The first high-mobility-group box of upstream binding factor assembles a cross-over DNA junction by basic residues

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Upstream binding factor (UBF) is a eukaryotic RNA polymerase I-specific transcription factor. Its predominant DNA-binding motif, ubfHMG box 1, preserves DNA assembling activity that can bind two or more DNA duplexes simultaneously to form a crossover DNA junction. Here we investigate the basis of cross-over DNA-assembling activity of ubfHMG box 1 by extensive mutagenesis analyses and mobility shift assay. Although the ubfHMG box 1 preserves a high mobility group (HMG) core structure, changing a number of the consensus hydrophobic and aromatic residues to alanine did not inhibit its crossover-assembling activity. This indicates that these residues do not directly participate in protein–DNA interaction. However,

altering a series of basic residues in the helices 1 and 2 regions or the N-terminal extended strand of the ubfHMG box 1 motif had severe effects on DNA-assembling activity; however, certain non-specific DNA binding activity still remained. This suggests that the ubfHMG box 1 motif might extensively contact the backbone of a crossover junction through its multiple basic residues. Mutating a hydrophobic residue in the terminal dimerization domain inhibited the association of truncated *Xenopus* UBF, but had little effect on its crossover-assembling activity. This indicates that the UBF–crossover DNA complex is not established by the association of individual DNA-bound peptides.

INTRODUCTION

Upstream binding factor (UBF) is an RNA polymerase I-specific transcription factor that co-operates closely with a TATA-binding-protein-associated complex, SL1, to establish a stable preinitiation complex in the promoter region of rDNA genes [1–3]. The cDNA of UBF has been cloned from a number of vertebrate species, including human [4], rat [5], mouse [6] and frog [2,7]. The peptide contains a cluster of high-mobility-group (HMG) box-specific DNA-binding domains plus an N-terminal dimerization domain and a C-terminal acidic tail. The ubfHMG boxes share a number of hydrophobic and bulky aromatic residues with members of the HMG-box superfamily, such as lymphoid enhancer-binding factor LEF-1, sex-determining factor SRY, and the non-histone nuclear protein HMG-1 (Figure 1) [4,8–10]. Native UBF molecules are organized in a dimeric form by their N-terminal 107-residue dimerization domain [11–13]. To support optimal ribosomal gene transcription, the UBF and redundant rDNA repeats are assembled together in nucleolar organizing regions. Without specific nucleolar targeting signals, the HMG box 1 motif and the C-terminal acidic residues are required for nucleolar accumulation [14].

A highly conserved conformation of the HMG motif has been determined by NMR analyses in a number of HMG-box proteins [9,15–20]. Basically, the secondary structure of this motif consists of three non-contiguous helices and two extended termini. The feature of the HMG box–DNA complex has been investigated by multi-dimensional NMR analyses [17,18,20]. It seems that the HMG motif contacts the minor groove of the DNA duplex via the concave face of the L-shaped configuration.

Xenopus UBF (xUBF), like many other HMG-box proteins, recognizes a number of irregular DNA structures, such as four-way junctions (cruciforms) or cisplatin-intercalated kinks [21–25]. It also exhibits a highly specific affinity for supercoiled DNA [26]. These similar structure-specific DNA-binding activities probably all result from a common protein structure. However, by using two-dimensional electrophoresis mobility-shift assay and electron micrograph image analysis, Hu et al. [26] revealed that the UBF also preserves DNA-assembling activity. It was shown that the UBF could contact two or more DNA duplexes simultaneously, with low sequence specificity, to build a crossover DNA junction by its predominant DNA-binding domain, ubfHMG box 1. This was the first HMG motif to be characterized as a DNA-assembling factor. HMG 1 has been found to preserve similar DNA-assembling activity, as seen in electron microscope images [27]. Here we investigate the basis of the ubfHMG box 1–crossover DNA complex by site-directed mutagenesis and DNA mobility-shift analysis. Our results indicate that ubfHMG box 1 extensively contacts the backbone of the DNA junction via its multiple basic residues. This DNA assembling activity does not rely on protein–protein association.

MATERIALS AND METHODS

Protein expression and structure prediction

The recombinant ubfHMG box 1, which spans residues His¹⁰⁸–Lys¹⁹¹ of xUBF, was derived from full-length xUBF cDNA [26]. The recombinant dbHMG1 peptide contains the N-terminal 191 amino acid residues of xUBF. Site-directed mutagenesis was performed by a PCR-mediated method [28]. The recombinant

Abbreviations used: dbHMG1, peptide containing the N-terminal 191 residues of xUBF; EGS, ethylene glycol bis(succinimidylsuccinate); HMG, high mobility group; ubfHMG box 1, peptide containing the first xUBF HMG box; xUBF, *Xenopus* upstream binding factor.

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Figure 1 Sequence alignment of ubfHMG box 1 domain

Upper panel: diagram of xUBF structure. Full-length xUBF contains five HMG boxes flanked by an N-terminal dimerization domain and a C-terminal acidic tail. Recombinant ubfHMG box 1 spans the xUBF residues His¹⁰⁸–Lys¹⁹¹. Lower panel: sequence alignment of ubfHMG box 1 with members of the HMG-box superfamily. Abbreviations: ratHMG1A and ratHMG1B, the two HMG-boxes of rat non-histone nuclear protein HMG-1; HMG-D, *Drosophila* HMG protein; lefHMG and sryHMG, the HMG boxes of lymphoid enhancer-binding factor LEF-1 and sex-determining factor SRY respectively; a, aromatic residues; h, hydrophobic residues Leu, Ile, Val. The consensus HMG-domain residues are capitalized. Residues of the hydrophobic core in a conventional HMG domain are indicated by filled boxes, including the four aromatic residues at positions 12, 15, 45 and 56. The three non-contiguous helices are underlined. The secondary structure of ubfHMG box 1 was predicted by the EMBO-PHD program [30–33]. We speculate that the ubfHMG box 1, like other HMG domains, contains three putative helices (indicated by boxes) that span residues 119–134, 141–152 and 159–181 respectively. The crossover-assembling activity of those ubfHMG box 1 substitution derivatives is summarized in the top column. The mutations that did not alter crossover-binding specificity are labelled with open diamonds; mutations that defeated crossover affinity are labelled with filled diamonds and bold italic lettering in the sequences.

peptides were expressed in *Escherichia coli* BL21(DE3) strain by using the pET11d-derived expression vector pET6H [26]. Consequently, all recombinant proteins started with an MHHHHHHAMG/V decapeptide tag sequence and ended with G/RSGC residues. Recombinant HMG1-B, which includes residues Ala⁹²–Gly¹⁷⁴ of human HMG-1, was derived from full-length hHMG1 cDNA [29]. The secondary structures of the native ubfHMG box 1 peptide and its residue-substitution derivatives were predicted by the EMBO-PHD program [30–33].

Peptide purification was performed by Ni-nitrilotriacetic acid nickel-affinity chromatography as described elsewhere [26]. The eluted peptides were dialysed against 25 mM Hepes (pH 7.9)/20% (v/v) glycerol/100 mM KCl/0.2 mM EDTA/1 mM dithiothreitol. When dialysis was completed, samples were divided into aliquots and stored at -70°C .

Probe preparation and DNA mobility-shift assay

A 136 bp *PvuII*–*XhoI* fragment derived from pBluescript SK – (spanning nt 532–668) was used as a probe [26]. Gel-purified fragments were 5' end-labelled with [γ -³²P]ATP by using T4 polynucleotide kinase. The DNA-binding activity assay was performed as described previously [13,26]. The recombinant protein was mixed with the DNA probe in 8% (v/v) glycerol/40 mM KCl/10 mM Hepes (pH 7.9)/1 mM dithiothreitol/0.04 mM EDTA in a total volume of 10 μl and then incubated on ice for 20 min. The protein/DNA mixture was loaded on 4% (w/v) polyacrylamide gel (acrylamide-to-bisacrylamide ratio 29:1) in 25 mM Tris/borate buffer. The gel was run for 4 h at 13 V/cm and 4 $^{\circ}\text{C}$. After electrophoresis was complete, the gel was dried for autoradiography.

Chemical cross-linking

Ethylene glycol bis(succinimidylsuccinate) (EGS) was purchased from Pierce. The procedure of EGS cross-linking was followed as suggested by the manufacturer [13]. Protein was suspended in a reaction buffer containing 150 mM NaCl, 25 mM sodium phosphate and 10% (v/v) glycerol, pH 7.8. Cross-linking was performed by adding 10 μl of 100 mM EGS (in DMSO); the sample was then incubated for 30 min at room temperature. The reaction was stopped by adding 50 μl of 1 M Tris/HCl, pH 7.5, and the sample was incubated for an additional 15 min at room temperature. The protein was precipitated by 20% (w/v) trichloroacetic acid followed by two acetone washes. The cross-linked protein was analysed by SDS/PAGE.

RESULTS

Mutational analyses of ubfHMG box 1

To determine the physical boundary of the ubfHMG box 1 domain, we made a series of truncated ubfHMG box 1 peptides from which various regions of the ubfHMG box 1 motif were deleted. For instance, residues His¹⁰⁸–Phe¹²⁰, His¹⁰⁸–Ser¹³⁸, His¹⁰⁸–Glu¹⁵², Arg¹⁸⁰–Lys¹⁹¹ and Arg¹⁶⁷–Lys¹⁹¹ of ubfHMG1 were deleted to form the derivatives Δ 13, Δ 31, Δ 45, Δ 12 and Δ 25 respectively. Figure 2(A) shows that the wild-type ubfHMG box 1 bound the DNA probes and produced a number of shift bands that ran much more slowly than the conventional 1:1 protein–DNA complex. By using a mixture of different-sized DNA as probes, Hu et al. [26] showed that each of these slow-shift protein–DNA complexes contains two or more DNA

Figure 2 DNA-binding activity of truncated derivatives of ubfHMG box 1

The DNA-binding activity of recombinant ubfHMG box 1 (**A**) and its truncated derivatives CΔ12 (**B**), CΔ25 (**C**), NΔ13 (**D**), NΔ31 (**E**) and NΔ45 (**F**) were examined by DNA mobility-shift assay. The first shift band represents a single crossover; the higher shift bands represent the aggregates of dual or higher-level DNA junctions. The DNA-binding activity of the recombinant HMG-1B motif was also examined (**G**). The DNA concentration used in each reaction was 0.15 nM. Abbreviation: Ctl, free probe.

Figure 3 Site-directed mutagenesis analysis of ubfHMG box 1

The DNA-binding activity of ubfHMG1 and its residue-substituted derivatives was examined by DNA mobility-shift assay as described in the legend to Figure 2. (**A**) Alanine substitution on the conserved hydrophobic core Tyr¹¹⁹, Phe¹²², Tyr¹⁵⁰ and Tyr¹⁶¹ and consensus residues Pro¹¹², Pro¹¹⁵, Pro¹³⁵, Phe¹⁷² and Phe¹⁷⁹. (**B**) Alanine substitution for basic residues Lys¹¹³-Lys¹¹⁴, Arg¹²⁷, Lys¹⁴⁴, Lys¹⁴⁶, Lys¹⁴⁹, Lys¹⁵¹, Lys¹⁵⁶-Lys¹⁵⁷, Lys¹⁵⁸, Arg¹⁶⁷-Lys¹⁶⁹, Lys¹⁷⁴ and Lys¹⁷⁸-Lys¹⁸⁰. The DNA concentration used in each reaction was 0.15 nM.

Figure 4 Hypothetical structure of ubfHMG box 1

A highly compact structure of the HMG box is conserved in a number of HMG-containing proteins. It has been speculated that ubfHMG box 1 also preserves a similar structure [44]. The three-dimensional structure of the HMG box B of HMG1 is illustrated here as a representative of the HMG box [16]. Two residues of the putative hydrophobic core, Phe¹²² and Tyr¹⁵⁰, are circled between the helices. Alanine substitution at these positions caused the ubfHMG box 1 to lose DNA-assembling activity. Basic residues in ubfHMG box 1 that are important for DNA-assembling activity are labelled with filled circles. These DNA-contact residues are distributed in helices 1 and 2 and the N-terminal extended strand.

duplexes. Consequently the aggregated DNA duplexes slowed down the mobility of the protein–DNA complexes considerably in the electrophoresis field and gave a high-shift pattern. Figures

2(B) to 2(F) show that only the CA12 peptide could produce a similar high-shift pattern to that of ‘wild-type’ ubfHMG1 except with a weaker binding affinity. The rest of the truncated derivatives, including NΔ13, NΔ31, NΔ45 and CΔ25, failed to assemble the crossover DNA junction, except for weak, non-specific binding activity resulting from incomplete motif structures. In connection with these results, we have defined a minimum ubfHMG box 1 motif that spans 72 residues of the xUBF molecule between His¹⁰⁸ and Phe¹⁷⁹. Figure 2(G) shows that the recombinant hHMG1-B peptide bound the DNA in a non-specific manner and produced multiple low-shift ladders similar to those of a defective ubfHMG box 1 peptide. This indicates that the recombinant hHMG1-B motif alone is not sufficient to assemble a DNA intersection.

To investigate the molecular basis of the crossover-DNA assembling activity of the ubfHMG box 1 motif, we made a series of residue-substitution derivatives in which the conserved hydrophobic core and bulky aromatic residues Pro¹¹², Pro¹¹⁵, Tyr¹¹⁹, Phe¹²², Pro¹³⁵, Tyr¹⁵⁰, Tyr¹⁶¹, Phe¹⁷² and Phe¹⁷⁹ were replaced individually with alanine. Figure 3(A) shows that residues Phe¹²² and Tyr¹⁵⁰ in the ubfHMG box 1 motif were indispensable for its DNA-assembling activity. Altering residue Pro¹¹⁵ greatly decreased crossover-assembling activity and produced a series of non-specific low-shift bands. However, altering the rest of the selected consensus hydrophobic and aromatic residues did not change the specificity of the DNA assembling activity, but decreased its binding affinity. We speculate that these hydrophobic and aromatic residues do not participate directly in protein–DNA interaction.

Compared with other HMG motifs, the helices 1 and 2 regions of the ubfHMG box 1 contain more basic residues (32 %) than do the rest of the HMG motifs (12–26 %) (Figure 1). To

Figure 5 Protein–protein association is not required for establishment of the ubfHMG box 1–crossover DNA complex

A truncated xUBF, dbHMG1, was expressed as a recombinant peptide spanning xUBF residues Met¹–Lys¹⁹¹. (A) EGS cross-linking analysis of dbHMG1 and its residue-substituted derivatives. Recombinant protein (15 µg) was cross-linked by EGS as described in the text, and cross-linked proteins were analysed by SDS/PAGE. Lanes 1 and 2, wild-type dbHMG1; lanes 3 and 4, (Leu³⁸ → Glu)dbHMG1 (L38EdbHMG1); lanes 5 and 6, (Ile⁷⁵ → Glu)dbHMG1 (I75EdbHMG1). Odd lanes, protein before cross-linking with EGS; even lanes, protein after cross-linking with EGS. (B) DNA-binding activity of wild-type dbHMG1 and its residue-substituted derivatives. The DNA-binding activity of the recombinant peptides was analysed by DNA mobility-shift assay as described in the text. The low-shift bands between the crossover-type high-shift bands and free probe represent a 1:1 protein–DNA complex that was formed by the non-specific protein–DNA interactions resulting from the cluster of basic residues in front of the ubfHMG box 1 [13]. The probe concentration in each reaction was 0.35 nM. Abbreviation: Ctl, free probe.

investigate the roles of these basic residues in DNA-assembling activity, we made a series of substitution mutants in which residues Lys¹¹³-Lys¹¹⁴, Arg¹²⁷, Lys¹⁴⁴, Lys¹⁴⁸-Lys¹⁴⁹-Lys¹⁵¹, Lys¹⁵⁶-Lys¹⁵⁷, Lys¹⁵⁸, Arg¹⁶⁷-Lys¹⁶⁹, Arg¹⁷⁴ and Lys¹⁷⁸-Arg¹⁸⁰ were replaced individually or simultaneously with alanine. Figure 3(B) shows that only the Arg¹⁶⁷, Lys¹⁶⁹ → Ala, Arg¹⁷⁴ → Ala and Lys¹⁷⁸, Arg¹⁸⁰ → Ala derivatives, which are all positioned in helix 3, still preserved certain DNA-assembling activity and gave the high-shift pattern. It also shows that Lys¹⁴⁴ → Ala (positioned in helix 2) gave a distinct shift pattern that might result from an altered DNA-binding property. The rest of the substitution derivatives in the extended termini and helices 1 and 2 regions failed to assemble the crossover complex and only produced a number of non-specific low-shift bands. However, the conformation of these substitution derivatives did not exhibit significant changes, as evaluated by the EMBO-PHD secondary structure prediction program (results not shown). It seems that the multiple basic residues of ubfHMG box 1 in the N-terminal extended strand and the helices 1 and 2 regions might interact extensively with the backbone of a crossover DNA junction to give a solid protein-crossover DNA complex (Figure 4).

Protein-protein association is not required for ubfHMG box 1-crossover interaction

Native UBF molecules are organized in a dimeric form by the N-terminal dimerization domain. Mutation of the hydrophobic residues in the hydrophobic face of the multiple amphipathic helices in the dimerization domain inhibited peptide dimerization and consequently abolished its transactivation activity in rRNA transcription [13,26]. A question arises of whether crossover DNA-assembling activity of the ubfHMG box 1 is executed by the association of individual DNA-bound peptides. Here we made a truncated xUBF (dbHMG1) peptide that spanned the N-terminal 191 residues of xUBF, including a complete dimerization domain plus a lysine-rich basic domain (corresponding to residues 1–107) and an intact HMG box 1 (corresponding to residues 108–191) [26]. The feature of peptide association was analysed by EGS cross-linking. Figure 5(A) shows that wild-type dbHMG1 peptides were held in a dimeric form (lanes 1 and 2). However, altering residues Leu³⁸ and Ile⁷⁵, both in the dimerization domain, to glutamic residues inhibited peptide dimerization (lanes 3–6). The DNA-binding activities of these dbHMG1-substitution derivatives were examined by DNA mobility-shift analysis. Figure 5(B) shows that both (Leu³⁸ → Glu)dbHMG1 and (Ile⁷⁵ → Glu)dbHMG1 derivatives still preserved crossover-assembling activity and produced a similar high-shift pattern to that of wild-type dbHMG1. Apparently, defeating the dimerization domain in an xUBF derivative did not inhibit its crossover-assembling activity. This indicates that the formation of the crossover DNA junction by the ubfHMG box 1 does not rely on protein-protein association.

DISCUSSION

The ubfHMG box 1, like many other HMG motifs, recognizes a number of irregular DNA structures, such as cisplatin-intercalated DNA kinks and flat four-way junctions (cruciforms), with low sequence specificity [21–23]. It is speculated that these structure-specific DNA-binding activities all probably result from a common configuration preserved in various HMG motifs. Indeed, NMR studies of a number of HMG motifs have revealed a common conformation composed of three helices and two extended termini. This compact structure is stabilized by a number of intramolecular hydrophobic interactions [34]. It has

been found that the ubfHMG box 1 motif also preserves DNA-assembling activity that can bind two or more duplexes simultaneously to form a crossover junction [26]. Here we show that changing a number of the conserved hydrophobic and aromatic residues in the ubfHMG box 1 did not inhibit its DNA-assembling activity. This indicates that these hydrophobic and bulky aromatic amino acids are not directly involved in DNA-assembling activity. Similar results were also obtained in other HMG motifs. For instance, changing the conserved residues Phe¹⁹, Trp⁴⁹ and Tyr⁷¹ in the HMG1A motif decreased its protein stability but did not impair its DNA-binding specificity [35]. This is consistent with the previous observation that hydrophobic and bulky aromatic residues in the HMG motif serve critical roles in protein stability but do not directly participate in the DNA-binding activity [36]. Nevertheless, the failure of DNA assembly in the ubfHMG mutants Phe¹²² → Ala and Tyr¹⁵⁰ → Ala might arise from their impaired structures.

Compared with other HMG motifs, ubfHMG box 1 contains more basic residues than other motifs in helices 1 and 2 regions. Here we show that changing a number of these basic residues in helices 1 and 2, but not in helix 3, causes ubfHMG box 1 to fail to assemble the DNA junction. The structural impacts of these substitutions were evaluated by EMBO-PHD secondary structure prediction analysis. It seems that these substitutions in the ubfHMG box 1 did not change the conformation significantly. This speculation is consistent with the previous observation that the structure of HMG motifs is stabilized exclusively by the hydrophobic and polar residues [34,36]. Consequently we speculate that the multiple basic residues, especially those in helices 1 and 2 and the N-terminal extended strand in the ubfHMG box 1, might serve essential roles in extensively contacting the backbone of the DNA junction. However, we do not exclude the possibility that the failure of DNA assembling activity in some of these substitution derivatives (for instance Lys¹⁴⁴ → Ala) might result indirectly from the defeated conformation.

Native UBF is organized in a dimeric form in solution by the close hydrophobic interactions of the N-terminal dimerization domain [11]. It is not yet clear whether this N-terminal region of UBF participates in DNA-binding activity. Nevertheless, we demonstrate here that the DNA-assembling activity of UBF is independent of the protein-protein association. However, the residues in the dimerization domain of UBF are still indispensable for rRNA transcription [11]. Replacing or deleting the mid-region of the dimerization domain could abolish rRNA transcription activity completely, even it had no effect on peptide association. This indicates that the N-terminal region of UBF serves critical roles in rRNA transcription. It has been shown that the terminal dimerization domain of UBF is required to bend, wrap and supercoil the rDNA [37]. Apparently, more work is needed to understand the basis of the terminal dimerization domain in the UBF-mediated rRNA transcription.

Compared with sequence-specific HMG motifs, none of the base-contacting residues, such as residues Asn³⁰², Met³⁰⁵, Met³⁰⁸, Glu³²³, Ala³²⁵, Asn³²⁸ and Tyr³⁷⁰ in LEF-1 [18], and Asn⁸², Phe⁸⁴, Ile⁸⁵, Ser¹⁰⁵, Ile¹⁰⁷, Ser¹⁰⁸ and Tyr¹⁴⁶ in SRY [20], were preserved in the ubfHMG box 1 motif; neither did they have any significant role in the crossover-assembling activity. This suggests that the basis of crossover DNA-assembling activity of ubfHMG box 1 is quite different from that of sequence-specific HMG motif-linear DNA interactions.

In mammalian cells, UBF molecules and the redundant rDNA repeats are assembled together in the nucleolus. Without nucleolar targeting signals, the ubfHMG box 1 motif and C-terminal acidic domain are required for nucleolar accumulation [14]. Although the features of rDNA organization are not yet

clear, recent studies have suggested that UBF might act as an architectural factor that can assemble rDNA species effectively to give higher-order forms of nucleoprotein complexes in the nucleolus [37,38]. In addition to UBF, a similar crossover-binding or crossover-assembling activity is also preserved in other non-HMG-box DNA-binding proteins, such as a variety of DNA junction-resolving enzymes and the linker histone H1. The histone H1 is a nucleosome-compacting factor that interacts with DNA junctions flanking the histone cores by its basic-rich C-terminal residues [39–41]. Here we speculate that the UBF might act like H1 to assemble the redundant rDNA repeats effectively in the nucleolar region. To support this speculation, it was discovered that the UBF could displace linker histone H1 effectively from its binding site on a preassembled nucleosome *in vitro* [42]. In the DNA polymerase I-specific transcription, the UBF acted as an anti-repressor both *in vivo* and *in vitro* to counteract histone H1-mediated repression in ribosomal gene transcription [23]. In addition, various types of UBF-induced DNA looping were observed *in vitro* by electron microscopic analyses [26,38,43].

In addition to UBF, HMG-1 also preserves a similar crossover-assembling activity, as seen on electron microscopic images [27]. At high ratios of protein to DNA, full-length HMG-1 assembled a number of DNA duplexes to produce various types of protein–DNA complex image. However, our DNA mobility-shift analysis shows that the recombinant HMG-1B motif alone did not produce a crossover-type high-shift pattern (Figure 2G). This suggests that HMG-1 might need both HMG boxes (A and B) to hold the arms of the DNA junctions effectively.

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