered the DNase digestion profile of the nucleosome core in regions overlapping those contacted by UBF (Fig. 5B, lanes 6 and 7), inducing new hypersensitive sites (arrows; this region is clearer at the bottom of Fig. 5C when the other strand is labeled) and protecting sequences proximal to the labeled end of the probe. In contrast, H1 did not alter the pattern of naked-DNA digestion in this region (Fig. 5C, lane 3). Interestingly, incubation of UBF and H1 together yielded the digestion pattern typical of a UBF-nucleosome core complex, eliminating all H1-induced footprints and hypersensitive sites. This suggests that UBF displaced H1 from its binding sites and possibly dissociated H1 from the nucleosome core entirely. It is noteworthy that substoichiometric molar quantities of UBF relative to H1 were used in this experiment (5 ng of ~21-kDa H1 in competition with ~20 ng of ~85-kDa UBF represents an ~2:1 molar ratio of histone H1 monomers to UBF dimers).

Next, we tested whether UBF could displace H1 from a nucleosome. Controls in which nucleosome cores alone or nucleosome cores plus UBF or histone H1 were incubated and then subjected to DNase I digestion were made (Fig. 5B, lanes 10 to 12). In lane 13, UBF and histone H1 were incubated together with the mononucleosome core, but in lane 14, histone H1 was allowed to bind first to form a nucleosome and then UBF was added. In both lanes 13 and 14 of Fig. 5B, the characteristic histone H1-induced changes in the digestion profile were not apparent following addition of UBF. Instead, only the characteristic UBF-induced changes to the footprint were observed, indicating that UBF had displaced, and possibly dissociated, histone H1 on the nucleosome.

To better examine the interactions of UBF and H1 with the region of the probe indicated at the top of Fig. 5B, we labeled the opposite strand and repeated the competition experiment (Fig. 5C). UBF footprints on naked DNA are shown in lanes 2 and 3 (compare to the control in lane 1). Again, the nucleosome core footprints in the absence (lane 4) and the presence (lanes 5 and 6) of UBF were very similar. However, UBF induced a strong hypersensitive site. Histone H1 footprinted a region in Fig. 5C which was relatively insensitive to DNase digestion and thus required a long exposure to be visualized (expanded detail in Fig. 5C). Incubation of the nucleosome core with both UBF and histone H1 again resulted in a pro-

FIG. 5. UBF outcompetes histone H1 for nucleosome core and nucleosome binding. (A) Assembly of nucleosome cores into nucleosomes and ability of UBF to interact with both as demonstrated by gel mobility shift assay. Lanes 1 and 2 show the mobility of free DNA and nucleosome cores assembled on the 222-bp 60-2 XP probe. Addition of 5 to 8 ng of purified histone H1 to nucleosome cores causes a slight shift (decrease) in mobility (lanes 3 to 6) indicative of nucleosome formation. Lane 1 is from the same gel as lanes 2 to 6. UBF (+, 5 ng; ++, 10 ng) binds to both nucleosome cores (lanes 8 and 9) and nucleosomes (lanes 10 and 11) to produce shifted complexes with the same mobility. (B) DNase I digestion of the top strand of DNA. In lanes 4 to 9, nucleosome cores assembled on the 60-2 XP enhancer probe were incubated with ~10 ng of UBF (lanes 5, 8, and 9) or histone H1 [6 (+) or 12 (++) ng; lanes 6 to 9] alone or together prior to subjection of protein-DNA complexes to DNase I digestion. The region of the probe interacting with core histones (compare lane 4 to lane 1) is shown with the hypersensitive sites spaced at ~10- to 11-bp intervals indicated (dots). The regions where both UBF and H1 produce footprints on the nucleosome core DNA are indicated. UBF-induced hypersensitive sites (asterisks) occur at the same sites in nucleosome core (lanes 5, 8, and 9) and free DNA (lane 2). Sites where both UBF and histone H1 alter the positions of hypersensitive sites flanking the region protected best by the histone octamer are indicated (arrows; this region is best seen at the bottom of panel C when the opposite strand is labeled). UBF footprints proximal to the labeled end of the probe are also shown (gray vertical bar). From another experiment, digestion patterns of nucleosome core DNA, nucleosome core plus UBF, and nucleosome core plus H1 are shown (lanes 10 to 12, respectively). The digestion patterns following incubation of nucleosome cores with UBF and H1 together for 40 min (lane 13) and after addition of UBF for the final 20 min after H1 had been allowed to prebind for 20 min to form a nucleosome (lane 14) are also shown. Only the UBF footprint is visible in lanes 13 and 14. (C) DNase I digestion of the bottom strand. UBF displacement of H1 is also apparent on the opposite DNA strand of the probe. A total of 10 (+) or 20 (++) ng of UBF or 6 (+) or 12 (++) ng of histone H1 was incubated with naked DNA or nucleosome cores (lanes 2, 3, 5, 6, 7, and 8). Addition of UBF and H1 together results in a footprint resembling that obtained with UBF alone (lane 9). A strong hypersensitive site induced by UBF (asterisk) and the H1-footprinted region proximal to the labeled end of the probe, visible upon long exposure (black vertical bar) (see expanded detail), are indicated.


A.
1 2 3 4 5 6
7 8 9 10 11 12
nucleosome
nucleosome core
free DNA
core histones: – + + + + +
ng H1: – – 5 6 7 8
UBF: – + + + + –
nuc. core
nucleosome
B.
1 2 3 4 5 6 7 8 9
10 11 12 13 14
UBF
H1
UBF: – + – – + – – + +
H1: – – + – – + + + +
– + – + +
– – + + +
DNA
Nucleosome core
together
H1 first
C.
1 2 3 4 5 6 7 8 9
Detail: long exposure of lower portion of gel
1 2 3 4 5 6 7 8 9
H1 protected region
UBF: – + + – + + – – +
H1: – – – – – – + + +
DNA
Nucleosome core

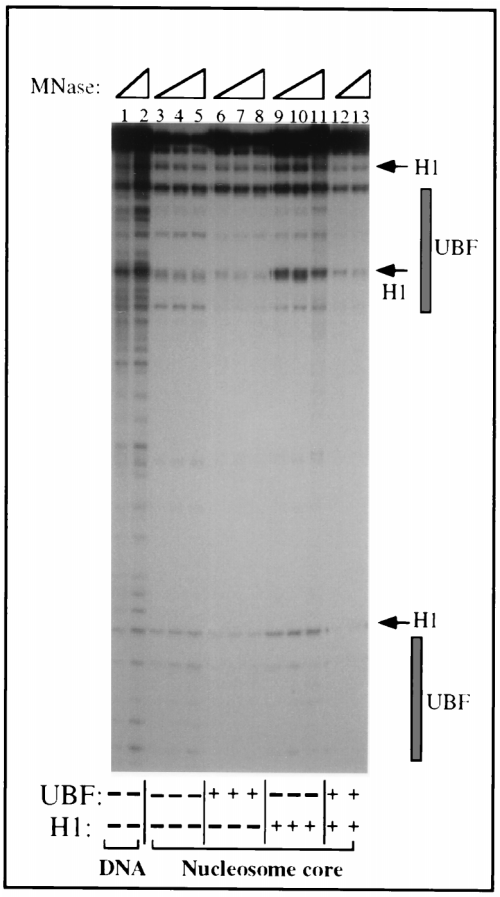


FIG. 6. Micrococcal nuclease footprinting of nucleosome cores assembled on the 222-bp 60-2 XP probe. The labeled strand (top strand) is the same as in the experiment whose results are shown in Fig. 5B. Free DNAs digested at two concentrations (0.4 and 1 U) of micrococcal nuclease are shown (lanes 1 and 2, respectively). Assembly of the probe into nucleosomes cores resulted in extensive protection of the DNA (lanes 3 to 5; 0.4, 1.0, and 2.5 U of nuclease, respectively). Incubation of 10 ng of UBF with nucleosome cores resulted in additional protection at the top and bottom (vertical bars) (lanes 6 to 8; same enzyme amounts as in lanes 3 to 5). Incubation with histone H1 (6 ng) resulted in the enhanced cleavage of several sites (arrows) but no additional footprints (lanes 9 to 11; 1.0, 2.5, and 5.0 U of nuclease, respectively). Incubation of UBF and H1 together (lanes 12 and 13; 2.5 and 5.0 U of nuclease, respectively) resulted in changes to the nucleosome core footprint identical to those caused by UBF alone. Similar results were obtained when the opposite strand was labeled (not shown). MNase, micrococcal nuclease.

tection pattern equivalent to that of UBF alone (Fig. 5C, lane 9).

The DNase I footprinting data of Fig. 5 suggest that UBF, like histone H1, interacts with DNA on both flanks of the histone octamer. This was also made apparent by exonuclease III footprinting which showed that UBF and H1 protected similar amounts of DNA on each end of the nucleosome core probe from exonuclease digestion (data not shown). Micrococcal nuclease footprinting also revealed UBF footprints overlapping the edges of the region protected by the histone octamer (Fig. 6, compare lanes 6 to 8 with lanes 3 to 5). Unlike the case with DNase I, H1 did not protect any sequences from micrococcal nuclease digestion (Fig. 6, lanes 9 to 11) but did cause several sites to be hypersensitive to digestion (arrows). In agreement with the results shown in Fig. 5, addition of both UBF and H1 resulted in a micrococcal nuclease digestion pattern identical to that produced by UBF alone (Fig. 6, lanes 12 and 13).

DISCUSSION

The data of Fig. 5 and 6, obtained by using two different nuclease protection assays, suggest that UBF will displace histone H1 when the two are in competition for binding to a nucleosome core. It seems likely that UBF completely dissociates H1 from the nucleosome, explaining the complete absence of footprints or changes that could be attributed to H1. This is reflected in the working model shown in Fig. 7, which is consistent with our findings to date. In the model, both UBF and histone H1 require linker DNA for nucleosome binding (Fig. 7A) and interact with similar sequences flanking the histone octamer. UBF outcompetes H1 for binding to nucleosome cores. Likewise, if H1 is allowed to prebind to form an intact nucleosome, UBF is still able to gain access, displacing (and probably dissociating) histone H1 (Fig. 7B).

In a previous study, it was shown that relatively short (280- to 560-bp) linear enhancer DNA fragments incubated with UBF and T4 DNA ligase were converted into closed circular DNA molecules containing as many as three positive supercoils (53). Though positive supercoiling could result from overtwisting of the DNA, the simplest explanation is that UBF wraps the DNA in a right-handed direction, completing 360° approximately once every ~140 bp (two enhancers). Consistent with the latter interpretation, structural studies of rat UBF using high-resolution electron microscopic imaging suggest that the UBF dimer resembles a U in which the free ends are offset to form a right-handed ramp (38). If the DNA follows the contour of the dimer, one would expect the DNA to be wrapped in a right-handed direction. Importantly, DNA is wrapped in a left-handed direction around the histone octamer, completing 1.75 turns for ~146 bp of DNA. Therefore, it was hypothesized in a previous paper that UBF binding

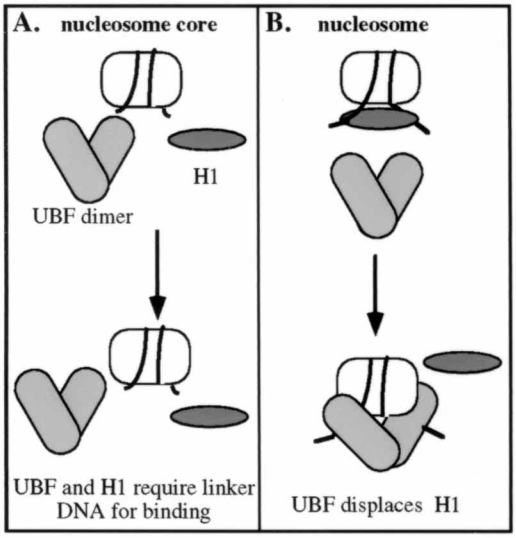


FIG. 7. Illustration of the linker dependence of UBF-nucleosome interactions and the ability of UBF to displace histone H1 on linker DNA. On the basis of our footprinting data, H1 is suggested to interact with the nucleosome near the DNA entry and exit points without contacting DNA at the dyad axis of the core particle (52, 74). For simplicity, H1 is suggested to be displaced and dissociated entirely from the nucleosome core by UBF, explaining the loss of all H1 footprints. However, complete dissociation of H1 has not yet been rigorously demonstrated in our study.

might disrupt assembled nucleosomes by wrapping the DNA in the opposite direction, thereby unwrapping the DNA from the core histones (53). In retrospect, this hypothesis was naive, given the fact that nucleosomes can be assembled on both negatively and positively supercoiled DNA and are not appreciably unfolded on the latter (6). Nonetheless, the SWI/SNF chromatin remodeling complex was shown to possess some of UBF's DNA binding characteristics, including sequence-tolerant, DNA length-dependent binding, binding to DNA cruciforms, and the ability to induce positive supercoils in DNA, suggesting that UBF's effects on nucleosome structure should be examined directly (55). The current study suggests that unlike SWI/SNF, UBF binds to nucleosome cores without significantly disrupting their structure. Noting that chromatin disruption by SWI/SNF requires ATP, we performed analogous experiments with UBF-nucleosome core complexes. No release of free DNA was observed in the gel mobility shift assay, nor was any change detected in nucleosome DNase I digestion patterns upon addition of ATP (data not shown). This is not unexpected, as UBF does not contain a putative ATPase domain analogous to that found in the SWI 2 subunit. However, it is interesting to speculate that nucleosome binding by UBF may play a role in targeting nucleosome-disrupting complexes to rRNA genes transcribed by RNA polymerase I.

UBF's ability to interact with nucleosomal DNA is consistent with results obtained with several other HMG proteins. For instance, a recent report examining HMG 1-nucleosome interactions suggested that HMG 1 competes with histone H1 for binding to linker DNA (40). HMG 1 and H1 have also been shown to compete for binding to four-way junctions (70) thought to mimic the arrangement of DNA entering and exiting the nucleosome. UBF also binds efficiently to four-way junctions (8, 19, 26). As with HMG 1 (40), UBF does not appear to affect the digestion pattern of DNA in the center of the nucleosome core but accesses DNA near the boundary with flanking linker DNA. HMG 17 and HMG 14 also bind nucleosomal DNA at sites that overlap those bound by histone H1 and coactivate transcription initiation or transcription elongation on chromatin templates (13, 45). In contrast, linker histones inhibit both initiation and elongation. The emerging picture is that HMG protein family members might act, in part, by antagonizing linker histone effects on chromatin structure (43). Our results with UBF are consistent with such a view. Interestingly, UBF has been shown to alleviate histone H1-mediated repression of rDNA transcription from naked-DNA templates in vitro (25). It will be important to determine if this result can be repeated with nucleosomal DNA rather than naked DNA to more accurately emulate native chromatin structure.

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