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Dimerization and HMG box domains 1–3 present in *Xenopus* UBF are sufficient for its role in transcriptional enhancement

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ABSTRACT

Transcription of *Xenopus* ribosomal genes by RNA polymerase I is directed by a stable transcription complex that forms on the gene promoter. This complex is comprised of the HMG box factor UBF and the TBP-containing complex Rib1. Repeated sequence elements found upstream of the ribosomal gene promoter act as RNA polymerase I-specific transcriptional enhancers. These enhancers function by increasing the probability of a stable transcription complex forming on the adjacent promoter. UBF is required for enhancer function. This role in enhancement is distinct from that at the promoter and does not involve translocation of UBF from enhancer repeats to the promoter. Here we utilize an *in vitro* system to demonstrate that a combination of the dimerization domain of UBF and HMG boxes 1–3 are sufficient to specify its role in enhancement. We also demonstrate that the acidic C-terminus of UBF is primarily responsible for its observed interaction with Rib1. Thus, we have uncoupled the Rib1 interaction and enhancer functions of UBF and can conclude that direct interaction with Rib1 is not a prerequisite for the enhancer function of UBF.

INTRODUCTION

Transcription of the repeated genes that encode 18S and 28S rRNAs by RNA polymerase I (RNA pol I) requires a series of regulatory elements that are located in the intergenic spacer (1). These include gene promoter and repeated enhancer elements. In *Xenopus*, enhancer elements comprise blocks of interspersed 60 and 81 bp repeats that have sequence homology to the gene promoter. *Xenopus* oocyte injection experiments demonstrate that blocks of these elements confer a competitive advantage on a linked promoter (2–6). Repetitive sequence elements are also found in the intergenic spacer, immediately upstream of the gene promoter, in rodent species. In the mouse these repeated elements are 140 bp in length and have been demonstrated to have enhancer activity in both mouse and *Xenopus* systems (7,8). Unlike *Xenopus*, mouse enhancer elements have no sequence similarity with the gene promoter.

Enhancers could function in principle by increasing the rate of transcription initiation from a linked promoter or by increasing the probability of a transcription complex forming on a linked promoter. A recent analysis, by electron microscopy (EM), of templates injected into *Xenopus* oocytes supports this latter model. EM demonstrated that active transcription units on enhancer-less templates are as densely packed with polymerases and nascent transcripts as those on enhancer-bearing templates. Furthermore, enhancer-bearing templates are 30- to 50-fold more likely to form such complexes (9).

RNA pol I transcription minimally requires the *trans*-acting factors upstream binding factor (UBF) and a second factor termed SL1 in humans (10), TIF 1B (11,12) or Factor D (13,14) in mouse and Rib1 in *Xenopus* (15). *Xenopus* UBF (xUBF) is comprised of an N-terminal dimerization motif, five HMG box motifs and a C-terminal acidic domain (15,16). Human UBF (hUBF) is highly related in sequence, the only major difference being the presence of an additional HMG box (17). UBF, which binds to DNA sequences within the promoter (10,18), has a remarkable propensity to bend and loop DNA (19–22). This so-called 'architectural' ability is conferred by the multiple HMG box DNA binding motifs in UBF (17,23,24). The importance of this architectural role is suggested by the observation that the precise spacial alignment of the promoter Upstream Control and Core elements is critical (25).

SL1 on its own binds very poorly to DNA, but in the presence of hUBF it binds tightly and specifically to DNA sequences within the human promoter (10). Similarly, Rib1 and xUBF can combine to form a stable transcription complex on the *Xenopus* promoter (15). Protein–protein interactions between mammalian UBF and SL1 or xUBF and Rib1 have been described (26–28). Thus it is generally considered that formation of a stable transcription complex is achieved through a combination of DNA bending by UBF, protein–protein contacts between UBF and SL1/Rib1 and direct interaction of SL1/Rib1 with promoter sequences.

In addition to binding to DNA sequences within the promoter, UBF can give rise to a DNase I footprint over *Xenopus* (18) and mouse (8) enhancer sequences. This suggested a role for UBF in enhancer function. Recently we developed a *Xenopus in vitro* system in which RNA pol I enhancers function in most respects as *in vivo* (29). This system was used to demonstrate that UBF does indeed function in enhancement. Enhancer-bearing templates out-compete enhancer-less templates by 30- to 100-fold *in vitro*. The principal requirement for enhancer function is the presence of a high concentration of UBF, 50- to 100-fold higher than normally present in transcription extracts. This system was used to demonstrate that enhancers act during stable complex formation by increasing the likelihood of complex formation on linked promoters. Once formed, transcription complexes function with equal efficiency

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irrespective of the presence of enhancers upstream, thus confirming the EM studies described above (9).

The *in vitro* system was also used to characterize the role of UBF in transcriptional enhancement. Previous models had proposed that enhancers may function by recruiting limiting amounts of UBF to the promoter (18). In contrast to this model, we demonstrated that enhancers function only when UBF is non-limiting. Additionally, forms of UBF that do not function at the *Xenopus* promoter, namely hUBF 1 and 2, can nonetheless function in transcriptional enhancement. We concluded that UBF must be performing a distinct role in enhancement that does not involve its translocation to the promoter. In order to further characterize the role of UBF in transcriptional enhancement, here we have performed a systematic mutagenic analysis of the domains within UBF that are required for enhancer function. We demonstrate that a combination of the dimerization domain of UBF and HMG boxes 1–3 are sufficient to specify its role in enhancement. Furthermore, we demonstrate that the previously described interaction of UBF and Rib1 (27,29) is mediated by the acidic C-terminus of UBF. We discuss models of enhancer action in the light of these results.

MATERIALS AND METHODS

Baculoviral expression of full-length and mutant UBFs

The expression and purification of full-length xUBF has been previously described (29). Mutant UBFs 1, 13, 15, 17, 18, 19, 21 and 25 were cloned as *Nco*I–*Xba*I fragments, derived from plasmids used for *in vitro* translation (24), into the *Nco*I–*Xba*I sites of the baculovirus transfer vector pBacHTa (Gibco BRL). Recombinant virus was produced for each of the above mutant clones using the Bac-to-Bac recombination system (Gibco BRL) following the manufacturer's instructions. For large scale protein production, Sf9 cells were grown under serum-free conditions (using SF900-II medium; Gibco BRL). Between 100 and 500 ml spinner cultures were infected with each mutant viral stock at a multiplicity of infection of 10. Cells were harvested 3 days post-infection. All the following manipulations were carried out at 4°C. Cell pellets were resuspended in 5 vol low salt buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT)] supplemented with protease inhibitors. Nonidet-P40 (N-P40) was added to a final concentration of 1% and the cell suspensions incubated end-over-end for 1 min, then briefly sonicated. The salt concentration in the lysates was adjusted to 0.5 M with KCl. After centrifugation at 25 000 r.p.m. for 2 h, cleared lysates were applied to a pre-equilibrated nickel column (Qiagen), washed and the bound protein was eluted in CB100 (25 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 20% glycerol and protease inhibitors) containing 200 mM immidazole and 5 mM DTT. Fractions containing mutant UBF proteins were pooled and subjected to ion exchange chromatography on BioRex 70 (Bio-Rad). The column was eluted with a linear salt gradient of from 200 to 1000 mM KCl. Mutant UBFs typically eluted at between 500 and 700 mM KCl. At this point, recombinant UBFs were on average 90% pure as judged by Coomassie staining of a SDS–polyacrylamide gel (see Fig. 1A). Mutant proteins were dialysed against and stored at –70°C in CB100.

Bacterial expression of truncated xUBF

Mutants 7 and 9 were constructed by cloning *Nco*I–*Bam*HI fragments, derived from plasmids used for *in vitro* translation

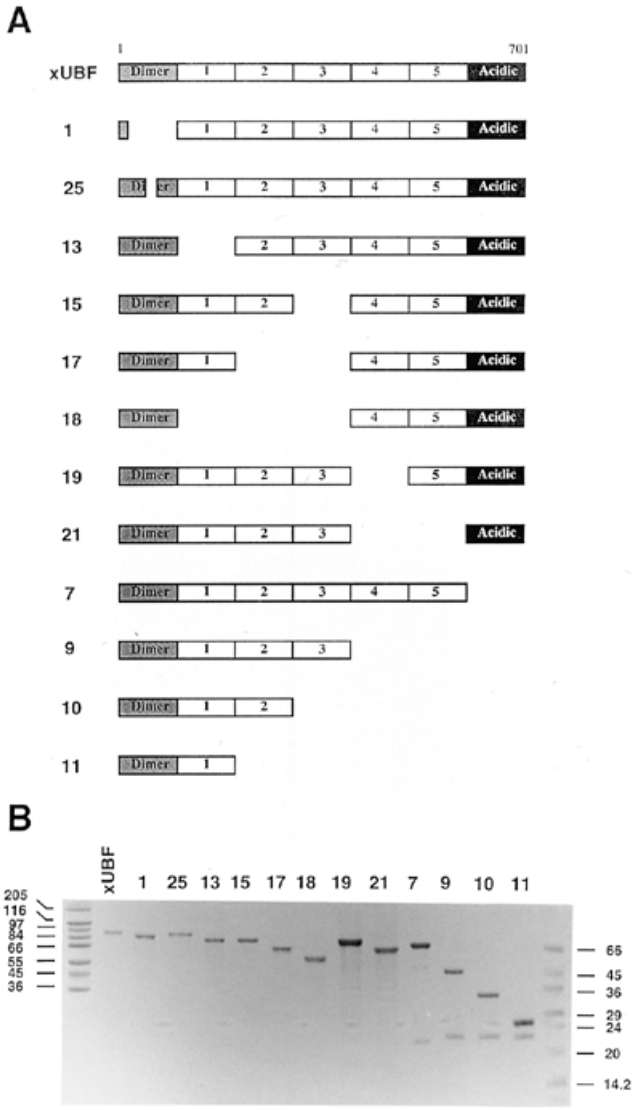

Figure 1. UBF mutants. (**A**) Structure of UBF mutants. xUBF, which is 701 amino acids, comprises an N-terminal dimerization domain (shaded box), five HMG boxes (open boxes) and a C-terminal acidic tail (solid box). The identity of each HMG box is denoted by a number within the box. The structure of each of the deletion mutants used in this study is shown in diagrammatic form with the identity of the mutant shown on the left. The precise boundaries of each deletion are described elsewhere (24). (**B**) Coomassie stained SDS–PAGE of UBF mutants. Approximately 1 µg full-length xUBF and each of the mutants used in this study was electrophoresed on a 10 % SDS–polyacrylamide gel stained with Coomassie blue. The size in kDa of molecular weight standards is shown on the side of the gel. The identity of each mutant protein is shown on top.

(24), into the *Nco*I–*Bam*HI sites of the bacterial expression vector pET11D (Novagen). These plasmids were transferred into the *Escherichia coli* strain BL21/DE3 pLys S (Novagen) for expression. Cultures (1.5 l) were grown at 37°C in L broth to an optical density at 600 nm of 0.5. After induction with 0.5 mM isopropyl-β-D-thiogalactoside, cultures were grown for a further 1.5 h. Cells were harvested by centrifugation and cell pellets were resuspended in 40 ml non-denaturing lysis buffer (10% glycerol, 0.5 M NaCl, 0.1% N-P40, 10 mM Tris, pH 7.9, 5 mM DTT and protease inhibitors). After repeated sonication on ice, extracts

were clarified by centrifugation at 16 000 *g* for 10 min. Supernatants were loaded directly onto a 20 ml BioRex 70 ion exchange column (Bio-Rad). The column was eluted with a linear salt gradient of from 500 to 1000 mM KCl in column buffer. Mutants 7 and 9 eluted from this column at ~700 mM KCl. Pooled fractions were adjusted to 200 mM KCl with CB0 and then loaded on a 5 ml HiTrap heparin column (Pharmacia). This column was then eluted with a 200–800 mM KCl linear gradient, with mutants 7 and 9 eluting at ~500 mM KCl. Pooled fractions were dialysed against CB100 and stored at –70°C.

The cloning, expression and purification of mutants 10 and 11 have already been described (OG and OH in 23).

For expression of the acidic C-terminus of xUBF as a GST–fusion protein, a *Bgl*II–*Eco*RI fragment encoding amino acids 606–701 of xUBF was excised from mutant K (described in 24). This fragment was cloned into the *Bam*HI and *Eco*RI sites of expression vector pGEX 2TK (Pharmacia). GST and GST–acidic tail fusion proteins were expressed and purified as described previously (27).

Transcription assays

The transcription templates used here, pGem40, pGem52 and pGem40EX, have been described elsewhere (29). pGem40 and pGem52 are promoter-only templates whose transcripts can be differentially detected using S1 nuclease protection. The template pGem40EX is based on pGem40 but includes sequences that extend upstream to –970 and include a single block of 60/81 bp repeats.

S100 transcription extracts in CB100 were prepared from the *Xenopus laevis* cell line XlK2 as described previously (29). Immunodepletion of UBF from S100 extract has been described elsewhere (29,30). Immunodepletion removes all detectable UBF, as judged by western blotting with anti-UBF antiserum (data not shown). In some experiments fractionated transcription extracts were employed. Heparin 0.4 M and Rib1 fractions have been previously described (15). The heparin 0.4 M fraction elutes from heparin–Sepharose with 400 mM KCl and contains both RNA pol I activity and UBF. The Rib1 fraction elutes from heparin–Sepharose with 600 mM KCl and contains no detectable RNA pol I activity or UBF.

In transcription reactions, immunodepleted extract (20 µl) was combined with baculovirus or bacterially expressed UBF (in 1 µl volume) and incubated with template DNA (400 ng total) for 10 min on ice. Reactions were initiated by addition of 20 µl transcription buffer (25 mM HEPES, pH 7.9, 80 mM KCl, 12 mM $MgCl_2$, 10 mM creatine phosphate, 1 mM DTT, 100 µg/ml α-amanatin, 1 mM nucleotide triphosphates) and incubation at 25°C. The final reaction conditions were 25 mM HEPES, pH 7.9, 90 mM KCl, 6 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 5 mM creatine phosphate, 50 µg/ml α-amanatin, 1 U/µl RNasin (Promega), 0.5 mM NTPs and 10 µg/ml template DNA. Reactions were allowed to proceed for 2 h, terminated and transcripts were detected using S1 nuclease protection with a probe prepared from pGem40 to detect transcripts from pGem40 and pGem40EX and a probe prepared from pGem52 to detect transcripts from that template (31). Transcription signals were quantitated by phosphorimaging using a GS-525 Molecular Imager (Bio-Rad).

Rib1 interaction with UBF-coated magnetic beads

A 100 µg aliquot of full-length xUBF and mutants 1, 7, 9, 10, 11, 18 and 21, in 0.1 M sodium phosphate (pH 7.4) at a concentration of 400 ng/µl was coupled to tosyl-activated M-280 magnetic beads (500 µg/coupling) following the manufacturer's protocol (Dynal UK). Following coupling the beads were stored at 4°C in CB100 containing 0.5 mg/ml bovine serum albumin (BSA) (100 µl).

In Rib1 interaction assays, 100 µl Rib1-containing fraction in CB100 plus 0.1% BSA was incubated with 20 µl UBF-linked beads. After incubation at 4°C for 15 min with occassional gentle mixing, the beads were removed using a Dynal MPC magnet and repeatedly washed in CB100 plus BSA (2 × 100 µl). The beads were finally resuspended in CB100 plus BSA (50 µl). In order to test for the presence of Rib1, transcription reactions were performed that contained either 7.5 or 15 µl of this bead suspension combined with 10 µl heparin 0.4 M fraction supplemented with 200 ng recombinant full-length xUBF.

Rib1 interaction with GST–acidic tail fusion protein

GST alone and GST–acidic tail fusion protein (50 µg each) were bound to glutathione agarose beads (50 µl). These beads were loaded onto a mini-column. The column was then equilibrated with CB100. Each column was then loaded with 300 µl S100 transcription extract. After loading, the column was washed with 2 × 250 µl CB100 buffer and bound proteins were then eluted with CB600 (150 µl). Following dialysis in CB100, each eluted fraction was tested for Rib1 activity by *in vitro* transcription. Reactions contained 10 µl eluate combined with 10 µl heparin 0.4 M fraction.

RESULTS

Production and purification of mutant UBF proteins

S100 extracts prepared from *Xenopus* culture cells support accurate and efficient transcription initiation by RNA pol I. A strict dependence of *Xenopus* RNA pol I transcription on xUBF has been demonstrated both by fractionation of these transcription extracts (15,24) and by immunodepletion of xUBF from unfractionated extracts (29,30). The amount of extract typically used in transcription reactions (20 µl) contains 5–10 ng xUBF. While this is sufficient xUBF for promoter function, enhancer function requires up to 400 ng xUBF per transcription assay (29). Previous studies have utilized *in vitro* translation in a rabbit reticulocyte lysate to investigate UBF promoter function (15,24). Enhancer function requires orders of magnitude more xUBF. This amount of UBF cannot be readily produced by *in vitro* translation. Therefore, we have chosen a combination of baculovirus and bacterial expression systems to produce sufficient protein for a mutational analysis of UBF in enhancement.

Previously described UBF mutants, numbers 1, 13, 15, 17, 18, 19, 21 and 25 (24) were transferred from *in vitro* translation vectors into the transfer vector pFastBacHTa (Gibco BRL) for baculoviral expression using the Bac-to-Bac system (Gibco BRL). Mutant UBF proteins contain an N-terminal six histidine tag and were purified from infected Sf9 cells using nickel–agarose followed by chromatography on BioRex 70 (see Materials and Methods for details).

The principal limitation to producing full-length xUBF in bacterial systems appears to be the presence of the acidic C-terminus (G.J.Sullivan and B.McStay, unpublished observation). Therefore, we have utilized bacterial expression to produce mutant proteins that lack this acidic tail. UBF mutants numbers 7 and 9 (24) were transferred from *in vitro* translation vectors into the bacterial expression vector pET11D (Novagen). Mutant

protein was purified by successive chromatography on BioRex 70 and HiTrap heparin. Two of the bacterially expressed mutant proteins, numbers 10 and 11, were purified using nickel–agarose as previously described (23). Mutant UBFs are depicted in diagrammatic form in Figure 1A and a Coomassie stained SDS–polyacrylamide gel loaded with purified full-length xUBF and each of the UBF mutant proteins is presented in Figure 1B.

Promoter function of mutant UBFs

Experiments using *in vitro* translated proteins have demonstrated that the N-terminal dimerization domain and HMG boxes 1–3 present in xUBF are absolutely required for its function at the promoter (24,30). Deletion of the acidic C-terminus or HMG boxes 4 and 5 had only modest effects on promoter function. In the above study it was calculated that between 2 and 5 ng UBF were added per transcription assay. These results are at odds with similar experiments in the human system. Jantzen *et al.* (32) reported that mutant hUBF proteins in which individual HMG boxes 1, 2 or 4 had been deleted functioned in human RNA pol I transcription. Even a hUBF mutant in which the dimerization domain was deleted functioned in transcription. We suspect that this discrepancy in results may be due to differences in the amount of UBF used in the two studies. In order to explore this possibility and to verify that mutant proteins produced in baculoviral and bacterial systems behaved in a manner consistent with previous observations with *in vitro* translated material, each was tested for promoter function at a range of input amounts (5–400 ng; Fig. 2). UBF immunodepleted *Xenopus* extract supplemented with mutant proteins was used in transcription reactions with the promoter-only template pGem40. At a low input amount (5 ng/transcription reaction) the results presented in Figure 2 are in good agreement with prior studies in the *Xenopus* system (24).

At high input amounts the domain requirements for promoter function are markedly different from those at lower inputs. Most notably, there appears to be some relaxation of the requirement for HMG boxes 1, 2 and 3. We observe that HMG boxes 4 and 5 can compensate for deletion of HMG boxes 1, 2 or 3. This point is illustrated by the following. At high input (400 ng), mutants lacking HMG box 1 (mutant 13) or 3 (mutant 15) function at 92 or 60% efficiency respectively, compared with that of full-length xUBF. Mutant 17, lacking HMG boxes 2 and 3, functions at 87% efficiency. In contrast, mutants 10 and 11, lacking HMG boxes 3–5 and 2–5 respectively, are inactive at all input concentrations. This compensatory ability of boxes 4 and 5 does not extend to deletion of boxes 1–3, since mutant 18 is inactive at all input amounts. These results, obtained with high inputs of mutant protein, more closely reflect those in the human system (32). Thus the original reported differences in the behaviour of UBF in both systems may be a function of the amount of UBF employed.

In summary, at low input amounts HMG boxes 1–3 are required for promoter function of xUBF. At high input amounts, mutant proteins that retain any three of the five HMG boxes present in xUBF are functional in promotion. The ability of HMG boxes 4 and 5 to compensate for deletion of the other HMG boxes should not be surprising, since they can bind DNA (23,32). In agreement with previous work, deletion of the acidic tail has a 5- to 10-fold effect at low inputs but little or no effect at high inputs. There is a strong requirement for the dimerization domain at all input amounts (Fig. 2, mutant 1), although at the highest input amount (400 ng) some promoter activity is observed. In mutant 25,

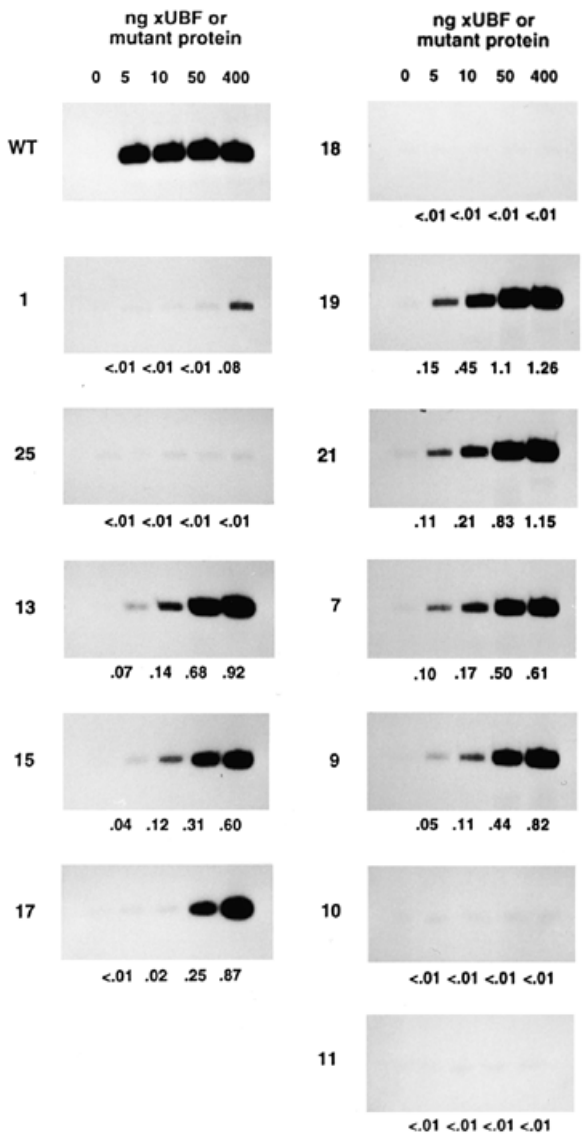

Figure 2. Promoter assays. Transcription reactions contained 20 µl xUBF-depleted extract, 400 ng template DNA (pGem40), 20 µl transcription buffer and the amount of wild-type xUBF or mutant protein indicated. Transcription signals were quantitated using phosphorimaging. The transcription signal obtained with each mutant protein was calculated relative to that observed with the same weight of wild-type xUBF (WT). This figure is shown under each reaction. The identity of each mutant is shown on the right.

sequences present in the centre of the dimerization domain have been deleted. This mutant retains dimerization activity (24), but here we show it is inactive in transcription at all input amounts (Fig. 2). We interpret this result as showing that the dimerization domain is responsible not just for dimerization but also for the precise positioning of HMG box 1 onto DNA.

Domain requirements for enhancer function

Transcription extracts supplemented with high levels of baculovirus-produced xUBF support enhancer function (29). This is demonstrated here by the observation that an enhancer-containing template, pGem40EX, out-competes a promoter-only template, pGem52, by 100-fold when transcribed in

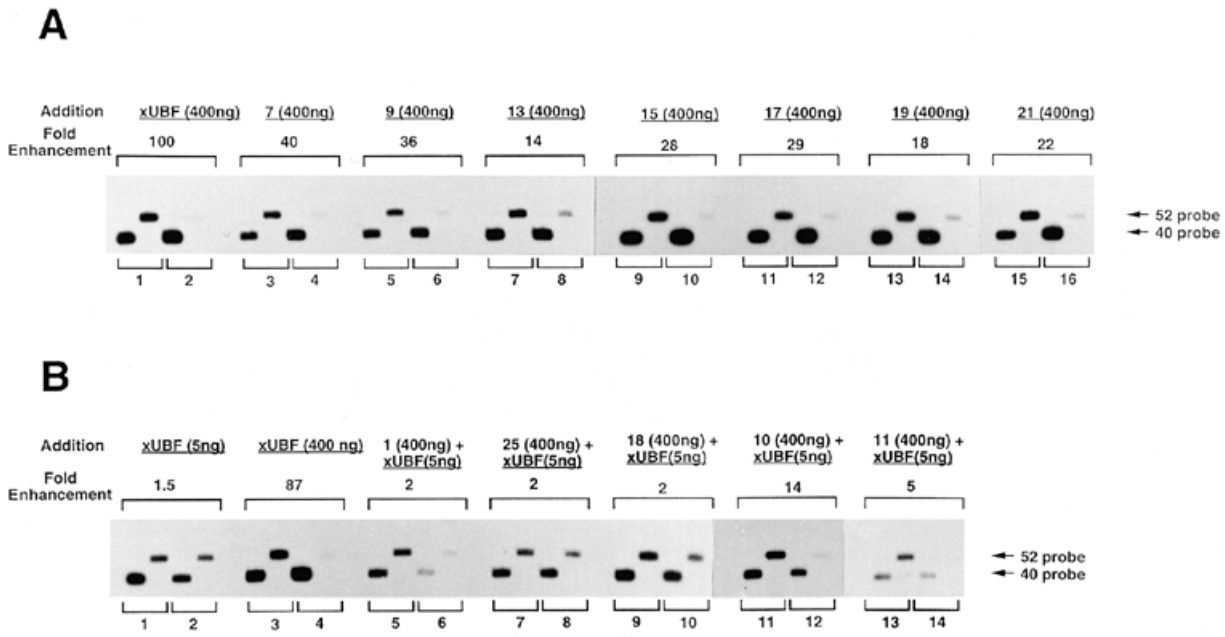

Figure 3. Enhancer assays. (**A**) Enhancer assays with mutants that function in promotion. Transcription reactions contained 20 µl xUBF-depleted extract, 400 ng template DNA, 20 µl transcription buffer and the amount of xUBF or mutant protein indicated. Reactions 1, 3, 5, 7, 9, 11, 13 and 15 contained an equimolar mixture of the templates pGem40 and pGem52. Reactions 2, 4, 6, 8, 10, 12, 14 and 16 contained an equimolar mixture of the templates pGem40EX and pGem52. Reactions were probed for transcripts from each promoter type (indicated by an arrow on the right side of the gel). The fold enhancement (calculated as the ratio of signal between pGem40EX and pGem52) is shown above each set of reactions as appropriate. (**B**) Enhancer assays with mutants lacking promoter function. Transcription reactions contained 20 µl xUBF-depleted extract, 400 ng template DNA, 20 µl transcription buffer and the amount of xUBF or mutant protein indicated. Reactions 1 and 2 contained 5 ng xUBF, 3 and 4 contained 400 ng xUBF, 5–14 contained 400 ng mutant protein indicated in addition to 5 ng xUBF. Reactions 1, 3, 5, 7, 9, 11 and 13 contained an equimolar mixture of the templates pGem40 and pGem52. Reactions 2, 4, 6, 8, 10, 12 and 14 contained an equimolar mixture of the templates pGem40EX and pGem52. The fold enhancement is shown above each set of reactions as appropriate.

UBF-depleted extract supplemented with 400ng baculoviral xUBF (Fig. 3A, reaction 2). Competing promoter-only templates pGem40 and pGem52 are transcribed with equal efficiency under the same conditions (Fig. 3A, reaction 1).

Using this assay, mutant proteins were tested for enhancer function in one of two ways. Mutants that function in promotion (Fig. 2, mutants 7, 9, 13, 15, 17, 19 and 21) were tested for enhancer function in the depleted extract in the complete absence of xUBF (Fig. 3A). Mutants devoid of promoter activity, even at high input levels (Fig. 2, mutants 1, 10, 11, 18 and 25), were tested for enhancer function in the presence of 5 ng xUBF. This amount of full-length xUBF is sufficient to support promoter function but does not support enhancer function to a significant degree (29) (Fig. 3B, reactions 1 and 2).

It is clear from these experiments that the domains within xUBF that are required for transcriptional enhancement overlap with those required for promoter function. As with promoter function, the deletion or alteration of the N-terminal dimerization domain (mutants 1 and 25 respectively) disrupts enhancer function (Fig. 3B, reactions 5–8). Mutant 1 also exhibits a dominant negative effect on total transcription. Deletion of the acidic tail (mutant 7) has little effect on enhancement (Fig. 3A, reactions 3 and 4). Deletion of individual HMG boxes 1, 3 or 4 (mutants 13, 15 and 19) results in mutant proteins that support 14-, 28- or 18-fold enhancement respectively (reactions 8, 10 and 14). Mutants deleted in HMG boxes 2 and 3 or 4 and 5 (mutants 17 and 21) enhance 29- and 22-fold respectively (reactions 12 and 16). A mutant deleted of boxes 4 and 5 as well as the acidic tail (mutant 9) enhances 36-fold (reaction 6).

Although similar to that required for promoter function, the HMG box requirement for enhancer function exhibits greater flexibility. This is demonstrated by the fact that mutant 10, with only HMG boxes 1 and 2 present, supports enhancement (14-fold; Fig. 3B, reaction 12) and even mutant 11, with only a single HMG box present, supports enhancement to a limited degree (5-fold; Fig. 3B, reaction 14). It should be noted that mutant 11, like mutant 1, appears to act as a partial dominant negative. The only HMG box deletion mutant that does not function in enhancement is number 18, in which HMG boxes 1–3 have been deleted (Fig. 3B, reaction 10).

In summary, this mutagenic analysis illustrates that the domains required for enhancer function fall entirely within those that are required for promoter function, but with a marked increase in flexibility with respect to HMG box requirements. This increase in flexibility should not be surprising, given that a diverse array of repeated sequences derived from the *Xenopus* intergenic spacer or that of the mouse ribosomal repeat can function in enhancement (5,8,29,33). This point will be further addressed in the Discussion.

Rib1 interacts with the acidic tail of UBF

Previously we and others have observed protein–protein interactions between UBF and Rib1 or its mammalian equivalent SL1 (26–28). Conceivably, this interaction could play an important role in either or both the enhancer and promoter functions of UBF. In order to determine the domain of UBF with which Rib1 interacts, we have covalently coupled xUBF and a selection of UBF mutants to tosyl-activated magnetic beads (M-280; Dynal UK). A previously characterized Rib1-containing fraction that elutes from heparin–Sepharose at 600 mM KCl (15,27) was

diluted to CB100. BSA was included as a non-specific competitor protein. This fraction was then incubated with UBF-coated beads. Following binding, the beads were washed repeatedly in CB100 plus BSA and finally resuspended in the same buffer. In order to determine if Rib1 had interacted with the beads, aliquots of the final bead suspension (7.5 or 15 µl) were tested for their ability to complement a heparin 0.4 M fraction (10 µl) in a transcription reaction. This heparin 0.4 M fraction contains RNA pol I and UBF but no Rib1 (15,27) (Fig. 4A, compare lanes 1 and 2). These reactions were further supplemented with full-length recombinant xUBF (200 ng) in order to compete Rib1 from the beads and make it available for transcription complex formation. Using this assay we clearly demonstrate the ability of Rib1 to specifically interact with xUBF. Rib1 activity is quantitatively recovered from xUBF beads but not from beads coated with BSA (compare reactions 3 and 4 with 5 and 6). Mutant proteins that are deleted for the dimerization domain (mutant 1, lanes 7 and 8), HMG boxes 1–3, (mutant 18, lanes 9 and 10) and HMG boxes 4 and 5 (mutant 21, lanes 11 and 12) retain the ability to interact with Rib1. All the mutants proteins in which the acidic terminus has been deleted (mutants, 7, 9, 10 and 11) have lost the ability to interact with Rib1 (lanes 13–20).

To further demonstrate that the acidic tail of UBF is both necessary and sufficient to specify UBF–Rib1 interactions, we have constructed a GST–acidic tail fusion protein. A column consisting of glutathione–agarose beads coated with GST–acidic tail fusion protein or GST alone as a control was loaded with transcription extract. The columns were repeatedly washed in CB100 and then bound proteins were eluted with CB600. After dialysis to CB100, the eluted fractions were tested for Rib1 activity by complementation of the heparin 0.4 M fraction in transcription reactions (Fig. 4B). In this experiment we observe Rib1 activity binding to GST–acidic tail beads (lane 3) but not to GST beads (lane 2). From these experiments we conclude that the acidic tail present in xUBF is the major site of interaction with Rib1 *in vitro*.

DISCUSSION

RNA pol I enhancers function during assembly of a stable transcription complex on a linked promoter (29). Enhancers function by increasing the likelihood of complex formation at a promoter rather than by increasing the rate of transcription initiation from that promoter (9,29). The stable transcription complex on the *Xenopus* promoter is minimally composed of UBF and Rib1 (15). Thus, in principal, enhancers could act by recruiting either or both Rib1 and UBF to the promoter. We have shown previously that forms of UBF (hUBF 1 and 2) that cannot function at the promoter retain enhancer function (29). Thus we concluded that enhancers do not act by recruiting UBF for complex formation at the promoter. Instead, UBF has its own distinct role in enhancer function. This conclusion is further strengthened by the work presented here. An array of UBF mutants that are severely impaired in promoter function retain enhancer function. For example, xUBF mutants that retain only two or even a single HMG box retain some enhancer function but are totally devoid of promoter function.

As enhancers do not act by recruiting UBF to the promoter, it seemed plausible that they act by recruiting Rib1. In accordance with this notion, we previously demonstrated that Rib1 could make protein–protein contacts with UBF both in solution (27) and when UBF is bound to enhancer DNA (29). With the work presented here, however, we can rule out Rib1 recruitment by direct protein–protein contact with enhancer-bound UBF as a model for enhancer action. This is because we describe UBF mutants that uncouple Rib1 interaction and enhancer function. We identify the acidic tail of UBF as the principal Rib1 interacting domain and show that UBF mutants in which these sequences have been deleted retain both promoter and enhancer function.

We and others have previously proposed that Rib1 is recruited at the promoter by the combined architectural and protein–protein interaction abilities of UBF (19,20,21,30). The results presented here strongly suggest that the architectural role of UBF is dominant over its ability to interact with Rib1.

The question then remains, how do enhancers function if not by directly recruiting Rib1? We can envisage two possible mechanisms. In the first mechanism, it is possible that the function of enhancer sequences is to inhibit the repressive effects of chromatin in a UBF-dependent manner. Indeed, in mouse and *Xenopus in vitro* systems it has been demonstrated that UBF can function to overcome the repressive effects of adding histone H1 (34;

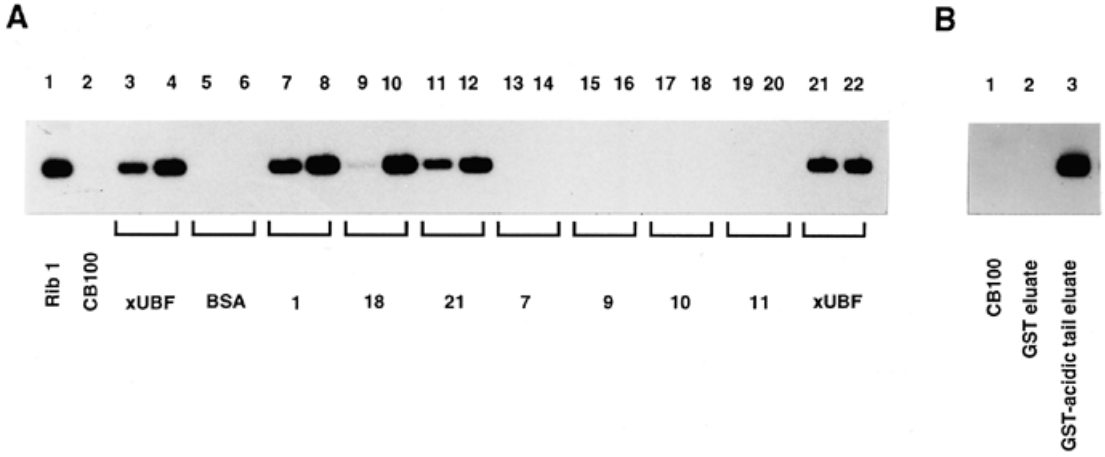

Figure 4. Rib1 interaction with immobilized UBF. (**A**) Rib1 binding to UBF-coated magnetic beads. Rib1 was incubated with Dynal beads coated with UBF, BSA or mutant UBF protein, washed and resuspended as described in Materials and Methods. Transcription reactions were then performed that contained 10 µl heparin 0.4 M fraction, 200 ng baculovirus-produced xUBF and either 7.5 (lanes 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21) or 15 µl (lanes 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22) of the final bead suspension (indicated above). As positive and negative controls the reactions shown in lanes 1 and 2 contained 10 µl heparin 0.4 M fraction, 200 ng xUBF and 10 µl starting Rib1 fraction or CB100 buffer respectively. (**B**) Rib1 binding to GST–acidic tail fusion protein. GST and GST–acidic tail columns were loaded with S100 transcription extract, washed and eluted as described in Materials and Methods. Subsequent transcription reactions contained 10 µl heparin 0.4 M fraction, 200 ng baculovirus-produced xUBF, 10 µl CB100 buffer (lane 1) and 10 µl eluate from a GST column (lane 2) or 10 µl eluate from a GST–acidic tail column (lane 3).

G.J.Sullivan and B.McStay, unpublished observation). However, the timing of enhancer action observed both *in vitro* and *in vivo* argues strongly against such a model. We observe in our *in vitro* system that enhancers function only during stable transcription complex formation (29). Enhancer action is completed during a 10 min preincubation of templates and extract on ice in the absence of nucleotide triphosphates and magnesium. These are conditions that are inconsistent with chromatin assembly to a significant degree. Furthermore, in oocyte microinjection experiments it has been observed that enhancers act within the first 4 min after microinjection (35). Even in oocytes, chromatin assembly has considerably longer kinetics than this. Furthermore, we have observed that addition of histone H1 to our *in vitro* system, if anything, preferentially inhibits enhancer-bearing templates (G.J.Sullivan and B.McStay, unpublished observation).

The second possibility is that a factor other that UBF or Rib1 is specifically required for enhancer function. Support for this hypothesis comes from the following observation. The heparin 0.4 M and Rib1 fractions combined support high level promoter activity but do not support any enhancer function, even in the presence of added recombinant UBF (G.J.Sullivan and B.McStay, unpublished observation). Such a factor would be expected to have the characteristic of facilitating capture of Rib1 by the promoter in a manner that is dependent on UBF being bound to upstream enhancer repeats. These fractionated components should provide an assay system for identifying and purifying this presumptive factor.

One of the intriguing observations concerning RNA pol I transcriptional enhancement is that a diverse array of sequences can function as enhancers. Polymerized sequences derived from the enhancer homology region or core elements of the gene promoter and from other repeated sequences found further upstream in the *Xenopus* intergenic spacer function in enhancement (5,18). Mouse enhancer elements can function in the *Xenopus* system (8,29). This flexibility of enhancer function also extends to the UBF utilized. hUBF (both hUBF 1 and 2) can function in enhancement of *Xenopus* RNA pol I transcription (29) and here we show that UBF mutants severely impaired in promoter function retain enhancer function. We must conclude, therefore, that any architectural function UBF has in enhancement exhibits a remarkable in-built flexibility. This is possibly a consequence of the repeated nature of RNA pol I enhancer elements.

The remaining puzzle concerns the role of the acidic tail of UBF and the role of the UBF–Rib1 interaction. One potential role for the acidic tail that we do not address in our *in vitro* system is the subcellular localization of UBF. Maeda *et al.* (36) have shown that the acidic tail is required for nucleolar accumulation of mouse UBF. Likewise, in microinjection experiments we have demonstrated that the acidic tail of xUBF is required for accumulation of UBF in the amplified nucleoli of oocytes (G.J.Sullivan and B.McStay, unpublished observation). It is possible, therefore, that the acidic tail of UBF not only directs nucleolar accumulation of UBF, but, by virtue of its interaction, is also responsible for the nucleolar accumulation of Rib1. It is worth noting that Rib1 is an unstable complex that freely dissociates into the TBP and TAF components and that it can be stabilized by a high concentration of UBF (27).

Finally, it has been demonstrated that the acidic tail of UBF is a target for phosphorylation by a casein kinase II-like activity (37). Alterations in the phosphorylation status of the acidic tail correlate with changes in RNA pol I transcription (38,39). Accordingly, the acidic tail may have a role in growth regulation of RNA pol I transcription involving alterations in Rib1 affinity that is not recapitulated in our *in vitro* system.

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