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The RNA Polymerase I Transactivator Upstream Binding Factor Requires Its Dimerization Domain and High-Mobility-Group (HMG) Box 1 To Bend, Wrap, and Positively Supercoil Enhancer DNA

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Upstream binding factor (UBF) is an important transactivator of RNA polymerase I and is a member of a family of proteins that contain nucleic acid binding domains named high-mobility-group (HMG) boxes because of their similarity to HMG chromosomal proteins. UBF is a highly sequence-tolerant DNA-binding protein for which no binding consensus sequence has been identified. Therefore, it has been suggested that UBF may recognize preformed structural features of DNA, a hypothesis supported by UBF's ability to bind synthetic DNA cruciforms, four-way junctions, and even tRNA. We show here that full-length UBF can also bend linear DNA to mediate circularization of probes as small as 102 bp in the presence of DNA ligase. Longer probes in the presence of UBF become positively supercoiled when ligated, suggesting that UBF wraps the DNA in a right-handed direction, opposite the direction of DNA wrapping around a nucleosome. The dimerization domain and HMG box 1 are necessary and sufficient to circularize short probes and supercoil longer probes in the presence of DNA ligase. UBF's sequence tolerance coupled with its ability to bend and wrap DNA makes UBF an unusual eukaryotic transcription factor. However, UBF's ability to bend DNA might explain how upstream and downstream rRNA gene promoter domains interact. UBF-induced DNA wrapping could also be a mechanism by which UBF counteracts histone-mediated gene repression.

The tandemly repeated rRNA genes are located in the nucleolus and are transcribed by the enzyme RNA polymerase I (pol I) to produce a primary transcript processed into 18S, 5.8S, and 28S rRNAs. The RNA pol I transcription system is highly active, accounting for the majority of all nuclear transcription in a rapidly growing eukaryotic cell (reviewed in reference 50). Three to five transcription factors, some of them known to be multiprotein complexes, are needed to transactivate pol I. The best characterized thus far is upstream binding factor (UBF). First purified from a human cell fraction required for RNA pol I transcription in vitro (4), UBF was independently purified from *Xenopus laevis* as an rRNA gene enhancer-binding protein (11, 42) and has since been purified and cloned from human, frog, rat, and mouse cells (1, 3, 17, 19, 31, 34, 43, 45, 57). Immunological evidence suggests that a UBF homolog is also present in protozoa and plants (51). The predicted protein sequence of *Xenopus* UBF (xUBF) includes five reiterated ~80-amino-acid domains (1), termed high-mobility-group (HMG) boxes, with similarity to the DNA-binding domains of HMG proteins 1 and 2 (HMG1 and -2) (19), an amino-terminal dimerization domain (20, 32, 35), and a highly acidic carboxyl terminus (Fig. 1).

Numerous studies have demonstrated that UBF greatly stimulates transcription, apparently by acting early in the assembly of stable preinitiation complexes (4, 24, 25, 31, 53, 57). UBF apparently serves a dual role as an activator and antirepressor, facilitating transcription complex assembly by stabilizing promoter binding by selectivity factor 1 (SL1 [28]) and/or RNA pol I while preventing repression by histones or other DNA-binding proteins (24, 25). SL1 in human extracts has been shown to consist of TATA-binding protein (TBP) and three TBP-associated factors (8). An equivalent activity has been characterized in other systems and has been named SL1, TFID, factor D, Rib-1, or TIF-1B (7, 31, 33, 48, 57–60). Human SL1 does not bind to the human rRNA gene promoter on its own but interacts with UBF to form a complex whose DNase I footprint is much larger than the footprint produced by UBF alone (4, 5). Therefore, UBF appears to be the primary DNA-binding transcription factor in the human system (3, 4). In contrast, mouse SL1 is capable of binding directly to the promoter in the absence of UBF, and in vitro studies have suggested that UBF may be nonessential for basal-level transcription in rodents (24, 54). Nonetheless, UBF and SL1 interact to form extended footprints on the mouse promoter (3) just as they do on the human promoter.

UBF is unusual among eukaryotic transcription factors in that it is a highly sequence-tolerant DNA-binding protein (18, 10). No DNA consensus sequence has emerged from comparison of footprinted regions of various probes (42) or from allowing UBF to select preferred binding sites from a population of oligonucleotides with randomized sequences (10). Methylation interference analysis using probes modified by dimethylsulfate or CpG methylase has also failed to reveal critical nucleotides required for UBF-DNA interactions (10). Finally, tRNA and low-complexity DNA polymers, including poly(dG) · poly(dC) and poly(dA) · poly(dT), have been shown to be good competitors of UBF-enhancer interactions, further underscoring the sequence-tolerant nature of UBF-nucleic acid interactions (10). However, like other HMG box proteins, UBF can bind synthetic DNA cruciforms (10, 26), four-way junctions (18), and even tRNA (10), suggesting that UBF may

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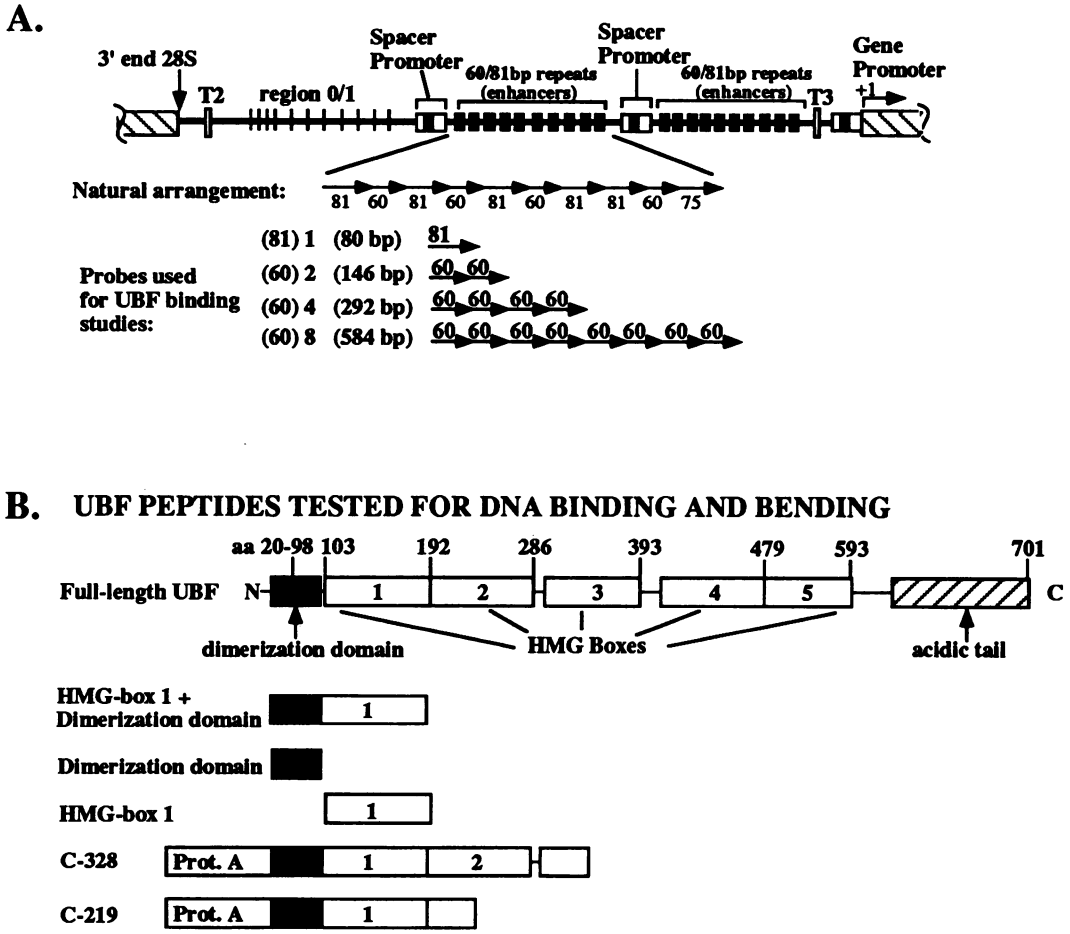

FIG. 1. (A) Enhancer probes and their relationships to natural enhancers within the *X. laevis* intergenic spacer. Natural enhancers exist as blocks of alternating 60- and 81-bp repeats. The 81-bp elements consist of a complete 60-bp element with an additional 21 bp added to the 3′ end. Intact 60-bp enhancers and slightly truncated 81-bp enhancers (76 bp) were subcloned as *Hae*III-*Pst*I fragments (44). The (60)*n* probes were generated by linking cloned 60-bp enhancers via flanking compatible *Sal*I and *Xho*I sites. The size of each probe is shown in parentheses. (B) UBF polypeptides used in the study. Full-length xUBF is 701 amino acids long and consists of a dimerization domain (102 amino acids), five HMG boxes, and an 87-amino-acid acidic tail. Recombinant polypeptides overexpressed in *E. coli* were used to determine the UBF domains involved in DNA bending. C-328 is a carboxyl-terminal truncation to amino acid 328, leaving the dimerization domain, HMG box 1, and HMG box 2 intact. In C-219, the dimerization domain and HMG box 1 are intact. Both C-328 and C-219 are fusions to the immunoglobulin binding domain of protein A in the expression vector pRIT2T (Pharmacia). The HMG box 1-plus-dimerization domain polypeptide is a C-terminal truncation having only the dimerization domain and HMG box 1 (peptide OG of Hu et al. [18]). The dimerization domain peptide has no HMG domains attached (peptide O1 of Hu et al. [18]), and the peptide labeled HMG1 consists solely of HMG box 1 (peptide FG of Hu et al. [18]).

recognize structural features of nucleic acids rather than specific sequences.

Despite UBF's promiscuous DNA-binding behavior, UBF binding to rRNA gene enhancer sequences is thought to be biologically significant (41–43, 47). Supporting evidence is that enhancers will compete in *trans* against an rRNA gene promoter both in vitro and in injected oocytes (27, 42), and addition of highly purified UBF can overcome this enhancer competition phenomenon in vitro (42). Therefore, we have used enhancer probes to study UBF-DNA interactions in some detail (10, 42, 47). UBF binds the *X. laevis* enhancers (and other probes of equal length) with a stoichiometry of one dimer for every two enhancers (~140 bp). On probes consisting of four or more enhancers, UBF dimers bind cooperatively presumably as a result of protein-protein interactions, conformational changes in the target DNA that affect the free energy of UBF binding, or both (47). Single UBF dimers bind probes varying in length from ~60 to 150 bp but fail to bind DNA fragments shorter than about 60 bp (47). However, within the 60 to 150-bp size range, the strength of UBF binding is proportional to probe length (47).

Pondering the importance of DNA length, but not sequence, on the strength of UBF-DNA interactions led us to consider two hypotheses. One is that UBF may need to bend or otherwise manipulate the DNA to bind stably, making longer probes favorable because of their improved flexibility relative to short DNA fragments. Second, stronger interactions with longer probes could be due to the binding of multiple HMG domains. xUBF has five HMG boxes (Fig. 1B) (1); thus, a UBF dimer has 10 potential DNA binding domains. It remains controversial how many HMG boxes actually contact the DNA, though the prevailing view is that multiple HMG boxes are involved (2, 18, 19, 29, 32, 47).

In this study, we used ligase-mediated DNA circularization (56) to show that UBF bends and wraps DNA, independently confirming the recent results of Bazett-Jones et al., who used

electron spectroscopic imaging to visualize UBF-induced DNA loops of approximately 180 bp (2). However, our data extend the conclusions drawn from the electron spectroscopic images by showing that UBF can circularize DNA fragments at least as short as 102 bp and can wrap the DNA of a 584-bp fragment multiple times. Furthermore, in contrast to the models of Bazett-Jones et al. (2) and Leblanc et al. (29), we show that the dimerization domain and HMG box 1 alone are necessary and sufficient to account for UBF's DNA bending and wrapping activity. All UBF polypeptides that induce DNA bending give rise to DNase I footprints virtually identical to those produced by the full-length UBF protein, whereas peptides that fail to bend the DNA also fail to produce footprints, suggesting that stable binding is coincident with bending. On long probes to which UBF can bind cooperatively, UBF wraps the DNA such that self-ligation results in circles that are positively supercoiled, suggesting that wrapping occurs in a right-handed direction. The implications of UBF's ability to alter DNA structure are discussed in relation to the protein's role in RNA pol I enhancer and promoter function.

MATERIALS AND METHODS

Full-length xUBF purification. UBF was purified from isolated nuclei of an *X. laevis* kidney cell line (Xlk2) by DEAE-Sepharose, Biorex 70, and Mono Q chromatography as described previously (42, 43, 47).

DNase I footprinting conditions and probes. DNase I footprinting assays were performed with a slightly truncated (76 bp) version of an *X. laevis* 81-bp rRNA gene enhancer probe (44). The probe was a *Sal*I-*Xho*I fragment end labeled at the *Sal*I end, using T4 polynucleotide kinase and gamma-labeled ATP. Probe preparation and conditions for the 100-μl footprinting reactions were performed exactly as described previously (42, 43). Approximately 15 ng (180 fmol) of full-length UBF purified from nuclei or 30 ng of the recombinant polypeptides (~1 to 1.5 pmol, depending on polypeptide size) was used in each reaction mixture that contained protein. Each reaction mixture contained approximately 10 fmol (~0.5 ng) of labeled probe DNA.

UBF-induced DNA circularization assay. Highly purified nuclear UBF (~5 ng [~60 fmol] unless otherwise noted in the figure legends) was added to 50-μl reaction mixtures containing ~20 fmol (~0.8 to 3.2 ng, depending on the length of the probe) of end-labeled probe DNA, using conditions similar to those of Verrijzer et al. (62). The reaction mixtures generally contained 30 mM Tris-HCl (pH 7.8), 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 12% glycerol, 57 mM KCl, 10 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, and 0.5 mM EDTA. After a 10-min incubation at room temperature, 1 U of T4 polynucleotide ligase (Promega) was added. Reactions were stopped by addition of an equal volume of 0.5% sodium dodecyl sulfate (SDS)–50 mM EDTA followed by heat treatment at 65°C for 10 min to denature the UBF and ligase. Reaction mixtures were then phenol-chloroform extracted, ethanol precipitated, washed with 70% ethanol, and resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 8.0). The reaction mixtures treated with exonuclease III (U.S. Biochemical), topoisomerase I (U.S. Biochemical), or DNA gyrase (Gibco BRL) received 0.5 to 1.0 U of enzyme and were incubated at 37°C for 30 min in 10- to 25-μl volumes. The enzymes were then inactivated at 65°C for 10 min prior to addition of marker dyes (xylene cyanol and bromophenol blue) in glycerol (52). Samples were loaded on 5% polyacrylamide gels (acrylamide-bisacrylamide, 30:1) made and run in 50 mM Tris-borate buffer (52). Gels run in a single dimension were run at approximately 10 V/cm. For the two-dimensional gels, gels were run at 10 V/cm (distance between buffer tanks) in the first dimension (100 V on a vertical gel box), soaked in 50 mM Tris-borate buffer containing 2 μM ethidium bromide, and then run at 25 V/cm (300 V in a submerged horizontal gel apparatus) in a second dimension perpendicular to the first.

xUBF deletion constructs overexpressed in *Escherichia coli*. The xUBF cDNA clone pxUBF (31) was cut with *Ava*I and *Eco*RI to yield a 1,730-bp (3′ end) fragment and with *Dde*I to yield a 616-bp (5′ end) fragment. An *Eco*RI adapter (made by annealing 5′ AATTCGGGTCCTCGTGC 3′ and 3′ GCCCAG GAGCACGAGT 5′) was ligated to the *Dde*I fragment. The product was digested with *Ava*I to yield a 430-bp fragment, which was then ligated to the 1,730-bp *Ava*I-*Eco*RI fragment. The product of this ligation was cloned into the *Eco*RI site of pBlueScript SK- (Stratagene) to yield pAKUBF1. A 1,010-bp *Eco*RI-*Pst*I fragment encoding the amino-terminal 328 amino acids of xUBF was then cloned in frame with the immunoglobulin binding domain of protein A in the expression vector pRIT2T (Pharmacia) to yield the plasmid pC-328. The C-219 (C-terminal deletion to amino acid 219) truncation was made by digesting pC-328 at the internal *Sph*I site and at the *Pst*I site (in the 3′ flanking plasmid polylinker), blunting the ends, and religating. Three highly purified polypeptides that consisted of HMG box 1 attached to the dimerization domain, the dimerization domain alone, or HMG box 1 alone were generously provided by Chin-Hwa Hu and Ronald H. Reeder (Fred Hutchinson Cancer Research Center, Seattle, Wash.) and correspond to peptides OG, O1, and FG in their nomenclature (18).

Purification of protein A-xUBF fusion proteins overproduced in *E. coli*. Fusion proteins C-328 and C-219 were overproduced in *E. coli* DH5α. Cells were collected by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.5)–300 mM NaCl–0.05% Tween 20, and lysed by sonication. The sonic extract was clarified by centrifugation, and the fusion proteins were purified by chromatography over Biorex 70 (0.3 to 1.0 M KCl step elution) followed by a Mono S linear gradient. Both C-328 and C-219 eluted from Mono S as symmetrical peaks at about 260 mM KCl; peak fractions were greater than 95% pure, as estimated from Coomassie blue-stained SDS-polyacrylamide gels.

RESULTS

UBF bends linear DNA fragments to facilitate their ligation into circles. Proteins that bend DNA can bring the two ends of a DNA fragment into proximity, facilitating their ligation to produce a circle under dilute conditions (56). Therefore, we tested UBF's ability to increase the rate of circularization of a probe consisting of two full-length 60-bp *X. laevis* rRNA gene enhancers linked head to tail [(60)2 probe]. The (60)2 probe is 146 bp long and has compatible *Sal*I and *Xho*I ends for self-ligation. This probe was chosen as our starting point because previous gel-shifting experiments showed that a range of UBF to (60)2 probe ratios produced only a single protein-DNA complex, consistent with binding of a single dimer (47). Figure 2A shows the ligation products of the (60)2 probe preincubated with no protein, 5 ng of highly purified UBF, or 20 ng of bovine serum albumin (BSA). In the absence of UBF (lanes 1 to 4), the (60)2 probe does not circularize appreciably. However, in the presence of UBF (lanes 5 to 8), a ligation product is formed that is resistant to the action of exonuclease III (lane 8), consistent with the novel DNA being covalently closed. The mobility of this ligation product was unchanged by eukaryotic topoisomerases I and II (data not shown), suggest-

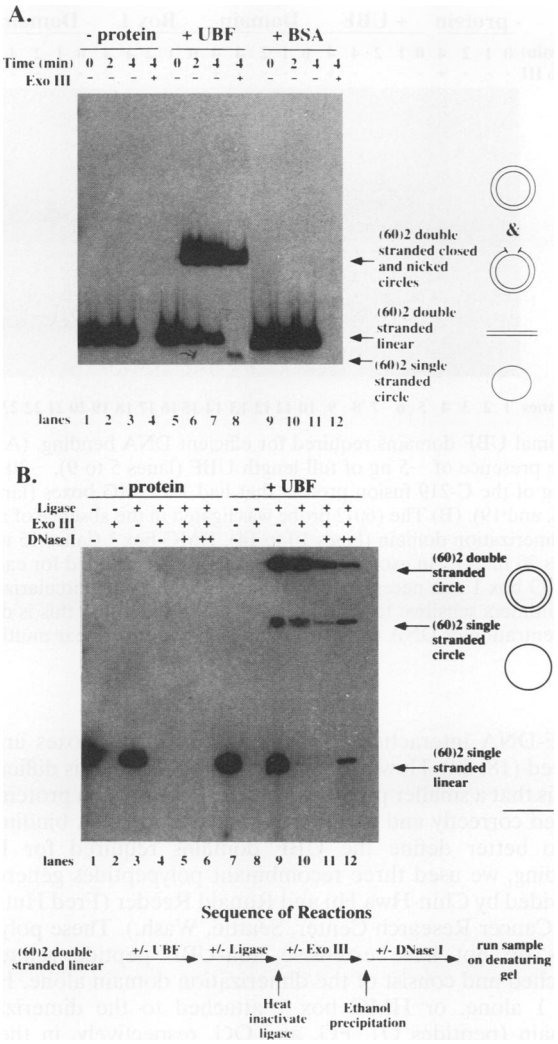

FIG. 2. UBF bends linear DNA upon binding. (A) UBF bends a two-enhancer probe to allow circularization by DNA ligase. Approximately 20 fmol (~0.8 ng) of end-labeled (60)2 probe (146 bp) was ligated for 0, 2, or 4 min in a volume of 50 μl in the absence of added protein (lanes 1 to 4) or in the presence of ~5 ng (~60 fmol) of UBF (lanes 5 to 8) or 20 ng of BSA (lanes 9 to 12); ~10 μl of reaction mixture was loaded in each lane of the native 5% polyacrylamide gel. In lanes 4, 8, and 12, the DNA was treated with exonuclease (Exo) III to degrade linear molecules. The ligation product formed in the presence of UBF is resistant to exonuclease, indicating that it is a circular product. (B) Analysis and identification of ligation products on an 8% denaturing polyacrylamide gel. The (60)2 probe was preincubated as in panel A in the presence or absence of UBF and then ligated with T4 DNA ligase for 4 min. Without UBF, no products are observed (lane 3). In the presence of UBF, the probe was efficiently ligated to yield two upper bands (lane 9) that we interpret to be the double-stranded circular monomer and a single-stranded circular monomer (half of a nicked double-stranded circle). Exonuclease III treatment reveals that the two ligation products in lane 9 are circular (lane 10). Nicking the ligation products with increasing amounts of DNase I (lanes 11 and 12) converts the double-stranded product to approximately equal amounts of single-stranded circular and single-stranded linear fragments, consistent with the interpretations of the various DNA species.

ing that it was a relaxed covalently closed circular molecule. However, a small portion of the UBF-dependent ligation product was accessible to exonuclease III, yielding a faster-migrating digestion product (bottom of lane 8). This digestion product represents the single-stranded circle that remains if the other strand is nicked and is therefore susceptible to exonuclease degradation. The nicked, single-stranded linear fragment can be resolved from the single-stranded circle and covalently closed double-stranded circle on an 8% denaturing urea-polyacrylamide gel (Fig. 2B). Note that although circularization appears complete by the 2-min time point, the reason for including longer time points is that preliminary experiments showed that by 4 min, the amount of nicked double-stranded circles reaches a minimum in favor of circles in which both strands are covalently closed. Presumably, this is because ligations of the two strands are independent events, though ligation of the second strand can be expected to proceed more rapidly than the initial ligation event that brings the ends of the DNA together. Preliminary experiments also showed that DNA circularization was dependent on both the concentration of UBF and the time of ligation (data not shown).

The exact nature and size of the ligated products observed on the native gel shown in Fig. 2A were determined by analysis of equivalent ligation products on a denaturing urea-polyacrylamide gel (Fig. 2B, lanes 7 to 12) adjacent to a complete set of control reactions to which no UBF was added (lanes 1 to 6). UBF-dependent ligation products migrated as two distinct bands (lane 9), both of which were resistant to exonuclease III treatment (lane 10). The top band corresponds to the covalently closed double-stranded circle whose strands remain entangled upon denaturation. The second band from the top of the gel corresponds to the single-stranded circle resulting from denaturation of a nicked double-stranded circular monomer and dissociation of the nicked complementary strand which runs as a single-stranded linear DNA with the same mobility as the denatured, unligated probe. Recall that the covalently closed and nicked double-stranded circular ligation products comigrated on the native gel (Fig. 2A, lanes 6 to 8) unless the nicked strand was removed by treatment with exonuclease III (Fig. 2A, lane 8). The identity of each of the different circular DNA forms was confirmed by treatment of the ligation products with exonuclease III to remove linear DNA (Fig. 2B, lane 10) followed by treatment for 30 s with trace amounts of DNase I to nick the DNA (10 and 100 ng/ml; lanes 11 and 12, respectively). As is best seen in lane 12, at the higher of the two DNase I concentrations tested, the double-stranded circular form was converted into approximately equal amounts of the single-stranded circle and linear DNA upon nicking by DNase I, as expected. The size of the linear DNA fragment released by DNase I nicking of the circular ligation products was the same as the starting probe (146 nucleotides; molecular weight markers were present on an adjacent lane [not shown]), as expected if the circular DNAs were monomers. If two probe molecules had been ligated together prior to circularization, DNase I nicking would have produced linear fragments of 292 nucleotides. The possibility that the UBF-induced ligation products might be catenated monomers was also considered but is inconsistent with number of DNA forms generated by the DNase I nicking experiment shown in Fig. 2B. Two catenated rings would be expected to yield at least five products upon DNase I nicking. These would consist of four, three, or two interlocked single-stranded circles, single circles, and linear monomers. We considered this possibility because of the recent report of Hu et al. (18) that UBF can bind two pieces of DNA simultaneously, presumably by stabilizing the

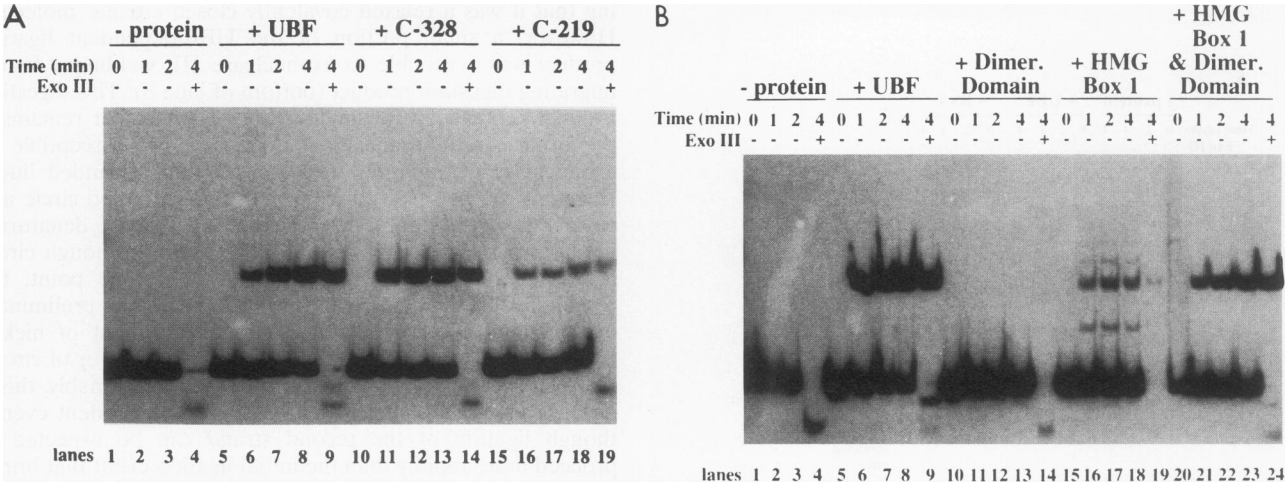

FIG. 3. The dimerization domain linked to HMG box 1 constitutes the minimal UBF domains required for efficient DNA bending. (A) The (60)2 probe was ligated in the absence of added protein (lanes 1 to 4) or in the presence of ~5 ng of full-length UBF (lanes 5 to 9), ~30 ng of the C-328 fusion protein containing 2.5 HMG boxes (lanes 10 to 14), or ~30 ng of the C-219 fusion protein that had 1.5 HMG boxes (lanes 15 to 19). An exonuclease (Exo) III reaction was included for each set (lanes 4, 9, 14, and 19). (B) The (60)2 probe was ligated in the absence of added protein (lanes 1 to 4) or in the presence of full-length UBF (lanes 5 to 9), the dimerization domain (lanes 10 to 14), HMG box 1 (lanes 15 to 19), or the polypeptide consisting of HMG box 1 plus the dimerization domain (lanes 20 to 24). An exonuclease III reaction was included for each set (lanes 4, 9, 14, 19, and 24). Note that the dimerization domain attached to HMG box 1 was necessary and sufficient to promote circularization. The HMG box 1 peptide facilitated ligation of the probe mostly into linear multimers sensitive to exonuclease III. We think that this is due to aggregation of HMG box 1 bound to DNA causing an increase in the local concentration of DNA ends, promoting ligation into linear multimers.

junctions where two DNA strands cross one another in the shape of an X. Ligation of DNAs involved in such a junction could yield catenanes or circular multimers. Because neither were detected, we conclude that UBF bends linear DNA upon binding, facilitating the self-ligation of relatively short probes into covalently closed DNA circles. Hu et al.'s demonstration using the gel mobility shift assay that HMG box 1 can assemble complexes containing two different DNA fragments may be due to stable association of two HMG box 1 peptides each bound to DNA (discussed below).

The dimerization domain and HMG box 1 of UBF are necessary and sufficient for DNA bending. The number of HMG boxes involved in DNA binding, as determined by gel mobility shift analysis and DNase I footprinting, remains controversial. The most recently published models suggest that at least HMG boxes 1 to 3 are involved, placing a minimum of six HMG boxes of the dimer in direct contact with DNA (2, 29). Therefore, we used the ligase-mediated circularization assay as an independent method to address the UBF domains involved in DNA binding or bending, comparing highly purified nuclear UBF with truncated recombinant polypeptides overproduced in *E. coli* (Fig. 1B). Initially, we used the Pharmacia pRIT2 expression vector to produce proteins bearing 30 kDa of *Staphylococcus* protein A at the amino terminus fused in frame to UBF amino acids 1 to 328 (C-328) or amino acids 1 to 219 (C-219). Both fusion polypeptides were able to efficiently catalyze the circularization of the (60)2 probe (Fig. 3, lanes 11 to 14 and 16 to 19) and produce DNase I footprints virtually identical to those produced by full-length UBF (data not shown, but see Fig. 5, showing even smaller polypeptides). Lack of interference from the ~30-kDa of protein A linked directly to the UBF dimerization domain suggests that the dimerization domain at the amino terminus of UBF may project away from the HMG boxes. Note that C-219 was about threefold less efficient than C-328 in promoting circularization, consistent with previous observations that the strength of UBF-DNA interactions is decreased as HMG boxes are removed (18, 29). However, another possibility that is difficult to test is that a smaller proportion of the C-219 fusion protein was folded correctly and was therefore active in DNA binding.

To better define the UBF domains required for DNA bending, we used three recombinant polypeptides generously provided by Chin-Hwa Hu and Ronald Reeder (Fred Hutchinson Cancer Research Center, Seattle, Wash.). These polypeptides do not have extensive non-UBF peptide sequences attached and consist of the dimerization domain alone, HMG box 1 alone, or HMG box 1 attached to the dimerization domain (peptides O1, FG, and OG, respectively, in the nomenclature of Hu et al. [18]). The peptide containing HMG box 1 plus the dimerization domain (OG) was found to be necessary and sufficient to reproduce full-length UBF's ability to efficiently circularize the (60)2 probe in the ligation assay (Fig. 3B; compare lanes 21 to 24 with lanes 6 to 9). The peptide containing the dimerization domain alone (O1) had no effect in the ligation assay (lanes 11 to 14). Interestingly, the HMG box 1 recombinant polypeptide (FG) facilitated ligation of the probe into linear multimers susceptible to exonuclease III (lanes 16 to 19) but yielded only a trace of circular product (lane 19). Formation of linear multimers was surprising because the DNA concentration is purposely kept low in the reactions to favor self-ligation while discouraging bimolecular ligation. HMG box 1 somehow increases the effective concentration of DNA fragments to allow them to be ligated together. Our interpretation is that HMG box 1 must be able to interact with DNA and with other HMG box 1 polypeptides bound to DNA, thereby bringing probe molecules together for bimolecular ligation into multimers. This interpretation might also explain the observation of Hu et al. that HMG box 1 could stably bind two pieces of DNA simultaneously (18). It is possible that interacting HMG box 1 polypeptides, each bound to a different piece of DNA, associated stably in the gel mobility shift assay (18). Interestingly, high concentrations of

full-length UBF can also lead to formation of linear multimers in the ligation assay (see Fig. 9, lanes 5 and 6). At amounts of UBF greater than 20 ng per 50-μl reaction (not shown), circularization is suppressed and only linear multimers accumulate, suggesting that linear forms are probably formed by virtue of protein aggregation. Therefore, it is important to note that all experiments performed in this study (other than lanes 4 to 6 of Fig. 9) used an amount of UBF well below the threshold at which such aggregates are presumed to occur. In other studies in which high amounts of UBF were used, effects of protein aggregation might have contributed to the results, leading to interpretations different from those that we present (2, 18).

Though our data suggest that HMG box 1 must be capable of interacting with DNA to promote ligation of multimers, in the absence of the dimerization domain, HMG box 1 does not efficiently bend DNA sufficiently to catalyze its self-ligation. It follows that the dimerization domain is key to forming a strong bend in the DNA, presumably by positioning the HMG box 1 domains bound to DNA in such a way that the ends of the DNA are brought into proximity (see Fig. 10). Nonetheless, it is important to point out that HMG box 1 apparently does bend DNA somewhat, because a small amount of circular product was produced upon ligation in the presence of HMG box 1 (Fig. 3B, lane 19), whereas none was produced in its absence (lane 4). This result is consistent with other studies that have shown single HMG boxes to be capable of bending DNA (13, 16, 39, 46).

UBF will catalyze the ligase-mediated circularization of nonenhancer probes. UBF is known to be a highly sequence tolerant DNA-binding protein; however, it is possible that UBF recognizes cryptic architectural features of enhancer and promoter sequences (10, 18). Therefore, we tested whether a monomer (SS90) or dimer (SS90)2 of a cloned 90-bp DNA fragment derived from the bacterial plasmid pBR322 could be circularized by either UBF or the peptide consisting of HMG box 1 linked to the dimerization domain (Fig. 4). Polymers of the SS90 fragment were previously shown to lack enhancer activity when cloned adjacent to an rRNA gene promoter and injected into *Xenopus* oocytes (41). The SS90 DNA fragments had self-compatible *SalI* and *XhoI* sticky ends such that the actual lengths of the probes were 102 bp for the monomer and 204 bp for the (SS90)2 dimer. The HMG box 1-plus-dimerization domain polypeptide (peptide OG; ~30 ng was used) was able to efficiently circularize both the 102- and the 204-bp SS90 plasmid DNA fragments (Fig. 4A, lanes 4 and 5; Fig. 4B, lanes 4 and 6), whereas BSA had no effect (lanes 2 of both panels). Full-length UBF (~5 ng) was less efficient (approximately three- to fourfold on a protein mass basis) than the HMG box 1-plus-dimerization domain polypeptide in promoting circularization of the 102-bp DNA (Fig. 4A, lanes 3 and 6). However, in the twin reaction (made from the same reaction mix, the only difference being the probe), full-length UBF efficiently catalyzed circularization of the 204-bp probe (Fig. 4B, lanes 3 and 5). The fact that the HMG box 1-plus-dimerization domain polypeptide could efficiently circularize the 102-bp circle whereas full-length UBF was inefficient suggests that HMG boxes 2 to 5 may actually sterically hinder the ends of the 102-bp DNA molecule or that the degrees of DNA bending are different with the two proteins. However, the latter possibility is disfavored by the results of Fig. 9, showing that full-length UBF and the HMG box 1-plus-dimerization domain polypeptide wrap the 584-bp (60)8 probe to the same degree (see below). Considering the results of Fig. 3 and 4 together, it is interesting that the same amount of full-length UBF (5 ng) helped circularize enhancer DNA probes more

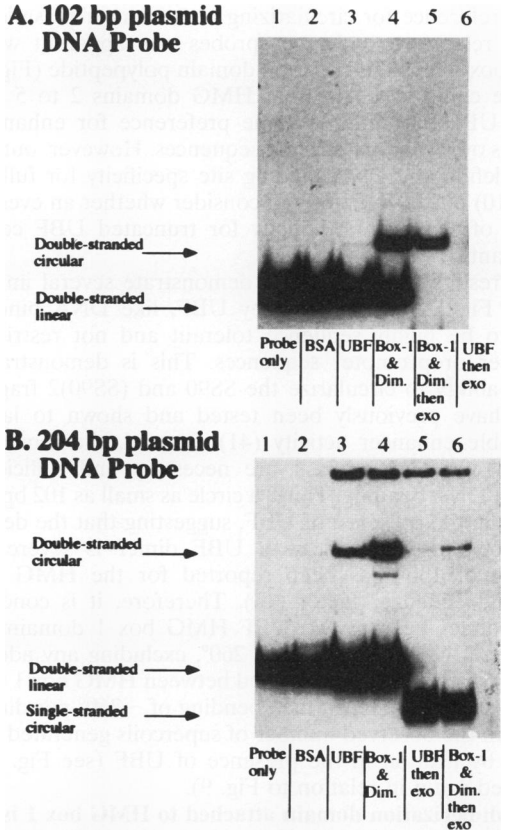

FIG. 4. DNA bending by UBF is not restricted to enhancer sequences. (A) UBF and the HMG box 1-plus-dimerization domain polypeptide differ dramatically in the ability to bend a 102-bp DNA fragment (SS90) derived from the bacterial plasmid pBR322 to promote ligase-mediated circularization. The SS90 probe (lane 1) was ligated for 3 min at room temperature in the presence of 20 ng of BSA (lane 2), 5 ng of purified full-length UBF (lane 3), or ~30 ng of the dimerization domain-plus-HMG box 1 polypeptide (lane 4). Lane 5 shows a duplicate of the reaction run in lane 4 after treatment with exonuclease III (exo). Lane 6 is an exonuclease-treated reaction identical to that shown in lane 3. Note that full-length UBF produces only a trace of circular ligation product (lane 3), whereas the smaller polypeptide efficiently catalyzes DNA circularization (lanes 4 and 5). The SS90 probe is the *SphI-SalI* fragment of pBR322 to which an *XhoI* linker was attached to the *SphI* end. The fragment has compatible *XhoI* and *SalI* ends to allow sticky-end ligation. (B) Both full-length UBF and the dimerization domain-plus-HMG box 1 polypeptide promote the efficient circularization of a 204-bp plasmid DNA probe [(SS90)2]. This probe has two of the plasmid DNA fragments used in panel A linked head to tail. Reaction mixtures contained no ligase (lane 1), 20 ng of BSA (lane 2), ~5 ng of full-length UBF (lane 3), ~30 ng of HMG box 1-plus-dimerization domain polypeptide (lane 4), a duplicate of the reaction run in lane 3 (UBF) after treatment with exonuclease III (lane 5), or a duplicate of the reaction run in lane 4 (dimerization domain-plus-HMG box 1 polypeptide) after exonuclease treatment. Note that most of the circular ligation products were nicked (only one of the two strands ligated) so that they were sensitive to exonuclease treatment, yielding mostly single-stranded circles that comigrated with one or more exonuclease products derived from the linear probe. However, the amount of exonuclease-resistant circular DNA was variable between repeats of this experiment. The bands at the top of the gel in lanes 3 to 6 are at the position of the well and may represent protein-DNA aggregates. Such labeled material at the position of the well was occasionally observed with several different probes and was always UBF dependent. The reason for its variable occurrence is not clear.

efficiently than nonenhancer plasmid DNA probes. In contrast, some preference for circularizing nonenhancer plasmid DNA probes relative to enhancer probes was apparent with the HMG box 1-plus-dimerization domain polypeptide (Fig. 3 and 4). One could speculate that HMG domains 2 to 5 of full-length UBF may impose some preference for enhancer sequences over random plasmid sequences. However, our inability to define any DNA binding site specificity for full-length UBF (10) makes it difficult to consider whether an even lesser degree of sequence specificity for truncated UBF could be significant.

The results of Fig. 3 and 4 demonstrate several important points. First, DNA bending by UBF, like DNA binding, is likely to be highly sequence tolerant and not restricted to enhancer or promoter sequences. This is demonstrated by UBF's ability to circularize the SS90 and (SS90)2 fragments, which have previously been tested and shown to lack any detectable enhancer activity (41). Second, the dimerization domain and HMG box 1 are necessary and sufficient for efficient DNA bending. Third, a circle as small as 102 bp can be formed in the presence of UBF, suggesting that the degree of DNA bending induced by a UBF dimer is severe. DNA bending of 130° has been reported for the HMG box of lymphoid enhancer factor (16). Therefore, it is conceivable that a dimer bearing two UBF HMG box 1 domains could bend the DNA by as much as 260°, excluding any additional bending due to the angle formed between HMG box 1 and the dimerization domain. In fact, bending of ~260° per dimer fits well with the observed number of supercoils generated in long probes by ligation in the presence of UBF (see Fig. 6 to 9; discussed again in relation to Fig. 9).

The dimerization domain attached to HMG box 1 is necessary and sufficient to produce DNase I footprints like those of full-length UBF. We next determined the relationship between the UBF domains required for DNA bending and those required for stable DNA binding as measured by DNase I footprinting. On a probe corresponding to an 81-bp *X. laevis* enhancer [probe (81)1], 15 ng of nuclear UBF and 30 ng of recombinant UBF produced very similar, though not quite identical, footprints, provided that the dimerization domain was linked to HMG box 1 (Fig. 5; compare lane 2 with lane 8). The twofold-greater mass of recombinant UBF needed to produce a footprint equivalent to that produced by full-length nuclear UBF is consistent with other reports that the binding affinity of UBF is decreased as sequences downstream of HMG box 1 are removed (18, 29). However, as was mentioned in relation to the data of Fig. 3, the possibility that some of the recombinant polypeptides might be folded incorrectly could also contribute to the need to use more recombinant protein to obtain strong footprints. Note that footprints were not observed with HMG box 1 alone (lane 4) or with the dimerization domain alone (lane 6). Recall that these domains alone were also insufficient to catalyze DNA circularization. Therefore, there is a close correlation between the UBF domains required for footprinting and ligation-mediated circularization (Fig. 3). It is worth noting that the footprints produced by the C-328 and C-219 fusion proteins were identical to the footprint produced by the HMG box 1-plus-dimerization domain polypeptide (not shown). These footprinting results are in general agreement with other published reports that HMG box 1 accounts for the majority of UBF's footprinting activity but that the dimerization domain is critical for efficient DNA binding (20, 29, 30). We conclude that the dimerization domain and first HMG box are responsible for UBF-enhancer interactions and that stable DNA binding by UBF is likely to coincide with DNA bending.

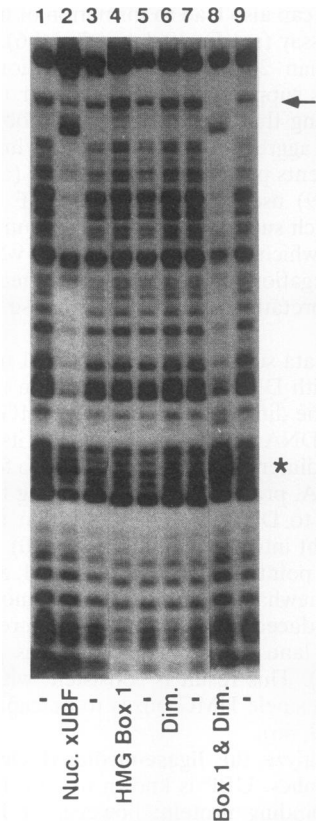

FIG. 5. The dimerization domain and HMG box 1 are necessary and sufficient for DNase I footprinting. Odd-numbered lanes show the DNA digestion pattern of the 81-bp enhancer probe in the absence of added protein. Lane 2 shows the protections conferred by full-length nuclear xUBF. Neither the HMG box 1 (lane 4) nor the dimerization domain (lane 6) polypeptide conferred any protection from DNase I digestion. However, the HMG box 1-plus-dimerization domain polypeptide was capable of making a typical UBF footprint (lane 8). Minor differences between the protection patterns conferred by full-length UBF and the HMG box 1-plus-dimerization domain polypeptide are a stronger hypersensitive site (asterisk) and an additional protected band (arrow) in the case of the truncated polypeptide. This result suggests that the absence of C-terminal domains downstream of HMG box 1 slightly affects the conformation of the DNA binding surface of HMG box 1.

Long probes can be wrapped and ligated into supercoiled circles in the presence of UBF. Because UBF dimers bind cooperatively to multiple enhancers and the apparent stoichiometry of UBF binding to such arrays is one UBF dimer per every two enhancers (47), we used the ligation assay to test the influence of UBF on the topology of tandemly repeated 60-bp enhancer arrays (Fig. 1) (44). Unlike the (60)2 or SS90 probe, but like the (SS90)2 probe, the (60)4 and (60)8 probes are long enough for their ends to be ligated into relaxed circular monomers in the absence of UBF (Fig. 6, lanes 10 to 12 and 18 to 20). However, in the presence of UBF, circularization was accelerated and one or more unique ligation products accumulated (lanes 14 to 16 and 22 to 24). The two novel UBF-dependent ligation products are most obvious with the (60)8 probe. These novel products migrated faster in the native gel than did the circular products formed in the absence of UBF, and they were resistant to exonuclease III, indicating that they were covalently closed (lanes 16 and 24). Subsequent analysis suggested that the novel UBF-dependent ligation

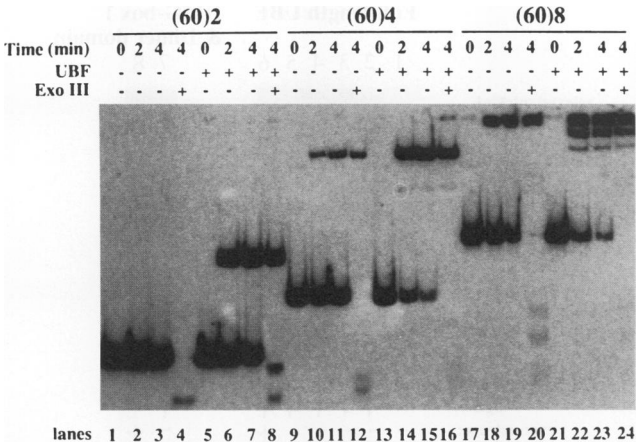

FIG. 6. UBF wraps a 584-bp DNA fragment to produce multiple circular ligation products. Probes consisting of two, four, or eight 60-bp enhancers in tandem [(60)2, (60)4, or (60)8 probe] were preincubated with ~5 ng of highly purified xUBF prior to addition of T4 DNA ligase (Promega). Reactions were then stopped at 0, 2, or 4 min after ligase addition, and a duplicate sample of the 4-min ligation was treated with exonuclease (Exo) III to degrade linear DNAs. Note that the (60)2 probe does not circularize in the absence of UBF (lanes 2 to 4). However, the (60)4 and (60)8 probes are long enough to be ligated into relaxed circles in the absence of UBF (lanes 12 and 20). In the presence of UBF, one (lanes 14 to 16) or more faster-migrating circular forms accumulate (difficult to see in lanes 14 to 16 but very obvious in lanes 22 to 24). Subsequent experiments suggest that these faster-migrating forms are supercoiled (see Fig. 7 and 8).

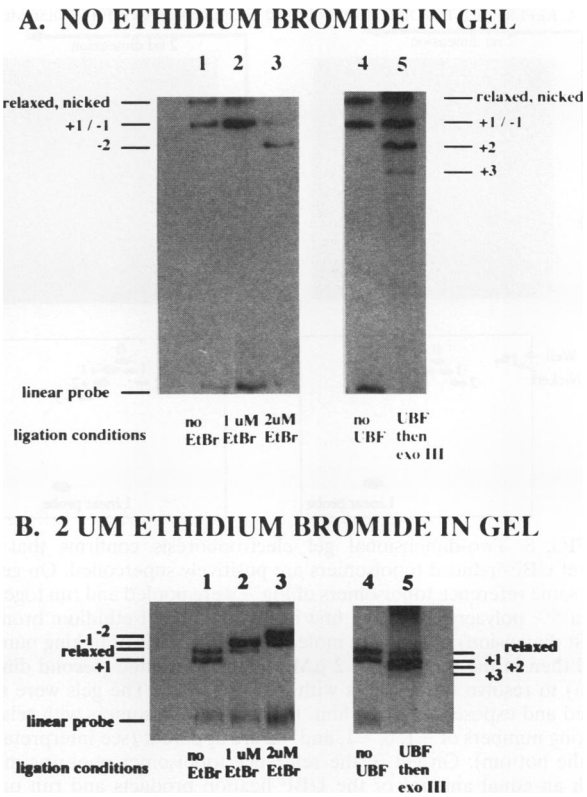

FIG. 7. Ligation of the (60)8 probe in the presence of ethidium bromide or UBF produces comigrating supercoiled DNAs twisted in opposite directions. For both panels, ~3.2 ng of (60)8 probe was ligated for 4 min at room temperature either in the presence of 0, 1.0, or 2.0 μM ethidium bromide (EtBr; lanes 1 to 3) or in the presence of 0 (lane 4) or 5 (lane 5) ng of UBF. The UBF-induced ligation products were then treated with exonuclease (exo) III to ensure that only circular products remained. Reaction mixtures were then phenol-chloroform extracted and ethanol precipitated. Upon resuspension, equal aliquots were loaded onto 5% polyacrylamide gels run in the absence (A) or presence (B) of 2 μM ethidium bromide. In panel A, topoisomers formed by ligation in the presence of ethidium bromide are negatively supercoiled (−1 and −2) when the intercalating agent is removed. The more negatively supercoiled DNAs run fastest in the gel. The two ligation products formed with this probe in the absence of ethidium or UBF (lanes 1 and 4) have relative linking numbers of 0 and +1 (apparent in Fig. 8). Topoisomers of +1 and −1 comigrate. The −2 topoisomer comigrates with one of the ligation products (labeled +2) produced in the presence of UBF. Lanes 1 to 5 are results from the same experiment and gel. (B) The negative supercoils of the DNAs ligated in the presence of ethidium bromide are unwound as expected, and their mobility is decreased in a gel containing ethidium bromide. In contrast, the mobilities of the topoisomers produced in the presence of UBF are increased, suggesting that UBF introduces positive supercoils into the DNA. Lanes 1 to 5 are results from the same gel.

products were covalently closed circles containing either two or three positive supercoils (Fig. 7 and 8). To determine this, a set of closed circular reference topoisomers was first created by untwisting linear (60)8 probe with increasing amounts of the intercalating agent ethidium bromide, self-ligating the undertwisted DNA at low concentration to form circles, and then removing the ethidium bromide (Fig. 7A, lanes 1 to 3). The linking number deficit that results upon removal of the intercalating agent is compensated by negative supercoiling, resulting in molecules that migrate faster than relaxed circles in a polyacrylamide gel (Fig. 7A). Note that in the absence of ethidium bromide or UBF in the ligation reactions (lanes 1 and 4), two circular ligation products were formed. Both were resistant to exonuclease III (not shown). Subsequent analysis suggested that the circle with the lowest mobility is a relaxed circle, whereas the faster-migrating circle formed in the absence of UBF or ethidium has one positive supercoil (+1; Fig. 8). Both persist after treatment with topoisomerase I or II (data not shown), suggesting that these are both topologically favored forms of the closed circle formed from this particular DNA. On a 5% polyacrylamide gel lacking ethidium bromide in the gel or buffer, one of the novel UBF-dependent, exonuclease III-resistant ligation products comigrated with the ethidium bromide-induced topoisomer containing two negative supercoils (Fig. 7A; compare lanes 3 and 5), whereas the other novel UBF-induced circular product had higher mobility still (lane 5; labeled +3 in Fig. 7). However, the sign of the UBF-induced supercoiling could not be determined directly from this experiment. Therefore, half of the ligation reactions visualized in Fig. 7A were subjected to electrophoresis through gels containing 2 μM ethidium bromide (Fig. 7B). Under these conditions, the negative supercoils formed in the reference topoisomers by ligating in the presence of ethidium bromide and then removing the intercalating agent were counteracted upon reintercalation of ethidium bromide, as expected, resulting in decreased mobility as the molecules returned to a relaxed form (Fig. 7B, lanes 2 and 3). In contrast, the relaxed and +1 circles became more positively supercoiled in ethidium bromide and ran faster (lane 1). Note that in the presence of ethidium bromide, the novel UBF-induced supercoils had higher mobility than the reference topoisomers (lane 5), as predicted for positively supercoiled DNA.

It is intriguing that positive (right-handed) coiling is oppo-

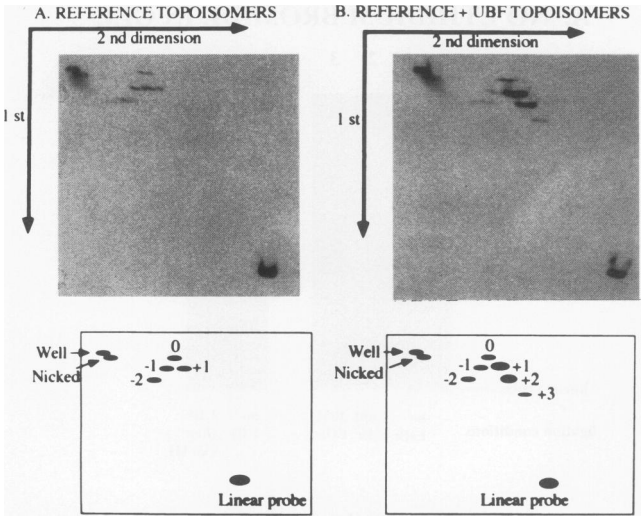

FIG. 8. Two-dimensional gel electrophoresis confirms that the novel UBF-induced topoisomers are positively supercoiled. On gel A, the same reference topoisomers of Fig. 7 were pooled and run together on a 5% polyacrylamide gel first in the absence of ethidium bromide (first dimension) to separate molecules on the basis of linking number and then in the presence of 2 μM ethidium bromide (second dimension) to resolve topoisomers with different signs. The gels were then dried and exposed to X-ray film. Reference topoisomers with relative linking numbers of +1, 0, −1, and −2 are apparent (see interpretation at the bottom). On gel B, the reference topoisomer pool was mixed with an equal amount of the UBF ligation products and run on an identical 5% gel. Both gels were run on the same power supply and handled in parallel. Note that the two novel UBF-dependent ligation products have apparent relative linking numbers of +2 and +3. The most intense band on gel B is the +1 band common to both sets of the mixed ligation reactions. Note that the −2 and +2 topoisomers comigrate in the first dimension, as is also shown in Fig. 7.

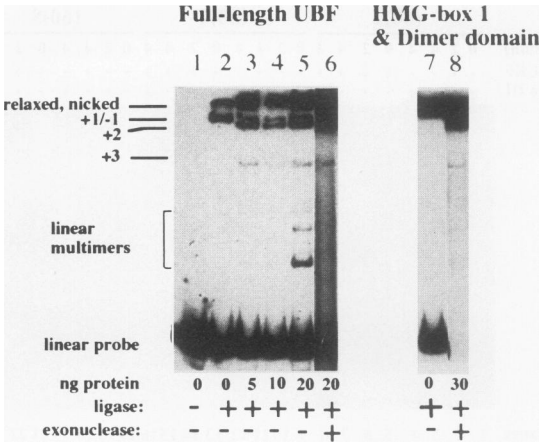

FIG. 9. Full-length UBF and the HMG box 1-plus-dimerization domain polypeptide wrap the (60)8 probe to the same degree. Lanes 1 and 2 show the probe only and probe-plus-ligase controls, respectively. Novel ligation products are produced upon ligation in the presence of 5 ng of UBF (lane 3; same products as shown in Fig. 6 to 8), 10 ng of UBF (lane 4), or 20 ng UBF (lane 5). Exonuclease treatment of an aliquot of the reaction loaded in lane 5 eliminates the linear multimers that accumulate in ligations in the presence of high UBF concentrations, revealing the circular products (lane 6; a threefold-longer exposure relative to lanes 1 to 5 was necessary). Circular ligation products identical to those formed with full-length UBF are made in the presence of the HMG box 1-plus-dimerization domain polypeptide (lane 8). The +1 and +2 bands in lane 8 are obscured by the need for a long exposure in order to see the +3 topoisomer, which is the critical topoisomer supporting the argument that DNA is wrapped to the same extent by full-length UBF and the HMG box 1-plus-dimerization domain polypeptide. All lanes shown are from the same gel.

site to the direction of DNA wrapping around a nucleosome or supercoiling induced by other HMG box proteins, including HMG1, HMG2, and mtTF1 (14, 21). Therefore, the direction of supercoiling was verified by two-dimensional electrophoresis (40). For this experiment, two identical two-dimensional 5% polyacrylamide gels (acrylamide-bisacrylamide, 30:1) were run. One was used to resolve the pooled ethidium bromide-induced reference topoisomers of Fig. 7 (Fig. 8A), whereas the second was used to resolve an equal mixture of reference topoisomers mixed with UBF-induced topoisomers that had been treated with exonuclease III (Fig. 8B). In the first dimension, the gels were run vertically at relatively low voltage in the absence of ethidium bromide. The gel plates were then pried apart, the gels were soaked for 45 min in 2 μM ethidium bromide, the top gel plate was replaced, and the gels were run at higher voltage in a second dimension with the electric field perpendicular to that of the first dimension. Ethidium bromide (2 μM) was also included in the running buffer for the second dimension. In the first dimension, the topoisomers resolve according to linking number (as in Fig. 7A), with supercoiled DNAs having higher mobility than the relaxed circle. In the second dimension, negatively supercoiled DNAs are retarded in their mobility relative to relaxed or positively supercoiled DNAs (as in Fig. 7B). As can be seen by comparison of Fig. 8A and B, the two novel bands in Fig. 8B are the UBF-dependent topoisomers that appear to contain two (+2) and three (+3) positive supercoils, respectively. Note, however, that we were unable to generate a reference topoisomer with more than two negative supercoils either by ligation in the presence of ethidium bromide or by treatment of relaxed closed circular probe with DNA gyrase in the presence of ATP (not shown). Therefore, the assignment of the +3 topoisomer generated by ligation with UBF is not as firm as the assignment of the +2 UBF-dependent topoisomer, which clearly comigrated in the first dimension with the −2 ethidium bromide-induced reference topoisomer (Fig. 8B). Nonetheless, we conclude that UBF can wrap the 584-bp (60)8 probe at least three times in a right-handed direction to produce a positively supercoiled molecule upon ligation of the free ends of the DNA.

We next examined whether full-length UBF and the HMG box 1-plus-dimerization domain polypeptide could wrap DNA to the same extent. As shown in Fig. 9, the HMG box 1-plus-dimerization domain polypeptide produced the same +2 and +3 topoisomers that were produced by full-length UBF based on one-dimensional analysis of exonuclease III-treated ligation products. In a previous study we showed that one UBF dimer on average binds to two 60-bp enhancers in a (60)*n* array, such that we can expect four dimers to bind the (60)8 probe (47). The production of three supercoils by UBF on this probe therefore suggests that each dimer can bend the DNA approximately 274°, a value close to the predicted value of 260° if each HMG box contributes 130° of bending as in the protein lymphoid enhancer factor 1 (16).

Both eukaryotic topoisomerase I and prokaryotic topoisomerase II (DNA gyrase) were able to convert the UBF-induced (60)8 ligation products and the pooled ethidium bromide-induced reference topoisomers to relaxed (0) and +1 circular DNAs of the same mobility as the circular DNAs formed by ligation in the absence of UBF or ethidium (data

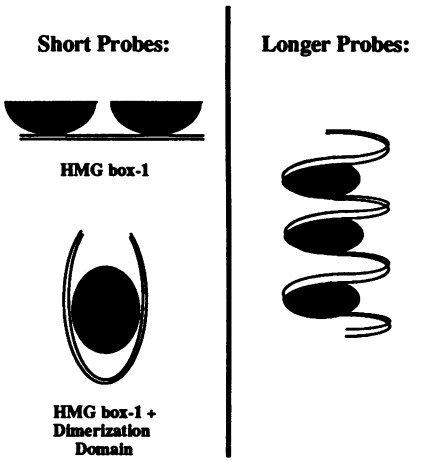

FIG. 10. Model for UBF binding. HMG box 1 can bind DNA but does not efficiently produce a circular product. When two HMG boxes are brought together through the action of the dimerization domain, DNA is stably bound and severely bent. On longer probes such as tandem enhancers arranged as in vivo, multiple UBF dimers can wrap the DNA into a right-handed coil.

not shown). Equal sensitivity to topoisomerases I and II further suggests that the UBF-induced (60)8 ligation products are simple positively supercoiled circles as opposed to knotted or other unusual structures.

DISCUSSION

UBF can bend and supercoil DNA. Using the (60)2 probe, on which a single UBF dimer can bind (47), we initially found that full-length UBF facilitated ligation of the probe into a relaxed circle (Fig. 2). In this regard, UBF is similar to other HMG box proteins that bind and bend DNA, such as HMG1, SRY, mtTF1, and lymphoid enhancer factor 1 (13, 14, 16, 39, 46). However, when longer probes were used, UBF organized the DNA such that self-ligation produced positively supercoiled molecules (Fig. 6 to 8). UBF could induce positive superhelicity by wrapping the DNA in a right-handed coil, by overtwisting the helix (the opposite effect of an intercalating agent such as ethidium bromide), or by stabilizing junctions where DNA strands cross one another. We favor the wrapping model (Fig. 10) because of UBF's ability to bend DNA to facilitate ligation of short probes into circles and because of the recently published electron spectroscopic images of Bazett-Jones et al. showing UBF-induced loops involving approximately 180 bp of DNA (2). It follows that long probes can be wrapped multiple times, resulting in supercoiling upon self-ligation.

Though our results are generally consistent with those of Bazett-Jones et al. (2), the direction of supercoiling that we demonstrate in this report is opposite the direction that these authors reported. Bazett-Jones et al. used DNA plasmids of several kilobase pairs to show that UBF at very high concentrations would stabilize negatively supercoiled DNA in the presence of topoisomerase. However, the protection of supercoils that they observed might not be due to DNA wrapping but might instead be due to UBF's ability to bind sites where two DNA strands cross one another. In fact, Hu et al. showed recently that UBF binds preferentially to either positively or negatively supercoiled plasmid DNA over relaxed DNA, presumably because both types of supercoiled DNA contain similar crossover junctions (18). Bazett-Jones et al. also showed that UBF could introduce supercoils into relaxed circular plasmid DNA in the presence of topoisomerase I (2). However, the direction of supercoiling is impossible to discern from the one-dimensional gel shown in their report because the negatively supercoiled plasmid DNA, the relaxed circular DNA, and the UBF-induced topoisomers all comigrate at the concentration of chloroquine used. This result could be the sum of slowed mobility of the negatively supercoiled plasmid control in the presence of the intercalating agent, increased mobility of the relaxed DNA molecules that become positively supercoiled in the presence of the drug, and unaltered mobility of already positively supercoiled topoisomers induced by UBF. Chloroquine is an intercalating agent that has the same effect as ethidium bromide but binds more weakly to DNA, making it preferable for analysis of numerous topoisomers of large DNA molecules such as plasmids but less useful for small molecules such as those that we have studied. Therefore, the use of chloroquine in the study of Bazett-Jones et al. (2) and ethidium bromide in our study cannot explain the different interpretations. Our use of short DNA fragments, a defined set of reference topoisomers, and two-dimensional electrophoretic analysis clearly shows that the UBF-induced topoisomers produced in the ligation assay are positively supercoiled.

Our simple model shown in Fig. 10 is similar to two others proposed for UBF interactions with the core promoter and the enhancers (2, 29). However, in these previously published models, the DNA is coiled in a left-handed direction and three to five HMG boxes of each UBF monomer are thought to be in direct contact with the DNA. In contrast, our model for UBF binding to the enhancers has the direction of coiling reversed (right-handed), and we detect no evidence for a direct role of HMG boxes other than HMG box 1 in binding, bending, or coiling of enhancer DNA. We suggest that alterations in the DNase I footprints that occur in xUBF upon removal of the acidic tail or downstream HMG boxes (Fig. 5 and references 2, 18, and 29) might be explained by changes in the folding of HMG box 1 when interactions with other domains are disrupted. Leblanc et al. have proposed that the UBF molecule is highly folded such that the acidic tail is probably in close proximity to the amino terminus and HMG box 1 (29). We find this hypothesis attractive, in part because we have found that antibodies raised against the fusion protein C-328 (the amino-terminal half of UBF; Fig. 1) can block phosphorylation of UBF's carboxyl-terminal acidic tail (35, 36, 63) by casein kinase II in vitro (10a). Stabilization of adjacent positively charged HMG domains by a shared interaction with the acidic tail is also intuitively appealing. Consequently, removing the acid tail or other downstream domains of UBF could change the DNase I footprint of HMG box 1.

UBF requires the dimerization domain and HMG box 1 to bind, bend, and coil DNA. Dependence on a distinct dimerization domain for UBF to efficiently bind and bend DNA may distinguish UBF from other HMG box proteins. With the possible exception of domain A of HMG1, which was unable to accelerate ligase-mediated circularization on its own (39) but which lacked residues toward the carboxyl terminus of the HMG box that may have been critical for the folding of the domain (49, 64), isolated HMG boxes can often bind and bend DNA (13, 14, 16, 39, 46). Furthermore, SRY and lymphoid enhancer factor 1 are thought to exist in solution and bind DNA as monomers (13, 16). It bears repeating that the weak DNA circularization activity of HMG box 1 on its own (Fig. 3B) is consistent with these other reports that individual HMG boxes can bend DNA, though in the case of UBF the extrinsic dimerization domain is clearly critical to strong DNA binding

(Fig. 5) and strong bending (Fig. 3B). A relevant observation is that the individual HMG boxes of HMG1 behave as dimers in solution (6). This finding suggests that HMG boxes themselves might interact when bound to DNA. If so, Hu et al.'s observation that UBF HMG box 1 can bind two pieces of DNA simultaneously may not be due to the binding of a single HMG box to two DNA molecules (18). Instead, an HMG box 1 dimer with each HMG domain bound to a separate piece of DNA could explain the data. Interaction of HMG box 1 polypeptides bound to DNA can also explain the bringing together of DNA fragments under dilute conditions to facilitate ligation of linear multimers (Fig. 3B).

Implications of DNA bending by UBF on enhancer and promoter function. In vitro, UBF binds cooperatively to the repeated enhancer elements located just upstream of the *X. laevis* ribosomal gene promoter (47) (Fig. 1) and footprints strongly on the analogous repetitive enhancers in mouse cells (43). The *Xenopus* enhancers are thought to act by facilitating transcription complex assembly (12, 37), but their precise mechanism of action remains unknown (see reference 50 for a review).

One possibility suggested by our finding that UBF can alter the topology of enhancer arrays is that UBF binding might displace nucleosomes or prevent nucleosome assembly. UBF appears to wrap linear enhancer DNA in a right-handed direction. This is opposite the direction of DNA wrapping around a histone octamer. It is tempting to speculate that UBF may prevent histones from binding and repressing an rRNA gene or that UBF may be capable of removing histones from key regulatory regions. The demonstration that UBF can prevent histone H1-mediated repression of ribosomal gene transcription is consistent with such a role (24), as is the observation that ordered nucleosomes are apparently absent from transcriptionally active rRNA genes (9). A structural role for UBF is also consistent with its abundance. UBF has been calculated to be present at 10,000 to 75,000 molecules per cell, depending on estimates of overall yield during purification (31, 42). Our data suggest that only the dimerization domain and HMG box 1 are required for DNA binding and bending, with no detectable role in DNA binding for the other HMG boxes. Interestingly, a recent report suggests that multiple HMG boxes are required for physical interaction between UBF and a specific subunit of RNA pol I (55). This finding suggests that if HMG box 1 contacts and bends the DNA, other HMG boxes may be available to interact with polymerase or other proteins.

As a transcription factor that binds the rRNA gene promoter, UBF's DNA bending properties suggest a possible similarity with DNA bending by TBP. Recent cocrystal structures of TBP bound to a TATA box recognized by the RNA pol II transcription machinery show that TBP bends the DNA dramatically (22, 23). No TATA box is present in the pol I promoters of most vertebrates, but TBP is known to be part of the multiprotein pol I transcription factor SL1 (8), which interacts with UBF to form extended DNase I footprints on the human, mouse, or *Xenopus* promoters (3–5, 8). UBF, like other HMG box proteins (16, 61) and TBP, apparently interacts with the minor groove (10). It is plausible that the pol I promoter, lacking a TATA box, uses UBF to bind the minor groove and bend the DNA, perhaps with the help of SL1, as a prerequisite to preinitiation complex assembly. Such bending may be crucial to allow direct interactions among transcription factors bound to proximal and distal promoter elements. Indeed, spacing changes that move the proximal and distal promoter domains out of register by half-helical turns severely alter rRNA gene promoter activity, suggesting a critical interaction between upstream and downstream promoter domains (38, 65). UBF may facilitate bending or wrapping of the promoter to allow interaction of upstream and downstream promoter domains and associated proteins. In this regard, UBF may function much like prokaryotic integration host factor, which also interacts with the minor groove and bends DNA to facilitate protein-protein interactions (15, 66).

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