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Analysis of the phosphorylation, DNA-binding and dimerization properties of the RNA polymerase I transcription factors UBF1 and UBF2

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ABSTRACT

The phosphorylation, DNA-binding and dimerization properties of both forms of the RNA polymerase I transcription factor UBF were studied and compared. Tryptic peptide maps of *in vivo* ^{32}P-labeled UBF contained four phospho-peptides. Two of these peptides are predicted to derive from the serine-rich, carboxyl-terminal of UBF. This region contains nine consensus phosphorylation sites for casein kinase II, and is one of the regions phosphorylated *in vitro* by casein kinase II. Analysis of the DNA-binding properties of recombinant forms of UBF1 and UBF2 by Southwestern blots revealed: (1) a role for the NH_2-terminal 102 amino acid domain of UBF1/UBF2 in DNA-binding; (2) the importance of the bases from −106 to −101 of the rat ribosomal DNA promoter for the binding of UBF; and (3) functional differences between UBF1 and UBF2. Glutaraldehyde cross-linking and overlay assays using recombinant forms of UBF1 and UBF2 demonstrated that the molecules can form both homodimers and heterodimers. These assays also demonstrated that the NH_2-terminal 102 amino acids of UBF plays a significant role in dimerization and that other domains contribute to dimerization. The dimerization properties of recombinant forms of UBF1 and UBF2 were different, suggesting that the HMG box 2 of UBF1, which is partially deleted in UBF2, also contributes to UBF dimerization.

INTRODUCTION

Transcription of the tandemly-repeated eukaryotic genes coding for ribosomal RNA involves protein:protein and protein:nucleic acid interactions between the rDNA promoter, RNA polymerase I and at least two defined transcription factors, SL-I and UBF (1–3). Whereas little detailed information is available on the structure and identity of SL-I and RNA polymerase I, considerable progress has been made on the identification and characterization of UBF. UBF has been purified from human (2), rat (1), mouse (3) and *Xenopus laevis* (4,5). In each case the factor purifies as a dimer. The human, rat and mouse proteins are 97 and 94 kDa, whereas the *X.laevis* forms are 82 and 85 kDa. More recently the cDNAs coding for 2 forms of UBF have been cloned from rat (6), human (6,7), mouse (6,8) and *X.laevis* (9–11). The two forms of human, rat and mouse UBF, UBF1 and UBF2, are almost identical and consist of an NH_2-terminal domain of 102 amino acids, succeeded by 4 domains called the HMG boxes and a serine-rich, acidic carboxyl tail (6–8). UBF2 has a deletion of 37 amino acids in HMG box 2 in comparison to UBF1 (6,8). Whereas the two forms of the mRNAs coding for rat, human and mouse UBF are believed to arise by differential splicing (6,8), the *X.laevis* forms of UBF are coded by two different genes (11).

We have recently established that UBF1 and UBF2 are phosphorylated *in vivo* on serine residues (12). Serum deprivation of Chinese Hamster Ovary (CHO) cells reduced the overall phosphorylation of UBF, had no significant effect on the total mass of UBF, and affected the subcellular distribution of UBF, with UBF redistributing between the nucleolus, the nucleus and the cytoplasm (12). Phosphatase-treated UBF demonstrated a significantly reduced ability to transactivate transcription *in vitro* (12). Taken together, these data suggested that the phosphorylation of UBF may be important in determining both the subcellular distribution of UBF and in regulating the transactivation properties of UBF.

The HMG boxes constitute the DNA binding domain of UBF (7). By UV-crosslinking experiments we have previously shown that both UBF1 and UBF2 are DNA-binding proteins as evidenced by their binding to the rat rDNA promoter (13).

In this manuscript we provide evidence that UBF is phosphorylated both *in vivo* and *in vitro* by casein kinase II. Tryptic peptide mapping of *in vivo* labeled UBF together with *in vitro* phosphorylation of recombinant UBF with casein kinase

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II has localized the site(s) of phosphorylation to both the serine-rich, acidic tail of UBF, which contains nine consensus casein kinase II recognition sites, and to the NH_2-terminal 102 amino acids of UBF. Analysis of the DNA binding properties and of the protein:protein interactions of UBF show that UBF1 and UBF2 differ in each of these properties.

MATERIALS AND METHODS

Materials

All radioactive compounds were purchased from NEN. DNA modifying enzymes and isopropyl-β-D-thio-galactopyranoside (IPTG) were purchased from Promega. PCR reagents were obtained from Perkin Elmer/Cetus. The expression vector pIH902 and the *Escherichia coli* host TB1 were from New England Biolabs. Low melt agarose was purchased from FMC. TPCK-treated trypsin and glutaraldehyde were purchased from Sigma. Protein size standards were purchased from BioRad and Sigma. Homogeneous *Drosophila* casein kinase II (CKII), was the kind gift of Dr Neil Osheroff, Department of Biochemistry, Vanderbilt University, Nashville, TN.

Methods

In vivo labeling, immunoprecipitation and tryptic peptide mapping of UBF

The culturing and *in vivo* labeling of CHO cells with [^{32}P]-orthophosphate, as well as the immunoprecipitation and blotting of UBF to nitrocellulose was performed essentially as described (12). Following blotting and autoradiography, the radiolabeled bands corresponding to UBF1 and UBF2 were excised and digested with TPCK-treated trypsin essentially as described (14). The digested products were fractionated by SDS-PAGE (15).

In vitro phosphorylation of UBF with casein kinase II

Either UBF purified from Novikoff hepatoma ascites cells (1) or recombinant maltose binding protein-UBF fusions (MBP-UBF, see below) were incubated in CKII assay buffer (16) (20mM Tris.Cl, pH8.0., 200mM NaCl, 0.5mM EDTA, 0.5mM dithiothreitol, 10mM $MgCl_2$, 0.05% Triton X-100, 10% glycerol) with casein kinase II (2μg/20μl reaction) and [γ-^{32}P] ATP (100 μCi) at 30°C for 20 min. The reaction mixtures were fractionated by SDS-PAGE (17), blotted onto nitrocellulose (18) and subjected to autoradiography.

Construction of MBP-UBF fusion proteins

A schematic representation of UBF1 and UBF2 is presented in Figure 2A together with the features of the expression vector pIH902 important for inducible expression of MBP-UBF fusion proteins in *Escherichia coli* (Figure 2C). A series of cDNAs coding for mutants of UBF1 and UBF2 were amplified by PCR using custom synthesized oligonucleotide primers and cloned in-frame into *BamH*1, *Sal*1 digested pIH902; the resulting series of constructs is presented in Figure 2B. For expression in the *Escherichia coli* host TB1, each clone was grown overnight at 37°C in LB, ampicillin (Ap) media (100μg/ml). The cultures were then diluted 1:50 into fresh LB, Ap media and grown at 37°C. After 2.5h, IPTG was added to 2mM final concentration and the cultures were grown at 37°C. After 2h, the cultures were centrifuged at 5,000 rpm (10 min), resuspended in solubilization buffer (17) (1/10th culture volume) and lysed by boiling at 100°C for 10 min. 10μl aliquots of each culture were analysed by SDS-PAGE (17). To prepare soluble proteins for *in vitro* labeling with casein kinase II, the cell pellets from induced cultures (1ml) were frozen at −70°C , thawed on ice, resuspended in 400μl CKII assay buffer, sonicated for 30 sec, and centrifuged at 15,000 rpm for 10 min. The resulting supernatants were stored at −70°C.

Southwestern blot analysis

Southwestern blotting was carried out essentially as described (18). To facilitate the analysis, 10μl aliquots of culture lysates of different size MBP-UBF fusion proteins were combined prior to electrophoresis on 10% SDS polyacrylamide gels. Following electrophoresis, the gels were blotted onto nitrocellulose (18), the filters were blocked in blocking buffer (1×PBS containing 2% BSA; 2×1 hr at room temperature), equilibrated in binding buffer (100mM Tris.Cl, pH 8.0, 10% glycerol, 50mM NaCl, 1mM $MgCl_2$, 0.1mM DTT; 2×30 min at room temperature) and incubated with the indicated probe (10^7 cpm) in binding buffer for 2 hours. The blots were then washed 2×20 min in 0.2M NaCl and subjected to autoradiography. The DNA probes used are presented schematically in Figure 2D. The DNA probes were prepared by PCR amplification from either the wild-type 45S promoter plasmid p5.1 E/X (1,19) or the linker-scanning mutant promoter BSM −106/ −101 (also called BSM 8) (1) using appropriate 5′ end-labeled primers. The amplified DNA probes were gel purified (18) from low melt agarose gels and resuspended in probe binding buffer.

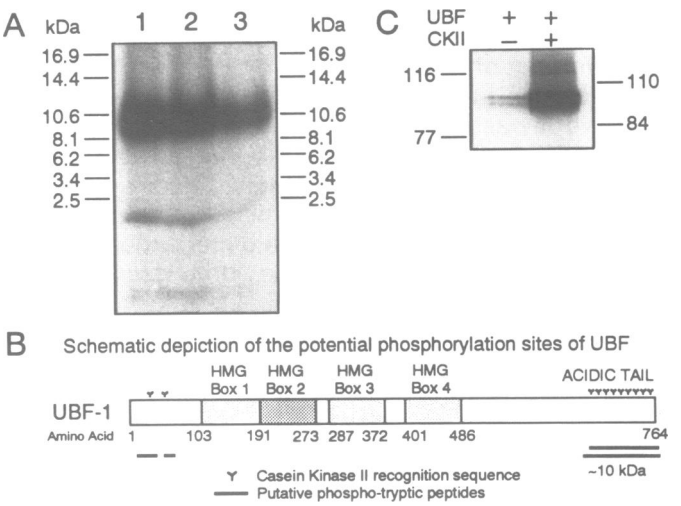

Figure 1. Analysis of the phosphorylation of UBF. (A) Tryptic peptide mapping of metabolically-labeled UBF. Following immunoprecipitation of ^{32}P−labeled UBF1/UBF2 from logarithmically growing (lane 1), serum-starved and refed (lane 2) and serum-starved (lane 3) CHO cells, the gel-purified UBF fractions were digested with TPCK-trypsin, and analyzed by SDS-PAGE (15) and by autoradiography. The positions of the molecular size standards are shown. (B) Schematic representation of the potential CKII phosphorylation sites in UBF. The potential CKII phosphorylation sites in UBF1 are shown together with the predicted phospho-tryptic peptides. Similar sites exist in UBF2 (not shown). (C) *In vitro* labeling of UBF with CKII. UBF, incubated with [γ-^{32}P] ATP in the absence (−) and presence (+) of CKII, was fractionated by SDS-PAGE and autoradiographed. The positions of the molecular size standards (in kDa) are shown.

DNase 1 footprinting on linker scanning mutants of the 45S rDNA promoter

The template derivatives of the 45S rDNA promoter used for DNase 1 footprinting were constructed from the plasmid p5.1 E/X (1,19). The linker scanning mutant series was constructed essentiallty as described (1,20). For DNase 1 footprinting, end labeled DNA probes were produced by labeling one of the two primers used to generate the PCR products to be footprinted with [γ-^{32}P] ATP and T4 polynucleotide kinase (18). The two primers used were 5'-CCATGGCCTCCTCGGTCT-3' (−208 to −191) and 5'-GGTGCAAGCCTCTTCCAACGTCC-3' (+61 to +39). The purification of the transcription factor UBF from Novikoff cells, and the conditions used for the DNase 1 footprinting, have been previously described (1).

Analysis of UBF dimerization by glutaraldehyde crosslinking

Soluble *Escherichia coli* cell lysates expressing the UBF1 and UBF2 derivatives 1−486 and 1−449, respectively, were incubated in 0.01% glutaraldehyde for 10 min at room temperature. Following the addition of lysine to 30 mM final concentration and solubilization buffer, the reaction mixtures were fractionated by SDS-PAGE, blotted onto Immobilon-P membrane and probed with the anti-UBF sera and [^{125}I]-goat anti-rabbit IgG, F(ab')$_2$ fragment, essentially as described (12).

Analysis of UBF-UBF interaction by overlay assays

Cell lysates of the IPTG-induced, recombinant MBP-UBF fusion cultures were fractionated by SDS-PAGE (17) and blotted onto nitrocellulose membranes (18). After the filters were equilibrated in buffer C20 (1) containing 1% w/w dry milk (2×1 hr at room temperature), they were overlayed for 2 hr in buffer C20/1% dry milk containing UBF which had been labeled *in vitro* with [γ-^{32}P] ATP by casein kinase II. The filters were then washed (3× 10 min in buffer C20/1% w/w dry milk and 3×10 min in buffer C20) and autoradiographed. To demonstrate that the radiolabel corresponded to labeled UBF, the radiolabeled bands on the filters were excised, boiled for 10 min in solubilization buffer, fractionated by SDS-PAGE, blotted onto nitrocellulose membrane and autoradiographed.

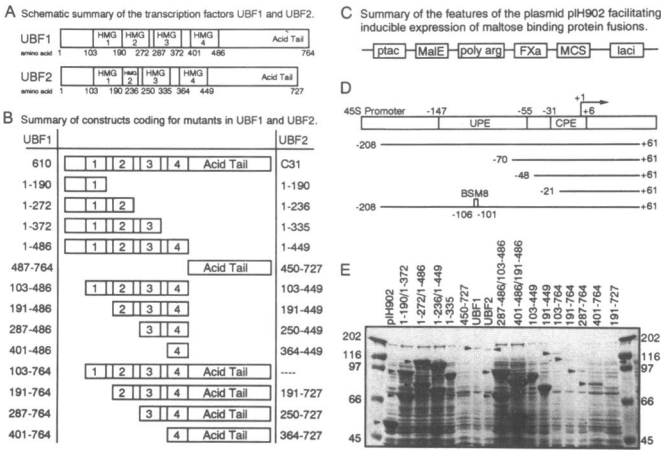

Figure 2. Construction and expression of mutants in UBF1 and UBF2. (A) A schematic representation of the transcription factors UBF1 and UBF2 is shown, detailing the amino acid positions of the 4 HMG boxes. (B) A summary of the constructs coding for mutants in UBF1 and UBF2. The numbers in the left and right hand columns represent the name of the mutants in UBF1 and UBF2, respectively. The four HMG boxes are denoted by the numbers 1,2,3 and 4 in the schematic. (C) A summary of the inducible expression vector pIH902 is shown. ptac represents the inducible tac promoter; MalE represents the coding region for the maltose binding protein (MBP); poly arg represents a run of 10 arginine codons; FXa represents the sequence coding for the amino acids ile.glu.gly.arg, the clevage site for the protease factor Xa; MCS represents a multiple cloning site; and laci represents the gene coding for the lacI repressor. (D) A summary of the rat 45S rDNA promoter is shown (top line) outlining the positions of the UPE, CPE and the transcription initiation site (+1). The series of DNA fragments, generated by PCR, used in the Southwestern blots are also shown. (E) SDS-PAGE analysis of the synthesis in *E.coli* of mutants in UBF1 and UBF2. The positions of the recombinant proteins in each lane are indicated by arrow heads. The constructs in each lane are indicated on the top line. Lanes 1 and 19, molecular size standards; lane 2, control MBP; lane 3, constructs 1−190 (bottom band) and 1−372 (top band); lane 4, constructs 1−272 (bottom) and 1−486 (top); lane 5, constructs 1−236 (bottom) and 1−449 (top); lane 6, 1−335; lane 7, 450−727; lane 8, UBF1 (610); lane 9, UBF2 (C31); lane 10, constructs 287−486 (bottom) and 103−486 (top); lane 11, constructs 401−486 (bottom) and 191−486 (top); lane 12, 103−449; lane 13, 191−449; lane 14, 103−764; lane 15, 191−764; lane 16, 287−764; lane 17, 401−764; lane 18, 191−727.

RESULTS

Phosphorylation of UBF by casein kinase II

We have recently shown that UBF1 and UBF2 are phosphorylated *in vivo* on serine residues (12) and that this phosphorylation is regulated in response to cell growth. We have analyzed the tryptic peptide map of *in vivo* [^{32}P]-orthophosphate-labeled UBF from logarithmically growing, serum starved, and serum starved and refed CHO cells. In each case we observed 4 tryptic peptides (Figure 1A), two of approximately 10 kDa size and 2 smaller peptides of less than 1 kDa size. The sequence of UBF predicts a large tryptic peptide of 97 amino acids corresponding to the C-terminal amino acids of UBF (Figure 1B). The two similarly sized 10 kDa tryptic peptides detected may arise either by partial trypsin digestion at the sequence LRGPNPKSSRTTLQSKSE (amino acids 622−639) which precedes the serine-rich, acidic

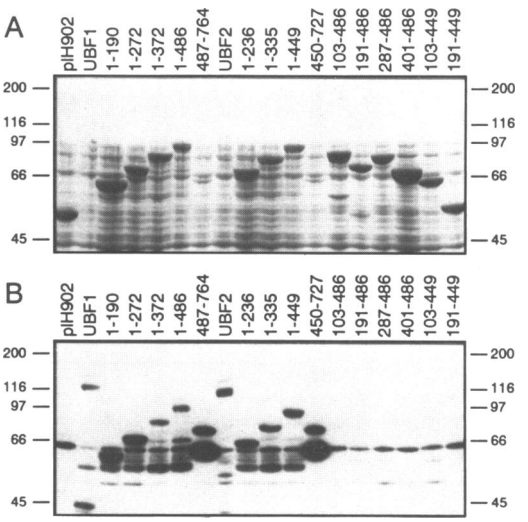

Figure 3. *In vitro* phosphorylation of mutants in UBF1 and UBF2 by CKII. (A) Total soluble lysates of *E.coli* expressing MBP:UBF fusion proteins were fractionated by SDS-PAGE and stained with Coomassie Blue. The identity of the MBP:UBF fusion protein expressed in each lysate is indicated as are the positions of the molecular size standards (in kDa). (B) Following treatment of soluble *E.coli* lysates with CKII, the lysates were fractionated by SDS-PAGE and autoradiographed. The MBP:UBF fusion lysates tested are indicated.

tail of UBF (and where the possible cleavage sites for trypsin are underlined) or by differential phosphorylation *in vivo* of one peptide. Examination of the predicted amino acid sequence for the serine rich, carboxyl-terminal acidic tail of UBF revealed 9 consensus casein kinase II recognition sites (XSXXD/E, where X is any amino acid) within this domain (Figure 1B) and we found that UBF, purified from Novikoff cells, can be phosphorylated *in vitro* by casein kinase II (Figure 1C).

To confirm that the acidic tail region of UBF can indeed be phosphorylated by casein kinase II, we analyzed the *in vitro* phosphorylation of truncated forms of both UBF1 and UBF2 by casein kinase II. We constructed a series of truncated forms of UBF1 and UBF2 fused to the maltose binding protein (summarized in Figure 2B), expressed the recombinant fusion proteins in *E. coli*, and tested their phosphorylation by CKII. For reference, we have presented a Coomassie Blue stained gel of the recombinant clones tested with CKII (figure 3A) and the corresponding autoradiogram following labeling with CKII and [γ-^{32}P] ATP (Figure 3B). Comparision of Figures 3A and 3B shows that CKII does not phosphorylate the maltose binding protein, but does phosphorylate full-length UBF1 and UBF2. As anticipated, CKII does label the acidic tail region of UBF (constructs 487−764 and 450−727). The radioactive, low molecular weight protein in each of these lanes may correspond to either a processed form of the MBP-UBF fusion or to a form of the MBP-acid tail fusion which arises by translational frameshifting or ribosome hopping (reviewed in 22). In addition, CKII phosphorylated those constructs of UBF1 and UBF2 which lacked the 277 amino acids of the carboxyl-terminus but retained the N-terminal 102 amino acid domain fused to HMG box 1 (construct 1−190), HMG box 1 & 2 (constructs 1−272 and 1−236, respectively), HMG box 1, 2 & 3 (constructs 1−372 and 1−335, respectively) or HMG box 1,2,3 & 4 (constructs 1−486 and 1−449, respectively) (Figure 3B). Constructs that lacked the 102 amino acids of the NH_2-terminus, as well as the carboxy-terminus (constructs 103−486, 191−486, 287−486, 401−486, 103−449 and 191−449), were not phosphorylated by CKII. This data suggests that there is a CKII phosphorylation site(s) in the NH_2-terminal 102 amino acid domain. The potential phosphorylation site(s) in the NH_2-terminal domain are the serine residues at positions 23 (DRWSQED) and 51 (TTESHMD) of the predicted amino acid sequence of UBF (6). The predicted tryptic peptides from this region may correspond to the two smaller tryptic peptides labeled *in vivo* (see Figure 1A).

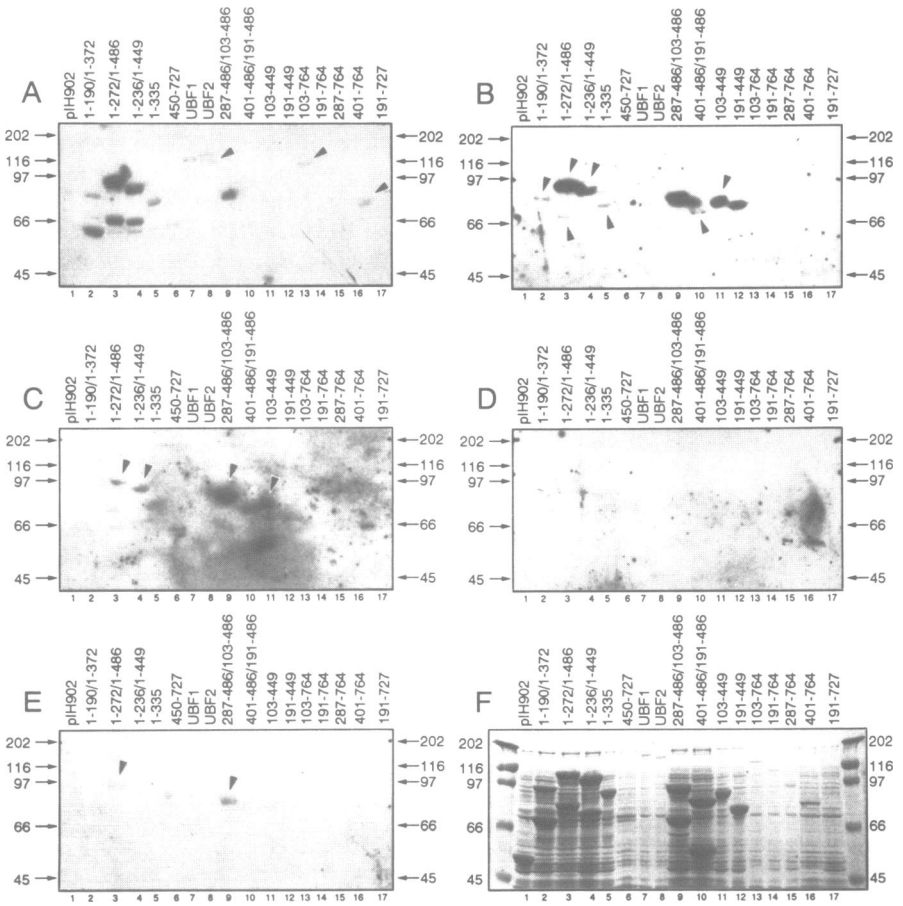

Figure 4. Analysis of the DNA-binding properties of mutants of UBF1 and UBF2 by Southwestern blots. (A-E) The indicated mutants were analyzed by Southwestern blot using the probes (see Figure 2D) derived from the wild-type rat 45S rDNA promoter (A-D) or the mutant 45S promoter BSM−106/−101 (also called BSM 8) (E). In each panel the positions of the molecular size standards (in kDa) are indicated, together with the constructs analyzed (top line) and lane numbers (bottom line). The probes used were: A, −208/+61; B, −70/+61; C, −48/+61; D, −21/+61; and E, −208/+61 from the linker-scanning mutant BSM−106/−101. (F) Analysis of the synthesis of mutants in UBF1 and UBF2 in *E.coli* by SDS-PAGE, same as in Figure 2E. In panels B, C and E the arrows indicate the position of protein bands which bind to the relavent probes.

Analysis of the DNA-binding properties of UBF

To further investigate the binding of UBF1 and UBF2 to the 45S rDNA promoter, the DNA-binding properties of the series of truncated forms of both UBF1 and UBF2 (Figure 2B) fused to the maltose binding protein on the vector pIH902 (Figure 2C) was examined (Figure 2D) by Southwestern blot analysis. A photograph of a typical gel displaying the recombinant forms of UBF is presented in Figure 4F. Several of the cell lysates coding for recombinant MBP-UBF fusions of different sizes were combined prior to SDS-PAGE. It should be noted that constructs which include the acidic tail region of UBF 1/UBF2 (Figure 4F, lanes 7,8, 13−17) were expressed at a very low level in *E.coli* (< 1% total cell protein), suggesting that the acid tail region of UBF is a poisonous sequence in *E.coli*.

Analysis of a Southwestern blot (Figure 4, panel A) in which the probe consisted of bases −208/+61 of the rat 45S rDNA promoter clearly shows that the fusion proteins 1−486 and 1−449 (lanes 3 and 4, upper bands), each of which retains the NH_2-terminus and 4 HMG boxes of UBF1 and UBF2, respectively, bind with the highest affinity. Removal of the NH_2-terminal domain (amino acids 1−102) reduced the binding of the respective truncated forms of UBF1 (103−486) and especially UBF2 (103−449) to the probe (Figure 4, panel A, lane 9, upper band, and lane 11). In addition, truncated forms of UBF1 and UBF2 which retain the NH_2-terminal domain together with either HMG box 1 (1−190, lane 2, bottom band), boxes 1 and 2 (1−272 and 1−236, bottom bands in lanes 3 and 4, respectively), or boxes 1,2 and 3 (1−372, lane 2, top band, and 1−335 lane 5) do not bind as efficiently as the constructs 1−486 and 1−449 which contain the NH_2-terminal domain and 4 boxes (upper bands in lanes 3 and 4, Figure 4, panel A). These data suggests that the 4 HMG boxes, together with the NH_2-terminal domain constitute the most effective DNA binding forms of UBF. Constructs coding for the acidic tail region of UBF (487−764) did not display any DNA-binding activity in the Southwestern blot (Figure 4A, lane 6), suggesting that the acidic tail region of UBF *per se* is not capable of binding DNA. Under these same conditions, none of the constructs tested bound to a probe corresponding to the CPE (probe −21/+61) (Figure 4, panel D).

When blots equivalent to that presented in Figure 4A were probed with partial deletions of the rat rDNA promoter (Figure 4, panels B and C) the binding of most of the UBF constructs was reduced significantly. Those constructs that contained the NH_2-terminal domain and the four HMG boxes still retained partial DNA-binding activity, [e.g. 1−449 and 1−486 (the upper bands in lanes 3 and 4, respectively), in panels B and C, Figure 4]. Unexpectedly, some of the mutants bound relatively better to these probes than they did to the wild-type promoter. For example, 103−486 bound better to the −48/+61 probe, than did either 1−486 or 1−449 (compare the indicated autoradiographic signals in lanes 9, 4, and 3, respectively, in Figure 4, panel C).

The binding of the UBF mutants to the linker-scanning mutant BSM−106/−101 (also referred to as BSM 8) was also tested (Figure 4, panel E). With the exception of two mutants of UBF1, 1−486 and 103−486, (the indicated autoradiographic signals in lanes 3 and 9, respectively), each construct tested failed to bind to the BSM−106/−101 probe. Most noticeable is the absence of binding of truncated forms of UBF2.

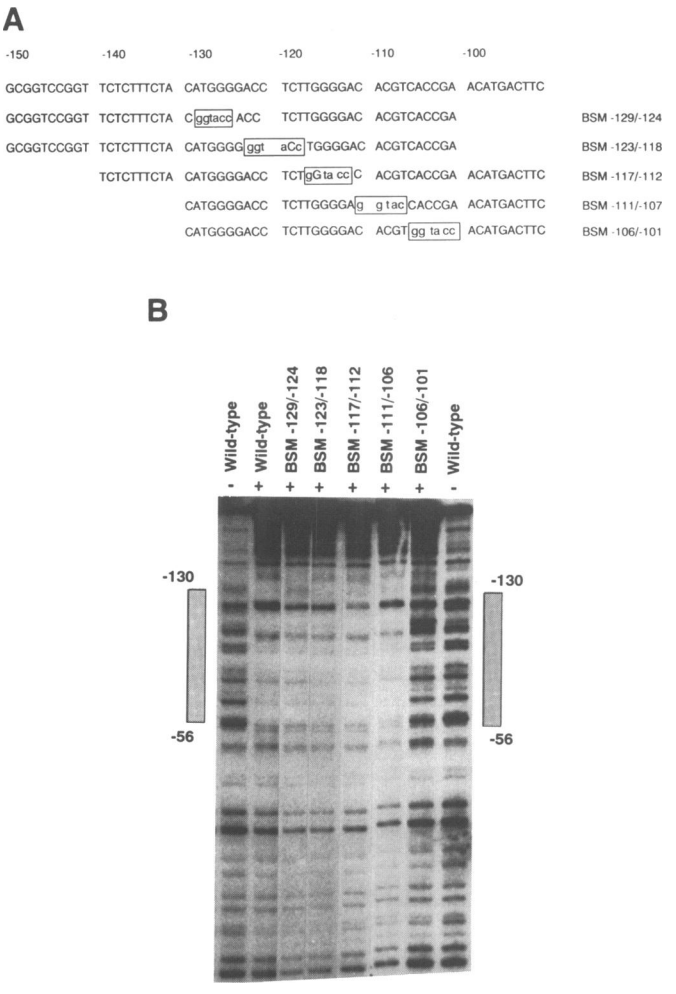

Figure 5. DNase footprinting of the linker-scanning mutants of the rat rDNA promoter with rat UBF. (A) A schematic representation of the linker-scanning mutants (BSM) of the rat 45S rDNA promoter used in the footprinting experiments. The corresponding wild-type rDNA promoter sequence is shown in the top line. (B) DNase 1 footprinting of the BSM mutants shown in panel A. In all cases the bottom strand was radiolabeled using 5′ end-labeled primer. The hatched bars alongside the autoradiograph indicate the region from −56 to −130 of the 45S rDNA promoter footprinted by UBF. The symbols − and + denote DNase 1 footprinting in the absence and presence of UBF.

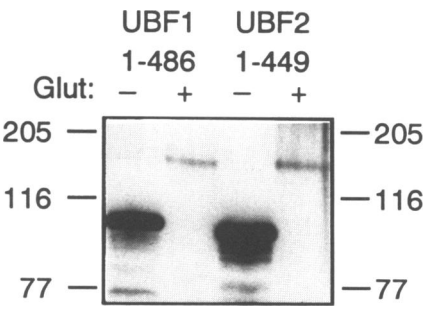

Figure 6. Analysis of UBF dimerization by crosslinking. Total soluble *E.coli* lysates expressing the UBF1 and UBF2 proteins 1−486 and 1−449, respectively, crosslinked in the absence (−) or presence (+) of glutaraldehyde (Glut), were fractionated by SDS-PAGE, Western blotted using the anti-UBF antisera followed by autoradiography. The positions of the molecular weight size standards (in kDa) are indicated.

Analysis of the DNase 1 footprint of UBF on the rat 45S rDNA promoter

We have previously shown that the binding of the transcription factor UBF to the rat 45S promoter can be demonstrated by DNase 1 footprint reactions, UV-crosslinking and *in vitro* transcription reactions (1,13). We tested the effect of a linker-scanning mutant series of the 45S promoter on the binding of UBF (Figure 5). Analysis of the DNase 1 footprints shown in Figure 5B shows that the mutants BSM−129/−124, BSM−123/−118, BSM−117/−112 and BSM−111/−106 did not interfere with the UBF footprint. Only one mutant, BSM−106/−101, disrupted the UBF footprint. Of these mutants, only BSM−129/−114 and BSM−106/−101 (1) interfered with UBF-dependent activation of *in vitro* transcription by RNA polymerase I (1, and Xie et al., manuscript submitted), and we have previously shown that BSM −129/−114, which mutates the distal SL-1 binding site, does not interfere with the binding of UBF to the promoter (1).

Analysis of UBF:UBF interactions by glutaraldehyde crosslinking

Glutaraldehyde crosslinking of both the UBF1 and UBF2 derivatives 1−486 and 1−449, respectively, followed by Western blot analysis using the UBF antisera, confirmed that UBF1 and UBF2 formed dimers (Figure 6) as evidenced by the appearance of crosslinked products of approximately 180 kDa size. The MBP alone was found not to crosslink under similar conditions (data not shown).

Analysis of UBF:UBF interactions by filter overlay

We developed an overlay assay to asses monitor UBF-UBF interactions. Recombinant MBP-UBF fusions present in *E.coli* cell lysates were fractionated by SDS-PAGE (Figure 7A), blotted onto nitrocellulose membranes and the membranes were probed with UBF radiolabeled (^{32}P) *in vitro* with CKII (Figure 7B). The *in vitro* labeled UBF binds to full-length UBF2 and to its derivatives which retain the NH_2-terminal 102 amino acids fused to HMG box 1 (1−190), HMG box 1,2 (1−236) and HMG box 1,2,3,4 (1−449), respectively. Deletion of the NH_2-terminal 102 amino acids abolishes the binding of radiolabeled UBF to the UBF2 derivatives 103−449 (boxes 1−4), 191−449 (boxes 2−4), 250−449 (boxes 3−4) and 364−449 (box 4). Similarly, the labeled UBF binds to full-length UBF1 and to its derivatives 1−190, 1−272, 1−372 and 1−486, which retain the NH_2-terminal 102 amino acid domain fused to the HMG boxes. However, deletion of the NH_2-terminal domain from UBF1 did not abolish the binding of the UBF1 derivatives 103−486 (boxes 1−4) and 191−486 (boxes 2−4) to labeled UBF in comparision to the results obtained with the analogous UBF2 derivatives (Figure 7B). This suggests that UBF1 and UBF2 differ in their respective dimerization properties. Interestingly, the box 3−4 and box 4 derivatives of UBF1 and UBF2 (287−486/250−449 and 401−486/364−449, respectively) are incapable of UBF:UBF interaction in this assay. Taken together, these results suggest that (1) the N-terminal 102 amino acids of UBF is important in facilitating UBF:UBF interaction; (2) the HMG box 2 of UBF1 contributes to UBF:UBF interaction more so than the equivalent HMG box in UBF2.

To determine if we were detecting homo or hetero dimers of UBF on the filters, the radiolabeled bands from the overlay filters were excised, boiled in solubilization buffer and fractionated by SDS-PAGE followed by autoradiography. In each case, the radiographic signals migrated as both UBF1 and UBF2 (Figure 7C) suggesting that UBF can form both homodimers and heterodimers.

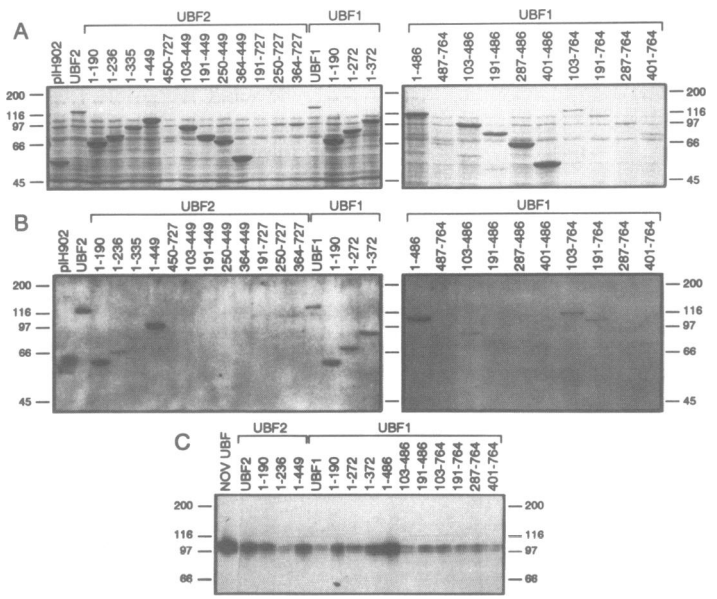

Figure 7. Analysis of UBF interactions by overlay assays. (A) Coomassie blue stained SDS-PAGE of total cell lysates of *E.coli* constructs expressing MBP:UBF fusion proteins. The identities of the MBP:UBF fusion in each lane are indicated as are the positions of the molecular size standards (in kDa). (B) Blots of gels equivalent to that presented in panel (A) were screened with authentic UBF labeled *in vitro* with CKII. The positions of the molecular size standards (in kDa) are indicated. (C) Radiolabeled protein which bound to MBP:UBF fusions in panel B were excised from the filters, solubilized, and fractionated by SDS-PAGE followed by autoradiography. UBF purified from Novikoff cells (NOV UBF) was labeled *in vitro* with CKII and fractionated on the same gel. The identity of the MBP:UBF fusion from which each UBF sample was eluted is indicated above each lane.

DISCUSSION

In this manuscript we have analyzed the phosphorylation, DNA-binding, and dimerization properties of the RNA polymerase I transcription factors UBF1 and UBF2. Our results indicate that UBF is phosphorylated by CKII *in vitro*. Furthermore, we would suggest that UBF1 and UBF2 are non-equal in their DNA-binding and dimerization properties.

Analysis of the tryptic digestion pattern of *in vivo* labeled UBF shows 4 tryptic peptides. Two of these peptides are similar in size (10 kDa) and undoubtedly are derived from the 97 amino acid, serine-rich, acidic, carboxyl-tail domain of UBF which has 9 consensus CKII phosphorylation sites, and which can be phosphorylated by CKII *in vitro*. The two *in vivo* labeled tryptic peptides from the acidic tail domain of UBF may arise by one of two means; either alternative digestion by trypsin *in vitro*, or differential phosphorylation *in vivo* by CKII or other kinases. There are three arguements in support of the latter possibility: (1) we have consistently detected these two 10 kDa peptides from *in vivo* labeled, immunoprecipitated UBF (Figure 1A where 3 different isolates of UBF were tested, and additional data not shown); (2) UBF1 and UBF2 purified from Novikoff cells (or directly immunoprecipitated from CHO cells) can be phosphorylated *in vitro* by CKII, pointing to the availability of 'unmodified' phosphorylation site(s) in UBF; (3) following serum starvation/refeeding, UBF is hyperphosphorylated in comparision to the degree of phosphorylation of UBF in logarithmically growing cells (12). Thus, it is tempting to speculate that there may be hierarchical stages of phosphorylation of UBF, and that the regulation of the stages of UBF phosphorylation is important in controlling its transcriptional activation of RNA polymerase I. Indeed, we have shown that phosphatase treatment of UBF reduces its transactivation properties *in vitro* (12).

In addition, by immunocytochemistry, we have shown that following serum deprivation UBF is redistributed from the nucleolus to the nucleolus, nucleus and cytoplasm (12). In this respect, it has been recently established that CKII, specifically the α' subunit of CKII, also preferentially relocates from the nucleus of logarithmically growing, interphase cells to the cytoplasm of serum-starved or metaphase cells (23,24). Thus, it may be that the nuclear phosphorylation of UBF by CKII may be a prerequisite in maintaining the preferential nucleolar localization of UBF in logarithmically growing cells. It will be of interest to determine if specific amino acid(s) of UBF are differentially modified *in vivo* under different growth conditions and to ascertain the significance of such differential phosphorylation(s) to the transactivation properties and subcellular localization of UBF. In addition to the *in vitro* phosphorylation of the acid tail region of UBF, we have also found that CKII will phosphorylate the NH_2-terminal 102 amino acid domain of UBF *in vitro*.

However, CKII may not be the only kinase which phosphorylates UBF *in vivo*. The *in vivo* phosphorylation of the C-terminus of UBF by CKII may in turn create substrate recognition sites for glycogen synthase kinase-3 (GSK-3) and casein kinase I, and similarly, phosphorylation by GSK-3 may introduce new GSK-3 recognition motifs (reviewed in 25). Consequently, initial phosphorylation by CKII may lead to multisite and hierarchical phosphorylations by other kinase(s), or vice versa, which in turn may have graded effects on UBF function.

Both our Southwestern and protein overlay assays depend upon the proper refolding of UBF following SDS-PAGE and blotting. Two lines of evidence suggest that UBF is refolding correctly (1). The DNA-binding properties of UBF observed in the Southwestern blotting experiments are similar to those predicted using authentic UBF (2). There is a distinct similarity in the overall pattern of the DNA and protein binding properties of mutant forms of rat UBF1/UBF2 in our experiments with that of *Xenopus* UBF (26 and see below). Thus, we consider it unlikely that UBF does not refold, following SDS-PAGE and blotting, to a conformationally active form.

Our Southwestern blotting results indicate that while the HMG boxes are necessary for DNA-binding, the NH_2-terminal 102 amino acid domain may exert both a quantitative and a qualitative role in DNA binding. The quantitative role of the NH_2-terminal domain becomes apparent when one compares the relative DNA-binding activities of the truncated UBF1 proteins 1−486 and 103−486 and the truncated UBF2 proteins 1−449 and 103−449. A suggestion that the NH_2-terminal domain of UBF plays a qualitative role is demonstrated by the finding that the UBF2 derivative, which lacks the NH_2-terminal domain, binds to the 5′ deletion rDNA probe −48/+61 but not to the wild-type promoter −208/+61. In addition, assuming that recombinant UBF is not a phosphoprotein, then the Southwestern blots demonstrate that the phosphorylation of UBF is not required for DNA binding, and that UBF1 is capable of binding to the rDNA promoter in the absence of UBF2 and vice versa.

McStay et al., (26) have recently stated that in the case of one form of *Xenopus* UBF (xUBF), they have 'observed a reproducible decrease in the DNA-binding ability' of a truncated form of xUBF lacking the NH_2-terminal domain, and that further deletions into the HMG boxes abolished DNA binding. Similar to our results for rat UBF1 and UBF2 they have found that when the HMG boxes of xUBF are retained, the amino terminus is not needed for DNA binding. However, if one or more of the HMG boxes of xUBF (or in our case rat UBF) are deleted, then the NH_2-terminal 102 amino acids must be present in order for DNA binding to occur, implying that the NH_2-terminal domain aids in DNA binding. This remarkable similarity in the DNA-binding properties of truncated forms of both rat and *Xenopus* UBF implies that rUBF and xUBF bind by similar mechanisms to their respective rDNA promoters. In addition, this may account for our finding that rat and *Xenopus* forms of UBF yield essentially identical DNase 1 footprints on each others rDNA promoters (27).

The lack of DNA-binding by truncated forms of UBF2, and by most truncated forms of UBF1, to the linker-scanning mutant BSM−106/−101 is interesting. This result again confirms that UBF1 and UBF2 differ in their quantitative DNA binding properties, and suggests that the region at or around −106/−101 of the 45S rDNA promoter is a major element of the binding site for UBF. Indeed, the lack of a DNAse footprint by UBF on the mutant promoter BSM−106/−101 together with the lack of transcriptional activation of BSM−106/−101 *in vitro* (1) confirms the reduced ability of UBF to bind to this mutant promoter. Interestingly the region between −106 and −95 apparently lies between two promoter domains that must be aligned stereospecifically (21).

Using a protein overlay assay we have found that UBF can interact with UBF. Such interaction is seen with full-length UBF1 and UBF2 and their deletion derivatives which retain the NH_2-terminal 102 amino acid domain and the 4 HMG boxes. Deletion of the NH_2-terminal 102 amino acids of UBF1 did not abolish these protein:protein interactions. In contrast, in the case of UBF2, deletion of this NH_2-terminal domain did abolish the

protein:protein interaction. These results demonstrate that the NH_2-terminal domain, especially with UBF2, is important for protein:protein interaction. In addition, the ability of the UBF1 derivatives that lack the NH_2-terminal 102 amino acids and the C-terminal 271 amino acids but contain either all 4 or at least 3 of the HMG boxes to dimerize indicates that regions other than the NH_2-terminus may be involved in the dimerization process. In contrast, the equivalent UBF2 constructs failed to demonstrate dimerization. This suggests that HMG box 2, which is the only difference between UBF1 and UBF2, also functions in UBF dimerization. Similarly, the acid tail region of UBF is implicated in facilitating UBF:UBF interactions. Taken together, this data strongly suggests that there are 3 regions in UBF important for dimerization or maintaining protein:protein interaction: (1) the NH_2-terminal 102 amino acids; (2) HMG box 2 as found in UBF1; and (3) the acidic tail region of UBF. In addition, these results demonstrate that UBF1 and UBF2 differ in their respective dimerization properties.

McStay et al., (26) have provided evidence that xUBF associates into dimers and that the NH_2-terminal 102 amino acid domain is necessary and sufficient for dimerization. The reported dimerization of xUBF is remarkably similar to the dimerization of rat UBF2. It will be of interest to determine if the dimerization properties of the second form of xUBF (9,11) will more closely resemble those of rat UBF1 reported here.

Considering that the Southwestern blot assays were carried out using immobilized UBF, it is not unrealistic to conclude that UBF is capable of binding to the rDNA promoter as a monomer. However, taken together the overlay data and the Southwestern data suggest the hypothesis that UBF either binds to the promoter as a dimer or dimerizes after binding.

One must also consider the possibility that domains of UBF, such as the serine-rich acidic tail, may interact with other components of the RNA polymerase I transcription system. For example, the general transcription factor TFIIB binds directly to the acidic activating region of VP16 (28). Further, the highly acidic, serine-rich regions of proteins such as UBF, Cdc68, SPT5 and SPT6 (25, 30, 31) have been hypothesized to function in (a) chromatin assembly or the modification of chromatin structure (reviewed in (32)); (b) tracking of DNA-binding factors along chromatin; (c) direct interaction with other chromatin proteins or transcription components; (d) targeting of proteins to the nucleus or nucleolus.

Interestingly, similar to UBF, Cdc68, SPT5 and SPT6 also have multiple, consensus CKII recognition sites. Phosphorylation of the acidic tail domain of each of these proteins presumably should increase the net negative charge of this domain or motif. We are currently analysing the interaction of different regions of UBF with different cellular components. Preliminary experiments (O'Mahony and Rothblum, unpublished) suggest that UBF does indeed interact with a specific group of proteins, including the HMG-like protein p16 (33) which footprints A:T rich regions located upstream and downstream of the UPE of the rat 45S rDNA promoter. It is tempting to speculate that the interaction of UBF with p16 in turn facilitates or directs the interaction of UBF with the UPE *in vivo* by one of the mechanisms outlined above. Taken together, our current data indicates that UBF consists of 3 overlapping, functional properties: (a) a dimerization domain which is primarily driven by amino acids 1–102; (b) a DNA-binding domain, consisting primarily of the 4 HMG boxes; and (c) a phosphorylated, acidic domain which may function as an activating domain similar to VP16. The differential and/or regulated phosphorylation *in vivo* of UBF in response to stimuli such as serum (12) may in turn ultimately regulate the interaction (or activation) of UBF with SL-I or RNA polymerase I and consequently control the rate of transcription initiation from the rDNA promoter.

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