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Upstream Binding Factor Stabilizes Rib 1, the TATA-Binding-Protein-Containing *Xenopus laevis* RNA Polymerase I Transcription Factor, by Multiple Protein Interactions in a DNA-Independent Manner

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Initiation of RNA polymerase I transcription in *Xenopus laevis* requires Rib 1 and upstream binding factor (UBF). UBF and Rib 1 combine to form a stable transcription complex on the *Xenopus* ribosomal gene promoter. Here we show that Rib 1 comprises TATA-binding protein (TBP) and TBP-associated factor components. Thus, Rib 1 is the *Xenopus* equivalent of mammalian SL 1. In contrast to SL 1, Rib 1 is an unstable complex that readily dissociates into TBP and associated components. We identify a novel function for UBF in stabilizing Rib 1 by multiple protein interactions. This stabilization occurs in solution in a DNA-independent manner. These results may partially explain the difference in UBF requirement between *Xenopus* and mammalian systems.

Transcription of the genes that encode 18S and 28S rRNAs requires, in addition to RNA polymerase I (Pol I), the *trans*-acting factors upstream binding factor (UBF) and Rib 1 in *Xenopus laevis* (23) or SL 1 in mammals (3, 9, 21). UBF has been cloned from human (16), rat (26), mouse (14, 32), and *X. laevis* (1, 23) cells. UBF binds to DNA sequences within the ribosomal gene promoter (3, 27). This binding activity is conferred by the presence of multiple high-mobility-group (HMG) box DNA binding motifs present in UBF (16, 22). One role of UBF appears to be in facilitating the association of SL 1 or Rib 1 with the promoter. SL 1 alone binds poorly to DNA, but in the presence of UBF it binds tightly and specifically to sequences within the promoter (3). Protein-protein interactions between UBF and SL 1 may play a role in recruiting SL 1 to the promoter (2, 13). Similarly, *Xenopus* UBF (xUBF) and Rib 1 can combine to form a stable transcription complex on the *Xenopus* promoter (23). These observations suggest that SL 1 and Rib 1 are equivalent factors. This view is further confirmed by the similar chromatographic properties of SL 1 and Rib 1 (21, 23).

It is now apparent that the TATA-binding protein (TBP) is required for transcription initiation by all three classes of eukaryotic RNA polymerase (see reference 28 for a review). Moreover, it has been shown that SL 1 is a stable multipeptide complex that is composed of TBP and three TBP-associated factors (TAF_Is) (7). Immunoprecipitation using anti-TBP antibodies demonstrates that SL 1 is composed of TBP and TAF_Is of 110, 63, and 48 kDa. This complex is sufficient to specify SL 1 activity. More recently, cDNA clones encoding each of these TAF_Is have been described and SL 1 activity has been reconstructed from recombinant components (8, 33). Here we demonstrate that Rib 1 is similarly composed of TBP and associated factors. In contrast to SL 1, however, it appears that Rib 1 is a markedly less stable complex that readily dissociates into TBP and TAF components.

Despite the overall similarity of *X. laevis* and mammalian systems, there appears to be a differential requirement for UBF in transcription initiation. Fractionated and immunodepleted transcription extracts have been used to demonstrate that UBF is an essential component of the *Xenopus* RNA Pol I transcription machinery (5, 23). A mutagenic analysis has demonstrated that xUBF function requires an amino-terminal dimerization domain and a precise arrangement and number of HMG box DNA-binding domains (22). Indeed, we have recently shown that the sole reason for the inability of human UBF to function in *Xenopus* RNA Pol I transcription is the presence of an additional HMG box (5).

In mammalian systems, however, it appears that UBF is an auxiliary transcription factor (3, 5, 17, 18, 30). Indeed, some experiments have indicated that the role of UBF is to negate the effect of RNA Pol I transcription inhibitors (18). Here we identify a novel role for UBF in stabilizing the Rib 1 complex in a DNA-independent manner. This is achieved by multiple protein contacts between UBF and Rib 1. We suggest that this may partially explain the difference in UBF requirement between *X. laevis* and mammalian systems.

MATERIALS AND METHODS

Antibodies. The following peptide sequences were chosen to generate anti-TBP antibodies: Pep 1 (TQQSTLQQGNQGSGQTPQL; residues 61 to 79 of xTBP), Pep 2 (ALRARNAEYNPK; 142 to 153), and Pep 3 (CTGAKSEEQSR LAARKY; 179 to 195). Rabbits were immunized with each peptide coupled to keyhole limpet hemocyanin. Each resulting antiserum was capable of detecting 1 to 5 ng of recombinant *Xenopus* TBP (xTBP) by Western blotting (immunoblotting).

Monoclonal antibody (MAb) 12CA5 recognizes the influenza virus hemagglutinin (HA) epitope YPYDVPDYA (11) and was purchased from Berkeley Antibody Company. MAb 9E10 recognizes the epitope MEQKLISEEDL present in human c-Myc (10).

Plasmids. Plasmid pGem 40 contains the *Xenopus* rRNA gene promoter positions −245 to +50 cloned as a *Sal*I-*Bam*HI restriction fragment in the vector pGEM-3 (Promega) (24). This plasmid was used as the template for *Xenopus* RNA Pol I transcription in vitro.

Plasmid pxTBP contains a 1.8-kb *Eco*RI fragment, encoding xTBP, cloned into the vector pGEM-7Zf(+) (Promega) (12). Site-directed mutagenesis (19) was used to create a novel *Bam*HI restriction site 6 nucleotides downstream of the translational stop codon in pxTBP. In addition, DNA sequences surrounding the translation initiation site were converted to either *Nco*I or *Nde*I restriction sites to facilitate cloning into in vitro translation or bacterial expression vectors, respectively. For in vitro translation, xTBP was fused to an IRES (internal

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ribosomal entry site) element from encephalomyocarditis virus. This IRES element, an *Eco*RI-*Nco*I fragment from the vector pCITE 1 (Novagen), was fused to TBP at the novel *Nco*I site described above. The resulting IRES-TBP fusion was cloned as an *Eco*RI-to-*Bam*HI fragment into the plasmid vector pBluescript SK^+ (Stratagene). This plasmid is called pCITE xTBP.

The following amino acid changes were generated in pCITE xTBP by site-directed mutagenesis: alanine 14 to arginine (A14R), serine 116 to arginine (S116R), valine 198 to serine (V198S), and lysine 295 to serine (K295S). In each of these mutations, the alteration of the coding sequence also resulted in the introduction of a novel *Bgl*II restriction site. These *Bgl*II sites were used to construct deletion mutants A to D. In mutant A, sequences between residues 15 and 116 inclusive have been deleted. In mutant B, sequences between residues 117 and 197 have been deleted. In mutant C, sequences between residues 198 and 294 have been deleted. In mutant D, sequences between residues 117 and 294 have been deleted.

A Myc epitope-tagged version of TBP in pCITE xTBP was generated by using the *Bgl*II site in A14R as the site of insertion for two copies of an oligonucleotide that encodes the peptide sequence MEQKLISEEDLN. An HA epitope-tagged version of TBP was generated by insertion of an oligonucleotide that encodes the peptide sequence YPYDVPDYA.

Plasmid pET xTBP, used for expression of TBP in bacteria, contains TBP cloned as an *Nde*I-*Bam*HI restriction fragment in the vector pET-11b (Novagen) (31). Plasmid pET Ha-xTBP is identical to pET xTBP except that it contains a single HA epitope tag inserted into A14R as described above.

Plasmid pGEXxTBP contains the entire xTBP open reading frame cloned into the bacterial glutathione *S*-transferase (GST) fusion protein expression vector pGEX 2TK (Pharmacia).

Bacterial expression and purification of recombinant xTBPs. Plasmids pET 11b xTBP and pET 11b Ha-xTBP were transfected into *Escherichia coli* BL21-DE3(pLysS) (31). Cultures (1.5 liters) of transfected bacteria were grown at 37°C in L broth to an optical density at 600 nm of 0.5. After induction with isopropyl-β-D-thiogalactoside (IPTG; 0.5 mM), cultures were grown for a further 1.5 h. Cells were harvested by centrifugation, and cell pellets were resuspended in 40 ml of nondenaturing lysis buffer (10% glycerol, 0.5 M NaCl, 0.1% Nonidet P-40, 10 mM Tris [pH 7.9], 5 mM dithiothreitol [DTT], protease inhibitors). After repeated sonication on ice, extracts were clarified by centrifugation at 16,000 × *g* for 10 min. Supernatants were dialyzed against 25 volumes of column buffer (CB; 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol) with 100 mM KCl (CB100). The dialyzed extract was then loaded onto a 20-ml Biorex 70 ion-exchange column (Bio-Rad). The column was eluted with a linear salt gradient from 100 to 1,000 mM KCl in column buffer. TBP eluted from this column at approximately 600 mM KCl. TBP-containing fractions were pooled, adjusted to 200 mM KCl, and then loaded on a 5-ml HiTrap heparin column (Pharmacia). This column was then eluted with a 200 to 800 mM KCl linear gradient, with TBP eluting at approximately 500 mM KCl. Column fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining or by Western blotting with anti-TBP antibodies. The resulting TBP was at least 80% pure. Typical yields of purified TBP were 500 μg at a final concentration of 100 μg/ml. The functionality of recombinant TBP was demonstrated by DNase I footprinting on the adenovirus major late promoter.

pGEXxTBP and pGEX 2TK were transfected into *E. coli* JM109, and 1.5-liter cultures were grown at 37°C in L broth to an optical density at 600 nm of 0.6. Protein expression was induced by addition of IPTG to a concentration of 0.5 mM. After 1.5 h, cells were harvested by centrifugation and the pellets were resuspended in 10 ml of phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.3]) containing 1 mM DTT and protease inhibitors. Cell suspensions were sonicated three times for 30 s each on ice and centrifuged at 16,000 × *g* for 10 min. Clarified extracts were incubated with 350 μl of preequilibrated glutathione-agarose beads (Sigma) for 30 min at 4°C on a rotator and then extensively washed with PBS containing 1 mM DTT. Beads were equilibrated with CB100 prior to use.

In vitro transcription and translation of TBP. Plasmid pCITE xTBP and its derivatives were digested with the restriction enzyme *Xba*I, which cuts in the polylinker downstream of the TBP open reading frame. Each of the digested plasmids was then transcribed with phage T7 RNA polymerase, and the resulting transcripts were translated in vitro in a rabbit reticulocyte lysate. Translation reactions were performed in a 25-μl volume containing 17.5 μl of rabbit reticulocyte lysate (Promega), 20 μM amino acids (including methionine), 20 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham), 20 U of RNasin (Promega), and 0.5 μg of synthetic TBP message. Reaction mixtures were incubated at 30°C for 90 min. Aliquots of each reaction mixture were electrophoresed in SDS–15% polyacrylamide gels. Following electrophoresis, gels were fixed in 40% methanol–10% acetic acid, dried, and autoradiographed.

Transcription assays. S100 transcription extracts were prepared from the *X. laevis* cell line X1K-2 as previously described (24). Transcription extracts (20 μl per reaction) alone or with additions (described in figure legends where appropriate) were combined with 400 ng of supercoiled pGem 40 template. Reactions were initiated by the addition of 20 μl of transcription buffer containing $MgCl_2$ and nucleotide triphosphates (NTPs). The final reaction conditions were 25 mM HEPES (pH 7.5), 90 mM KCl, 6 mM $MgCl_2$, 10 mM creatine phosphate, 0.5 mM NTPs, 1 mM DTT, and 100 μg of α-amanitin per ml. Transcription extracts were incubated at 25°C for 180 min. Reactions were terminated and analyzed by S1 nuclease protection as previously described (24). The probe used for detection of transcripts was the 5′-end-labeled coding strand of the −245 to +50 *Sal*I-*Bam*HI insert of pGem 40. Quantitation of transcription was performed with a phosphorimager (Bio-Rad).

In some transcription experiments, more purified protein fractions were used in place of S100 extract. These include Hep 0.4, Rib 1, and baculovirus-produced xUBF. The Hep 0.4 M fraction contains both RNA Pol I and UBF and has been described elsewhere (23). Briefly, S100 extract is chromatographed over DEAE-Sepharose. A fraction that elutes from this column with 0.35 M KCl is adjusted to 0.2 M KCl and loaded onto a heparin Ultrogel column. The Hep 0.4 M fraction is that which elutes from this column with 0.4 M KCl. Rib 1 was eluted from the heparin column with a 0.4 to 0.8 M KCl salt gradient (23). Fractions containing the peak of Rib 1 activity were pooled and dialyzed against CB100. Full-length xUBF was expressed in a baculovirus system and purified as previously described (15).

xTBP immunodepletion. xTBP was immunodepleted from transcription extract by using a cocktail of all three antipeptide antisera. Extract (2.0 ml) was incubated with 30 μl of anti-TBP antiserum on ice for 30 min and then chromatographed repeatedly over a 0.4-ml protein A-Sepharose Fast Flow column (Pharmacia). Alternatively, 300 μl of antiserum was incubated overnight with 130 μg of anti-rabbit immunoglobulin G magnetic beads (Dynal). Following exhaustive washing, these beads were then combined with extract (2.0 ml) and incubated on a rotator at 4°C for 1 h. Beads were removed as instructed by the manufacturer. Western blotting confirmed that immunoglobulin G was efficiently removed from depleted extracts.

UBF affinity chromatography. One milliliter of baculovirus-produced xUBF at 0.5 mg/ml in CB with 600 mM KCl (CB600) was incubated with 0.5 ml of prewashed Affi-Gel 10 affinity support matrix (Bio-Rad). The sample was split in two, and coupling was allowed to proceed for either 2 or 12 h at 4°C. The resins were then blocked by adding ethanolamine (pH 8.0) to a final concentration of 100 mM. The separate coupling reactions were then combined. Bovine serum albumin (BSA) Affi-Gel was prepared exactly as described above for UBF Affi-Gel. Transcription extract or in vitro-translated TBPs were chromatographed on columns of UBF or BSA Affi-Gel as described in figure legends.

RESULTS

TBP is a component of *Xenopus* RNA Pol I transcription. To investigate the role of TBP in RNA Pol I transcription in *X. laevis*, we have used anti-TBP antibodies raised against three peptide sequences derived from xTBP. The locations of these peptides are shown in Fig. 1A. Anti-TBP antibodies (α-Pep1, α-Pep2, and α-Pep3) can specifically inhibit RNA Pol I transcription in vitro. Addition of 1 μl of each antiserum completely inhibits transcription initiation in S100 extracts prepared from *X. laevis* culture cells (Fig. 1B, lanes 2, 5, and 8). The specificity of this inhibition is demonstrated by the fact that addition of an equal amount of either preimmune serum (lanes 1, 4, and 7) or immune serum in competition with the corresponding peptide (lanes 3, 6, and 9) has no effect on transcription. This result demonstrates that TBP is an essential component of *Xenopus* RNA Pol I transcription.

Endogenous TBP can be replaced with recombinant protein. Anti-TBP antibodies (α-Pep1, -2, and -3) were used in combination to immunodeplete TBP from an S100 transcription extract. This depleted extract cannot support RNA Pol I transcription (Fig. 2). Immunoprecipitation of TBP from transcription extracts results in depletion of TBP alone. This is demonstrated by the observation that addition of 2 μl of in vitro-translated TBP to depleted extract restores transcription to the level observed in a nondepleted extract (Fig. 2, lanes 1 to 3). This result suggests that immunoprecipitation of TBP has not coprecipitated to a significant degree any other component required for RNA Pol I transcription. This result also suggests that recombinant TBP can assemble into the Pol I preinitiation complex and restore transcription activity. To conclusively demonstrate this second point, we have reconstituted transcription activity with epitope-tagged TBP and shown that antibodies directed against this tag can subsequently inhibit TBP function in RNA Pol I transcription.

We have constructed modified forms of TBP containing

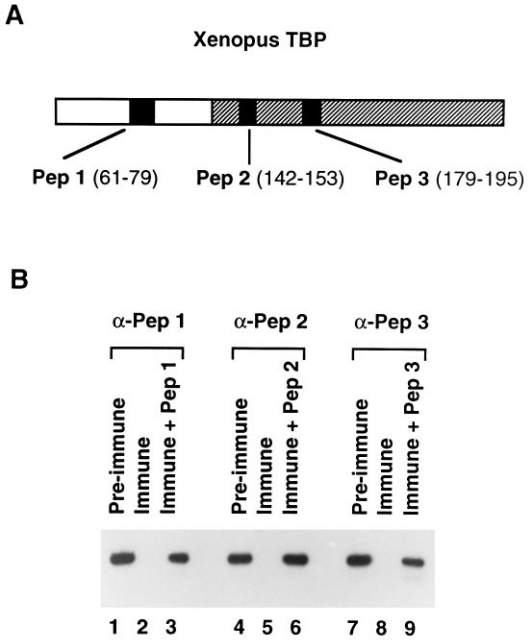

FIG. 1. Anti-TBP antibodies specifically inhibit *Xenopus* RNA Pol I transcription. (A) Peptides sequences. The structure of xTBP is shown in cartoon form. Open boxes denote the amino-terminal domain that is highly variable in sequence between species; cross-hatched boxes denote the highly conserved core domain of TBP. The locations of peptide sequences chosen for generating anti-TBP antibodies are shown by black boxes. The name and coordinates of each peptide are shown below. (B) Antipeptide antibodies inhibit RNA Pol I transcription. Transcription reaction mixtures in lanes 1, 4, and 7 contained 1 μl of preimmune serum for Pep 1, Pep 2, and Pep 3, respectively. Lanes 2, 5, and 8 contained 1 μl of immune serum that recognizes Pep 1, Pep 2, and Pep 3. Lanes 3, 6, and 9 contained 1 μl of immune serum that recognizes Pep 1, Pep 2, or Pep 3 preincubated with 1 μg of the corresponding peptide.

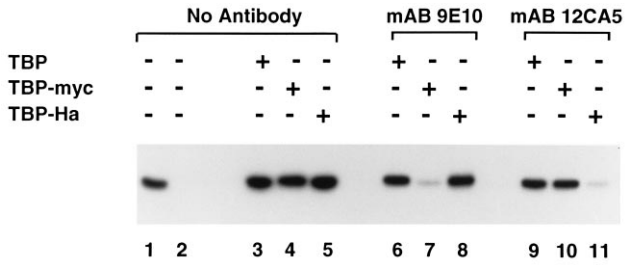

FIG. 2. Transcription activity can be reconstituted with recombinant TBP. Transcription reactions 1 and 2 were performed in untreated and TBP-depleted extracts, respectively. Transcription reactions 3 to 11 were also performed in TBP-depleted extract but were supplemented with 2 μl of in vitro-translated wild-type or epitope-tagged TBP. Reactions 6 to 8 contained, in addition, 2 μg of MAb 9E10. Reactions 9 to 11 contained 2 μg of MAb 12CA5. The form of TBP and MAb that was added to transcription reactions is shown above each lane.

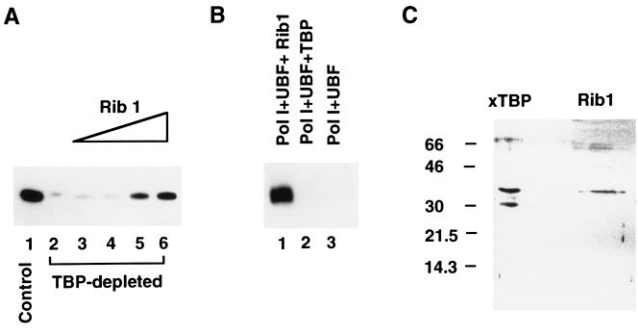

FIG. 3. Rib 1 contains TBP. (A) Addition of Rib 1 restores transcription activity to TBP-depleted extract. The transcription reaction shown in lane 1 was performed with an untreated transcription extract. Reactions shown in lanes 2 to 6 were performed with extract depleted of TBP as described in Materials and Methods. Transcription reaction mixtures in lanes 3 to 6 contained, in addition, 0.5, 1, 2, or 4 μl of a Rib 1 fraction. (B) TBP cannot substitute for Rib 1. Transcription reactions 1, 2, and 3 each were performed with 10 μl of a Hep 0.4 fraction that includes RNA Pol I and xUBF; 10 μl of a Rib 1 fraction was added to reaction 1, 100 ng of bacterially produced TBP in 10 μl of CB100 was added to reaction 2, and 10 μl of CB100 alone was added to reaction 3. (C) Western blot. A 20-μl aliquot of the Rib 1 fraction was subjected to SDS-PAGE (14% gel) alongside purified bacterially expressed TBP (10 ng). Western blotting was then performed with a commercial anti-human TBP antibody (Upstate Biotechnology Inc.) as instructed by the manufacturer. Positions of molecular mass markers, in kilodaltons, are shown on the left. The lower band present in recombinant TBP is a proteolytic fragment.

either Myc or influenza virus HA epitope tags, recognized by MAbs 9E10 and 12CA5, respectively. Insertion of these tags does not disrupt TBP function, since tagged forms of TBP function as efficiently in transcription as nontagged TBP (Fig. 2, lanes 3 to 5). Addition of MAb 9E10 inhibits transcription (approximately 10-fold) in a reaction performed with immunodepleted extract supplemented with Myc-tagged TBP (lane 7). MAb 9E10 does not inhibit transcription in reactions supplemented with either nontagged or HA-tagged TBP (lane 6 or 8, respectively). Likewise, addition of MAb 12CA5 inhibits transcription only in reactions performed with HA-tagged TBP (lanes 9 to 11). Thus, we conclude that immunoprecipitation removes only TBP and that epitope-tagged TBP can reassemble into a functional initiation complex.

TBP is a component of Rib 1. Rib 1 has been previously defined as a fraction that elutes from a heparin-Sepharose column (23). Rib 1 is an essential component of the *Xenopus* RNA Pol I transcription complex. On the basis of its chromatographic characteristics and the observation that it combines with UBF to form a stable complex on the *Xenopus* promoter, it has been argued that Rib 1 is the *X. laevis* homolog of the mammalian RNA Pol I factor, SL 1 (23). TBP has been previously demonstrated to be a key component of SL 1. Here we demonstrate that TBP is a component of Rib 1.

Addition of Rib 1 restores transcription activity to TBP-depleted cell extracts (Fig. 3A). Addition of either RNA Pol I or xUBF has no stimulatory effect (data not shown). We conclude that Rib 1 is the TBP-containing component of RNA Pol I transcription in *X. laevis*. This conclusion is supported by Western blotting experiments that also confirm the presence of TBP in Rib 1 (Fig. 3C).

To demonstrate that TBP, while essential, is only a single component of Rib 1, we have used fractionated transcription extracts. A Hep 0.4 M fraction contains both xUBF and RNA Pol I. This fraction on its own cannot support transcription initiation (Fig. 3B, lane 3). Transcription activity is reconstituted when Rib 1 is combined with the Hep 0.4 M fraction (lane 1). Transcription activity is not reconstituted when TBP is combined with the Hep 0.4 M fraction (lane 2). Thus, the Rib 1 fraction contains at least two components essential for RNA Pol I transcription, one of these being TBP.

Rib 1 activity is composed solely of TBP and TBP-interacting components. The human RNA Pol I transcription factor SL 1 is composed of TBP and three TAF_Is (7). It therefore seems likely that the non-TBP component of Rib 1, identified in the previous experiment, interacts directly with TBP and is the *X. laevis* equivalent of the SL 1 TAF_Is.

To demonstrate that Rib 1 activity is composed of TBP and TBP-interacting components, a Rib 1 fraction was incubated

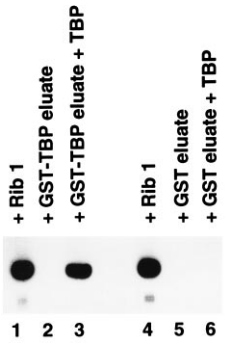

FIG. 4. Rib 1 is composed of TBP and TAF components. A Rib 1 fraction (400 μl) was incubated at 4°C with GST-TBP fusion protein on glutathione-agarose beads (50 μl) in CB100. Following a 1-h incubation, beads were washed with CB100 four times, each with 200 μl, and bound proteins were eluted with CB800 (100 μl). Eluted proteins were diluted to 100 mM KCl by the addition of CB (700 μl). Transcription reaction mixtures contained Hep 0.4 M fraction (10 μl) combined with the following: 10 μl of Rib 1 (lanes 1 and 4), 10 μl of the GST-TBP eluate (lane 2), 10 μl of the GST-TBP eluate supplemented with 100 ng of recombinant TBP (lane 3), 10 μl of eluate from GST-alone beads (lane 5), and 10 μl of eluate from GST-alone beads supplemented with 100 ng of recombinant TBP (lane 6). Rib 1 used in control reactions (lanes 1 and 4) is fourfold less concentrated than that applied to GST-TBP beads.

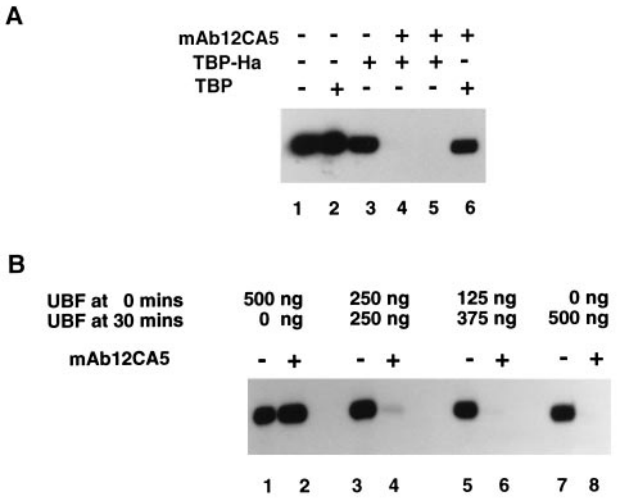

FIG. 5. UBF influences Rib 1 stability. (A) Rib 1 is an unstable complex. Transcription extract (20 μl) was incubated on ice for 30 min with 100 ng of bacterially produced TBP (lanes 2 and 6) or HA-tagged TBP (lanes 3 to 5). Following the incubation with recombinant TBP, MAb 12CA5 was added to reactions 4, 5, and 6. Reaction 4 was performed with 1 μg of antibody; reactions 5 and 6 were performed with 2 μg. Following these additions, transcription reactions were performed as described in Materials and Methods. A control reaction mixture (lane 1) contained neither added TBP nor antibody. (B) UBF stabilizes Rib 1. In reactions 1 to 8, transcription extract (20 μl) was incubated on ice for 30 min with 100 ng of bacterially produced HA-tagged TBP. In reactions 1 and 2, 500 ng of baculovirus xUBF was added prior to incubation of extract with HA-tagged TBP (at 0 min). In reactions 7 and 8, 500 ng of xUBF was added after incubation with TBP (at 30 min). In reactions 3 and 4, 250 ng of xUBF was added both before and after incubation with HA-TBP. In reactions 5 and 6, 125 ng of xUBF was added before and 375 ng of xUBF was added after incubation with HA-TBP. In reactions 2, 4, 6, and 8, 2 μg of MAb 12CA5 was added subsequent to all xUBF and HA-TBP additions. Transcription reactions were then performed as described in Materials and Methods.

with GST-TBP fusion protein on glutathione-agarose beads (see Materials and Methods). Following incubation, the beads were repeatedly washed with CB100, and bound proteins were then eluted with buffer containing 800 mM KCl (CB800). Eluate was converted to 100 mM KCl by dilution and tested for transcription activity (Fig. 4). The eluate from the GST-TBP beads on its own cannot support transcription initiation when combined with UBF and RNA Pol I (Hep 0.4 M fraction [lane 2]). However, the further addition of recombinant TBP restores transcription activity (lane 3). In control experiments, we show that this activity cannot be recovered by using GST beads (lanes 4 to 6). We can conclude from this experiment that Rib 1 activity can be fully reconstituted by a combination of TBP and a TBP-interacting component. This TBP-interacting component displays all of the characteristics attributed to SL 1 TAF_Is. We therefore conclude that Rib 1 is the true *X. laevis* equivalent of SL 1. In addition, this experiment demonstrates that there are no other essential activities present in the Rib 1 fraction. For reasons of clarity, we will now refer to Rib 1 as comprising TBP and TAF components.

Rib 1 is an unstable complex. Having demonstrated that Rib 1 is composed of TBP and TAF components (Fig. 4), it is somewhat surprising that anti-TBP antibodies can specifically deplete only TBP from Rib 1 (Fig. 2). Previous work with both human and mouse SL 1 has shown that anti-TBP antibodies can be used to immunoprecipitate the entire complex (7, 9). One possible explanation for this difference is that the antibodies used here disrupt the Rib 1 complex. An alternative explanation is that interaction of TBP with the TAF component of Rib 1 is dynamic rather than stable. The observation that the TBP-interacting component of Rib 1 can bind to GST-TBP beads suggests this to be the case (Fig. 4). To conclusively demonstrate that Rib 1 is an unstable complex, we show that exogenously added TBP can both exchange with endogenous TBP and interact with the endogenous TAF component to reassemble a fully functional Rib 1 complex.

We have produced both nontagged and HA-tagged TBP in bacteria and purified both proteins to apparent homogeneity. Addition of 100 ng (>50-fold molar excess over endogenous TBP) of either nontagged or HA-tagged TBP to a nondepleted extract has no negative effect on its transcription activity (Fig. 5A, lanes 1 to 3). Upon the subsequent addition of MAb 12CA5, we observe that transcription activity is abolished in reaction mixtures that contain HA-tagged TBP but not in those containing nontagged TBP (lanes 4 to 6). From this experiment, we conclude that the exogenously added TBP, which is in vast molar excess over endogenous TBP, can readily interact with the TAF component of Rib 1 and be recruited into a stable transcription complex. Thus, Rib 1 is a dynamic complex that can readily exchange its endogenous TBP with added recombinant forms.

Attempts at further purification of Rib 1 have also indicated how unstable the complex is. We have repeatedly observed that once Rib 1 has been separated from the other components of the transcription machinery (UBF and RNA Pol I), its activity becomes increasingly labile. It therefore seems likely that some other component of the RNA Pol I transcription machinery can stabilize Rib 1. Since UBF and Rib 1 combine to form a stable transcription complex on the promoter, we chose to examine if UBF could also influence Rib 1 stability in the absence of DNA.

UBF stabilizes the Rib 1 complex. We have shown above that when transcription extracts are incubated in the presence of a molar excess of tagged TBP, this TBP can functionally integrate into Rib 1. If, however, the extract is supplemented with a high concentration of xUBF prior to the addition of tagged TBP, we observe that transcription can no longer be inhibited by the addition of MAb 12CA5 (Fig. 5B). When 500 ng of xUBF (baculovirus produced) is added to transcription extract prior to the addition of tagged TBP, we observe com-

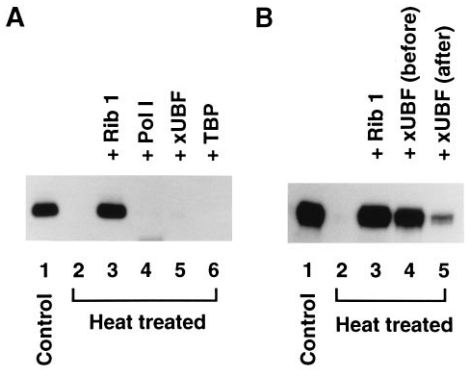

FIG. 6. UBF influences the heat stability of Rib 1. (A) Rib 1 is heat labile. S100 transcription extract was heat inactivated by incubation at 42°C for 15 min followed by incubation on ice for 10 min. Transcription reactions were performed with heat-inactivated extract (20 μl) alone (lane 2) or combined with 5 μl of Rib 1 (lane 3), 3 μl of RNA Pol I (lane 4), 500 ng of xUBF (lane 5), or 100 ng of TBP (lane 6). A transcription reaction performed with untreated extract (lane 1) served as a positive control. (B) UBF confers heat stability. Transcription reactions were performed with untreated and heat-inactivated extracts (lanes 1 and 2, respectively). In the reaction shown in lane 3, transcription was performed in heat-treated extract combined with 5 μl of Rib 1. In the reactions shown in lanes 4 and 5, transcription reactions were performed in S100 extract that contained 500 ng of xUBF added before and after heat treatment, respectively.

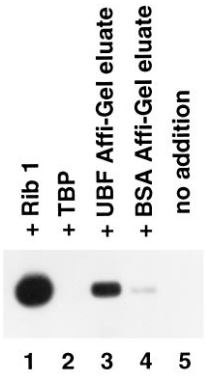

FIG. 7. UBF interacts directly with Rib 1. S100 extract (200 μl) was loaded repeatedly onto either UBF or BSA Affi-Gel columns (100-μl bed volume). Columns were washed with 10 bed volumes of CB100, and bound proteins were eluted with 200 μl of CB600. Eluted fractions were then dialyzed against CB100. Transcription reaction mixtures 1 to 5 each contained 10 μl of a Hep 0.4 fraction that includes RNA Pol I and xUBF; 10 μl of Rib 1 and 100 ng of TBP in 10 μl of CB100 were combined with the Hep 0.4 fraction in reaction mixtures 1 and 2, respectively, and 10 μl of high-salt eluate from the UBF and BSA Affi-Gel columns were combined with the Hep 0.4 fraction in reaction mixtures 3 and 4, respectively. As a final control, 10 μl of CB100 alone was added (reaction mixture 5).

plete protection from the inhibitory effect of MAb 12CA5 (compare lanes 1 and 2). Thus, no appreciable fraction of Rib 1 has been assembled around exogenously added TBP. When 500 ng of UBF is added after incubation of extract with tagged TBP, we observe that MAb 12CA5 can still totally inhibit transcription initiation (compare lanes 7 and 8). When lower amounts of UBF are added prior to incubation with tagged TBP, we observe intermediate effects (lanes 3 to 6). From this result, we conclude that when present at a high concentration, UBF can stabilize the Rib 1 complex, thereby preventing the incorporation of exogenously added TBP.

As an alternative approach to studying the stabilizing effects of UBF on Rib 1, we have investigated the heat stability of Rib 1 in both the presence and the absence of a high concentration of UBF. Transcription extract, when heated to 42°C for 15 min, is unable to support any transcription initiation (Fig. 6A; compare lanes 1 and 2). This inactivation is due to the irreversible denaturation of the Rib 1 complex. Addition of Rib 1 to heat-inactivated extract restores transcription activity to the level observed in the untreated extract (lane 3). Addition of RNA Pol I, xUBF, or TBP has no stimulatory effect on the heat-treated extract (lanes 4 to 6). Thus, Rib 1 is the most heat-labile component of the *Xenopus* RNA Pol I transcription machinery. The striking observation is that high concentrations of UBF can confer greater heat stability to the Rib 1 complex. Addition of 500 ng of xUBF to transcription extract prior to heat treatment results in levels of transcription activity comparable with that in the untreated extract (Fig. 6B; compare lanes 1 and 4). In contrast, when 500 ng of xUBF is added after heat treatment, we observe that the transcription signal is sevenfold lower than when it is added prior to heat treatment (compare lanes 4 and 5). As an additional control for nonspecific effects, we have demonstrated that addition of an equal amount of BSA has no protective effect against heat treatment (data not shown).

We have shown by using two approaches that UBF can influence the stability of Rib 1. We further conclude that this stabilizing influence of UBF can occur in the absence of DNA, since in the experiments described above, all of the effects of UBF on Rib 1 were observed to occur in the absence of template DNA. The most likely explanation of these results is that UBF makes direct protein-protein contacts with Rib 1 in solution.

UBF interacts with multiple components of Rib 1. To directly address UBF-Rib 1 interactions, we have generated an xUBF affinity column. Baculovirus-produced xUBF was covalently cross-linked to an Affi-Gel 10 matrix (Bio-Rad). Transcription extract was repeatedly loaded onto this affinity column. After being washed in buffer containing 100 mM KCl, bound proteins were eluted from the column with 600 mM KCl. In control experiments, transcription extract was loaded onto a column prepared with BSA. The eluate from the UBF affinity column contains Rib 1 activity, as judged by its ability to activate transcription when combined with RNA Pol I and xUBF (Fig. 7, lane 3). In contrast, the eluate from the BSA column contains little or no Rib 1 activity (lane 4). An additional control is that TBP alone cannot activate transcription when combined with RNA Pol I and xUBF (lane 2). This is the first demonstration that UBF interacts directly with Rib 1 in the absence of DNA.

We envisage that stabilization of Rib 1 by UBF requires multiple protein contacts. Indeed, interactions between components of SL 1 and UBF have already been described (2, 13, 20). Here we show that xUBF can interact directly with TBP. TBP was translated in vitro in the presence of [^{35}S]methionine and loaded onto a UBF affinity column. Bound TBP was eluted from the column with high salt and then visualized by SDS-PAGE. Using this protocol, we demonstrate that TBP binds efficiently to a UBF column (Fig. 8B; compare lanes TBP in left and right panels). To identify the domain of TBP that interacts with UBF, we have constructed a series of deletion mutants (Fig. 8A). These mutants were tested for the ability to bind to the UBF affinity column. Deletion of the nonconserved amino terminus of TBP (mutant A) has little or no effect on UBF interaction (Fig. 8B; compare lanes A in left and right panels). Proteins with deletions in the conserved core domain (mutants B, C, and D) cannot interact with UBF. These TBP mutants were also tested for the ability to stimulate transcription in a TBP-depleted transcription extract. Mutant A can

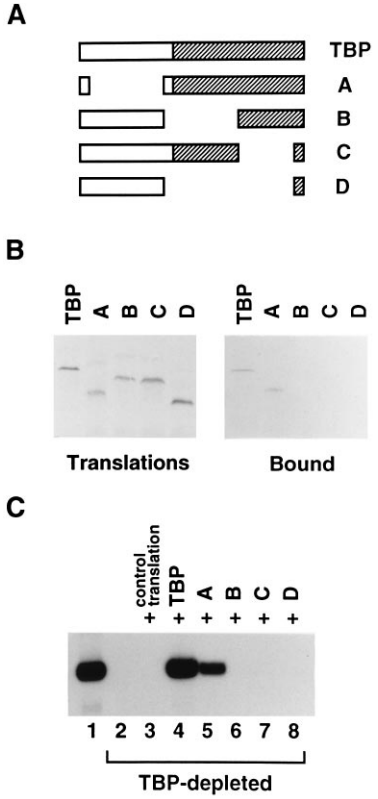

FIG. 8. UBF interacts directly with the core domain of TBP. (A) Structures of TBP mutants. Wild-type and mutant TBPs are shown in cartoon form. Open boxes denote the amino-terminal domain; cross-hatched boxes denote the highly conserved core domain of TBP. See Materials and Methods for a full description of these mutants. (B) Interaction of TBP with xUBF. Wild-type TBP and mutants A to D were translated in vitro in the presence of [^{35}S]methionine. All mutants were translated with approximately equal efficiency and gave rise to a product of the predicted size, as judged by SDS-PAGE of 1-μl aliquots of each translation (left panel). Five-microliter aliquots of each translation reaction diluted to 30 μl with CB100 were repeatedly loaded onto 30-μl UBF Affi-Gel affinity columns. Columns were washed with 5 bed volumes of CB100 and eluted with 40 μl of CB600; 50% of each elution was analyzed by SDS-PAGE (right panel). (C) Mutants tested in transcription. Transcription reactions 1 and 2 were performed with untreated or TBP-depleted extracts, respectively. Transcription reactions 3 to 8 were also performed with TBP-depleted extract but with the addition of 2 μl of a control translation reaction (lane 3), translated wild-type TBP (lane 4), or translated TBP mutants A to D (lanes 5 to 8, respectively).

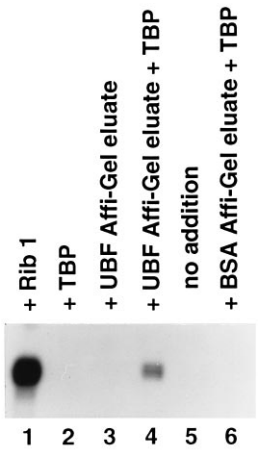

FIG. 9. UBF interacts with the TAF component of Rib 1. TBP-depleted S100 extract (200 μl) was loaded repeatedly onto either UBF or BSA Affi-Gel columns (100-μl bed volume). Columns were washed with 10 bed volumes of CB100. Bound proteins were eluted with 200 μl of CB600. The eluted fraction was then dialyzed against CB100. Transcription reactions 1 to 6 each were performed with 10 μl of a Hep 0.4 fraction that includes RNA Pol I and xUBF. In reactions 1 and 2, 10 μl of Rib 1 and 100 ng of TBP in 10 μl of CB100, respectively, were combined with the Hep 0.4 fraction. In reactions 3 and 4, 10 μl of high-salt eluate from the UBF Affi-Gel column was combined with the Hep 0.4 fraction. The mixture for reaction 4 also contained 100 ng of TBP. As controls, reactions 5 and 6 were performed with 10 μl of CB100 and 10 μl of the high-salt eluate from the BSA Affi-Gel column, respectively.

partially substitute for full-length TBP in transcription (Fig. 8C; compare lanes 4 and 5). Mutants B, C, and D are completely inactive in transcription (lanes 6 to 8). This finding is in agreement with previous observations which show that the conserved core of human TBP is sufficient for SL 1 function in human RNA Pol I transcription (29). Thus, the conserved core domain of TBP alone can support both interaction with UBF and RNA Pol I transcription activity.

In order for UBF to efficiently stabilize Rib 1, we predicted that in addition to contacting TBP, UBF would also interact with the TAF component of Rib 1. To identify such an interaction, we loaded TBP-depleted transcription extract onto both UBF and BSA affinity columns. The high-salt eluate from the UBF affinity column cannot alone restore transcription activity when combined with RNA Pol I and xUBF (Fig. 9, lane 3). However, the further addition of TBP results in restoration of transcription activity (lane 4). This result demonstrates that the entire TAF component of Rib 1 can bind to UBF in a TBP-independent manner. As a control for this experiment, we show that eluate from the BSA column combined with TBP cannot restore transcription (lane 6). This is the first demonstration that xUBF interacts not only with TBP but also with the TAF component of Rib 1 in a DNA-independent manner.

DISCUSSION

Rib 1 is an essential component of the *Xenopus* RNA Pol I transcription machinery. Previously, Rib 1 has been characterized only as a chromatographic fraction that combines with UBF to form a stable transcription complex on the promoter (23). Here we demonstrate that Rib 1 activity can be resolved into two essential components, TBP and a TBP-interacting or TAF component. Thus, Rib 1 must be the *X. laevis* equivalent of mammalian SL 1. While the number and nature of the polypeptides which combine with TBP to form a functional Rib 1 complex is unknown, it is clear that all of these peptides must interact either directly or together with TBP in the absence of DNA. The novel aspect of this work is that, unlike SL 1, Rib 1 is an inherently unstable complex which readily dissociates into its TBP and TAF components. This is most clearly demonstrated by the observation that recombinant TBP can freely exchange into the Rib 1 complex.

We show that a high concentration of UBF can profoundly increase the stability of Rib 1. This was demonstrated by using two different approaches. First, UBF can prevent free exchange of recombinant TBP into Rib 1, and second, UBF greatly increases the heat stability of the Rib 1 complex. We have also observed that in the presence of a high concentration of UBF, immunoprecipitation with anti-TBP antibodies results in depletion of at least part of the TAF component of Rib 1 in addition to TBP (4). While previous work has demonstrated that UBF and Rib 1 interact to form a stable transcription complex on the promoter, we show here that UBF influences Rib 1 stability even in the absence of DNA. Consistent with this finding, we show that UBF makes multiple protein contacts with Rib 1 in solution. These include direct contacts with

the core domain of TBP and the TAF component. Such interactions have also been observed between components of SL 1 and UBF. Human UBF has been shown to interact with both TBP (20) and TAF_I 48 (2). However these interactions were not studied in the context of the SL 1 complex.

Why does it take such a high concentration of UBF to stabilize Rib 1 in vitro? In standard transcription extracts, we have shown that the UBF concentration is approximately 0.5 ng/μl. To stabilize Rib 1, we have increased the UBF concentration 50-fold to 25 ng/μl. UBF is highly localized within intact cells. Immunostaining experiments have demonstrated that the vast majority of UBF in a cell is localized to the nucleolus (6, 16). When in vitro transcription extracts are prepared, subcellular architecture is destroyed and this high degree of UBF concentration is lost. We therefore believe that by supplementing transcription extracts with a high concentration of UBF, we are more closely mimicking the in vivo situation. Indeed, other aspects of RNA Pol I transcription display this strict dependence on a high concentration of UBF. We have recently observed that RNA Pol I transcriptional enhancers function in vitro only in the presence of such high UBF concentrations (25).

Combining the observations that in vivo, UBF is highly localized to the nucleolus and our in vitro data showing that Rib 1 is not a stable complex at low concentrations of UBF (0.5 ng/μl or less), we predict that in vivo, intact Rib 1 could exist only in the nucleolus. Rib 1 therefore would not be observed either in the cytoplasm or the nucleoplasm. If this indeed were the case, it would follow that the individual components of Rib 1 would independently target to the nucleolus, where the Rib 1 complex would be assembled and stabilized. It is conceivable that this localization is driven by the affinity that these components have for the high concentration of UBF present in the nucleolus.

Finally, there appears to be a lack of consensus as to the role of UBF in RNA Pol I transcription. In the *X. laevis* system, it is clear that UBF is an essential component of the transcription machinery (5, 23), while in mammalian transcription extracts, its requirement is less clearly defined (3, 5, 17, 18, 30). The results presented here suggest that this contrast in UBF requirement may at least in part be explained by differences in Rib 1 and SL 1 stability. Under certain conditions, it appears that SL 1 can specify transcription initiation in the absence of UBF, perhaps by virtue of its stable nature. However, we speculate that in *X. laevis*, Rib 1 cannot interact with the promoter without the stabilizing effect of multiple xUBF interactions.

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