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The Species-Specific RNA Polymerase I Transcription Factor SL-1 Binds to Upstream Binding Factor

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Transcription of the 45S rRNA genes is carried out by RNA polymerase I and at least two *trans*-acting factors, upstream binding factor (UBF) and SL-1. We have examined the hypothesis that SL-1 and UBF interact. Coimmunoprecipitation studies using an antibody to UBF demonstrated that TATA-binding protein, a subunit of SL-1, associates with UBF in the absence of DNA. Inclusion of the detergents sodium dodecyl sulfate and deoxycholate disrupted this interaction. In addition, partially purified UBF from rat cell nuclear extracts and partially purified SL-1 from human cells coimmunoprecipitated with the anti-UBF antibody after mixing, indicating that the UBF–SL-1 complex can re-form. Treatment of UBF-depleted extracts with the anti-UBF antibody depleted the extracts of SL-1 activity only if UBF was added to the extract prior to the immunodepletion reaction. Furthermore, SL-1 activity could be recovered in the immunoprecipitate. Interestingly, these immunoprecipitates did not contain RNA polymerase I, as a monospecific antibody to the 194-kDa subunit of RNA polymerase I failed to detect that subunit in the immunoprecipitates. Treatment of N1S1 cell extracts with the anti-UBF antibody depleted the extracts of SL-1 activity but not TFIIIB activity, suggesting that the binding of UBF to SL-1 is specific and not solely mediated by an interaction between UBF and TATA-binding protein, which is also a component of TFIIIB. These data provide evidence that UBF and SL-1 interact.

Despite a lack of sequence homology, the promoters of the mammalian 45S pre-rRNA genes (rDNA) consist of elements with similar functions (30, 34). The region of the promoter from −31 to +6 is referred to as the core promoter element (CPE). The CPE is necessary and sufficient for transcription in vitro and is necessary but insufficient to support transcription in vivo. An additional element, the upstream promoter element (UPE), has been shown to be required for transcription in vivo, for elevated levels of transcription in vitro under stringent conditions, and for formation of the stable preinitiation complex. Moreover, experiments with various types of mutations of the promoters have demonstrated a complex interaction between the UPE and the CPE, including evidence that the two elements must be stereospecifically aligned for optimum function (reviewed in references 30 and 34).

At least two *trans*-acting factors, upstream binding factor (UBF) and the species-specific factor SL-1, are required for efficient transcription by RNA polymerase I. SL-1 is a multimeric protein that contains TATA-binding protein (TBP) and three TBP-associated factors that are specific for transcription by RNA polymerase I (10). TBP is a component of the basal transcription machinery used by all three nuclear RNA polymerases (16). The three human TBP-associated factors have been cloned and found to have apparent molecular masses of 48, 63, and 110 kDa (11, 47).

SL-1 is required for correct initiation of rDNA transcription and confers species-specific promoter recognition upon RNA polymerase I (9, 23, 28, 29). The three characterized mammalian forms of SL-1 demonstrate very different affinities for their respective promoters. Both nuclease footprinting analyses and in vitro transcription assays demonstrate that the interactions of the various SL-1 forms with their respective promoters may require additional factors for the formation of the preinitiation complex (9, 23, 40).

Vertebrate UBF purifies as a doublet (4, 5, 35, 40). In humans, rats, and mice, these proteins, referred to as UBF1 and UBF2, have sizes of 97 and 94 kDa, respectively. The cDNAs for UBF have been cloned (1, 2, 17, 19, 27, 31). The deduced amino acid sequences of human and rat UBF1 are 98% identical. The two forms of human, rat, and mouse UBF, UBF1 and UBF2, consist of an amino-terminal domain of 102 amino acids, four domains referred to as high-mobility group boxes, and a serine-rich, highly acidic carboxyl tail (17, 18, 31). UBF2, which is identical to UBF1 except for a deletion of 37 amino acids from high-mobility group box 2, fails to activate rDNA transcription in vitro (17, 20, 39).

While UBF is not required for basal transcription in vitro (40), the addition of UBF to such assays increases the efficiency of transcription (30, 34). Unlike SL-1, the vertebrate forms of UBF can both bind to heterologous promoters and activate transcription in heterologous systems (4, 35). UBF has been demonstrated to bind to the UPE (24, 40). The acid tail does not appear to affect dimerization or DNA binding (30, 32, 34), but it may be essential for UBF function in transcription assays (18, 19, 42). It has been suggested that the highly charged acid tail may interact with other components of the rDNA transcription machinery (38).

There are several lines of evidence that are consistent with the hypothesis that UBF can interact with SL-1. First, the binding of SL-1 to the promoter is relatively weak (4–6, 24, 30, 34). Indeed, the initial characterization of the binding of human SL-1 to the human rDNA promoter demonstrated that human SL-1 did not bind to the promoter but extended the UBF footprint over the UPE (5, 24). This could also be interpreted as evidence that UBF stabilized the binding of SL-1. The binding site of rat SL-1 in the rat UPE is proximal to the region protected by UBF (40) and is probably analogous to the extension of the footprint produced by the combination of human UBF and SL-1 together (5, 24). Second, it has been

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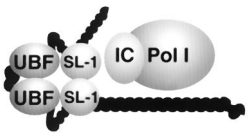

FIG. 1. Cartoon of the RNA polymerase I (Pol I) preinitiation complex on the 45S rDNA promoter. This model is based on experimental results which have been recently reviewed (30, 34). The molar ratios of UBF and SL-1 have not been determined. UBF probably binds as a dimer. However, it is not clear if there are one or two molecules of SL-1 bound per complex. IC, TFIC (34).

demonstrated that the SL-1 binding sites within the UPE and CPE must be stereospecifically aligned (30, 34, 44), and the binding of UBF to the promoter causes the promoter to bend (3), which would bring the SL-1 binding sites within the UPE and CPE into proximity. This would result in a structure (Fig. 1, consistent with the model presented in reference 3) in which UBF would be proximal to SL-1 bound to both the UPE and CPE and would be part of the structure that bridges those binding sites. Third, phosphorylation affects the ability of UBF to activate transcription but not the ability of UBF to bind to DNA, raising the possibility that phosphorylation regulates the interactions of UBF with one or more components of the rDNA transcription apparatus (32, 33, 42). Fourth, the stable association of UBF with the rDNA promoter appears to require additional DNA-binding proteins. In order-of-addition experiments, UBF by itself did not commit to the rDNA promoter, but UBF did commit to the promoter in the presence of SL-1 and other components of the rDNA transcription apparatus (40). Fifth, it has been recently demonstrated that TBP, as part of TFIID, is capable of associating with a number of transcription activators. TBP has been shown to associate with transcription activators such as VP16 (41), the adenovirus large E1A protein (25), and the product of the c-*myc* oncogene (15, 26).

One example of the interaction of UBF with other proteins is the recent demonstration that UBF can interact with the 60-kDa subunit of RNA polymerase I (38). In addition, we have recently demonstrated that UBF interacts with the protein product of the retinoblastoma susceptibility gene (Rb) (8). Indeed, Rb protein, by associating with UBF, blocked the UBF-dependent activation of rDNA transcription in vitro.

In light of these recent discoveries, it seemed likely that if UBF could bind to SL-1, it would do so in the absence of DNA. In this study, using epitope-tagged TBP as a reporter for SL-1, we provide evidence from coimmunoprecipitation experiments that UBF and SL-1 do interact. In addition, treatment of UBF-depleted extracts with an anti-UBF antibody depleted the extracts of SL-1 activity only if UBF was added to the extract prior to the immunodepletion reaction. Furthermore, SL-1 activity could be recovered in the immunoprecipitate. This interaction appears to be specific for SL-1, since immunodepletion of UBF did not lead to a loss of TFIIIB activity in the same extracts.

MATERIALS AND METHODS

Preparation of nuclear extracts. Nuclear extracts were prepared from Novikoff hepatoma ascites cells, N1S1 cells, and LTRα3 cells (46) essentially as previously described (36). Following the final dialysis, extracts were frozen in liquid N_2 and stored at −80°C. S-100 extracts were prepared from LTRα3 or N1S1 cells as previously described (43).

Fractionation of nuclear extracts. Nuclear extracts of Novikoff hepatoma ascites cells and LTRα3 cells were fractionated as previously described (40). The nuclear extracts (1.6 mg of protein per ml of column bed volume) were fractionated over DEAE-Sephadex columns (Sigma, St. Louis, Mo.) equilibrated in 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–5 mM $MgCl_2$–0.2 mM EDTA–0.5 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–20% glycerol (DE buffer) containing 50 mM ammonium sulfate (DE-50). RNA polymerase I and SL-1 activities were coeluted by using DE buffer containing 175 mM ammonium sulfate (DE-175). UBF was eluted by using DE buffer containing 500 mM ammonium sulfate (DE-500). The DE-175 fractions containing the peak of RNA polymerase I activity (UBF-depleted extract) and the protein peak from the DE-500 fractions (partially purified UBF) were pooled and dialyzed for 12 h against buffer C/20 (20 mM HEPES [pH 7.9], 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol). All fractions were stored at −80°C. For some experiments, UBF was purified through the heparin-Sepharose step as previously described (40).

Immunoprecipitation and Western blotting (immunoblotting) of nuclear extracts. All steps of the immunoprecipitations were carried out at 4°C unless otherwise specified. LTRα3 nuclear extract or S-100 extract (10 mg/ml) was diluted 1:1 with buffer C/0 (C/20 without glycerol) and precleared by incubation for 1 h with protein A-agarose beads (Sigma) while tumbling gently. The beads were removed by centrifugation for 5 s, and various amounts of precleared extract were added to 25 μl of packed protein A-agarose beads that had been incubated overnight with the anti-UBF antibody C-21 (31). The volume of the binding reaction mixture was adjusted to 200 μl with C/10 (C/20 buffer with 10% glycerol) and brought to a final concentration of 0.2% Nonidet P-40 (NP-40). The mixture was tumbled for 4 h, and the beads were washed three times with 1 ml of C/10–0.2% NP-40. The beads were finally resuspended in 40 μl of 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (22) and incubated for 10 min at 95°C. The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. UBF and TBP were visualized by standard immunoblotting techniques. UBF was detected by incubating the filter with a 1:10,000 dilution of a polyclonal anti-UBF antibody (raised to recombinant protein) followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibodies (Amersham). TBP was detected by incubating the filter with either antibody 12CA5 (anti-influenza virus epitope [FLU epitope] tag) at 1:2,000 or a monoclonal anti-human TBP antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) at 1:500. The immunoreactive proteins were visualized by incubation with horseradish peroxidase-conjugated anti-mouse antibodies and enhanced chemiluminescence (Amersham). For many experiments, we used extracts of LTRα3 cells, as they express FLU epitope-tagged TBP (46). This facilitates the immunodetection of immunoprecipitated hemagglutinin (HA)-tagged TBP with mouse monoclonal anti-HA antibodies because the secondary antibody is directed against mouse immunoglobulin G (IgG) and only weakly cross-reacts with the rabbit Ig heavy chain (IgHc) present in the immunoprecipitates. A secondary antibody directed against rabbit IgG would interfere with the detection of TBP on immunoblots of immunoprecipitates because SDS-PAGE does not sufficiently resolve the large amounts of IgHc, seen as a heavy band running immediately above TBP in some figures, and TBP in the immunoprecipitates. Similarly, IgHc dimer runs just above the immunoprecitated UBF doublet.

In vitro transcription. rDNA transcription reactions were carried out as previously described (7). For these assays, the template DNA consisted of *Eco*RI-linearized plasmid pU5.1 E/O, derived from plasmid pU5.1 E/X (7), which contains the rat 45S rDNA promoter (−286 to +520) and is designed to generate a truncated transcript of 520 nucleotides. The samples were incubated for 30 min at 30°C, and the reaction was stopped by the addition of 5 μl of 2.0% SDS–5 mg of tRNA per ml and a fragment of DNA end labeled with ^{32}P as an internal standard for the final recovery of nucleic acids. Five microliters of proteinase K (1 mg/ml) was added to the samples, and they were incubated for 15 min at 65°C. The samples were then extracted once with phenol-chloroform and twice with chloroform and precipitated with ethanol. The ethanol precipitate was collected by centrifugation, washed once with 70% ethanol, and dried. The samples were then resuspended in 3 μl of 0.1% SDS–27 μl of formamide, incubated for 10 min at 65°C, and resolved by electrophoresis on 4% urea-polyacrylamide gels. The gels were dried and exposed to film. 5S rDNA transcription reactions were performed essentially as described above, with modifications as previously described (45). The reaction products were processed and analyzed as described above except that the products were resolved on 10% urea-polyacrylamide gels, as the transcript of the Syrian hamster 5S gene is 118 nucleotides long.

Immunodepletion. For the immunodepletion experiments, all incubations were carried out at 4°C unless otherwise stated. Protein A-agarose or anti-UBF beads were pretreated by incubation for 30 min in 0.05% Tween 20 in C/20 and then for an additional 30 min in 0.5 mg of bovine serum albumin (BSA) per ml in C/20 to reduce nonspecific binding of proteins to the beads. All fractions were brought to 0.5 mg of BSA per ml, and 200 μl of UBF-depleted extract was mixed with 25 μl of either partially purified UBF or C/20 and incubated for 30 min. The samples were then added to 25 μl of packed, pretreated protein A-agarose or anti-UBF beads and incubated for 3 h. The beads were removed by centrifugation for 5 s, and the supernatants were tested for the ability to transcribe from the 45S rDNA promoter. For those experiments in which S-100 extracts were used, 50 μl of N1S1 S-100 extract (10 to 12 mg/ml) was incubated with the pretreated beads for 3 h. The remaining steps were carried out as described above, and the resulting supernatants were tested for the ability to transcribe either the 5S RNA gene or the 45S rDNA promoter.

Recovery of SL-1 following immunoprecipitation. The immunoprecipitation experiments were carried out by using a procedure similar to that previously described (10). An anti-UBF antibody covalently coupled to Affi-Gel 10 beads was used. The anti-UBF antibody was purified from 10 ml of anti-UBF antiserum on a 10-ml protein A column as instructed by the manufacturer (Pierce). Following an overnight dialysis against 0.1 M HEPES (pH 7.5), 5 mg of purified antibody was coupled to 2 ml of packed Affi-Gel 10 beads (Bio-Rad) according to the manufacturer's instructions. UBF-depleted N1S1 extract (1 ml) was adjusted to a final concentration of 0.1% NP-40 and incubated with 50 μl of packed anti-UBF/Affi-Gel 10 beads in the absence or presence of 50 μl of partially purified UBF for 2 h. The beads were pelleted and washed two times with 1 ml of C/20 containing 0.1% NP-40 and then with 1 ml of C/20. The beads were then resuspended in 100 μl of C/20 containing 2.5 M urea and 0.2 mg of cytochrome *c* per ml and incubated for 30 min. The beads were pelleted, and the eluted proteins were dialyzed for 12 h against C/20. The dialysates were harvested and tested for the ability to reprogram an S-100 extract of LTRα3 cells to transcribe from the rat 45S rDNA promoter.

RESULTS

Recent experiments have demonstrated that some transcription activators can directly interact with the RNA polymerase II transcription factor TFIID in the absence of DNA (15, 25, 26, 41). In the context of rDNA transcription, SL-1 is the functional equivalent of TFIID. Thus, by analogy, it is formally possible that UBF, an activator of rDNA transcription, can interact with SL-1. To examine this hypothesis, we determined if TBP (as a reporter for SL-1) could be coimmunoprecipitated with UBF (Fig. 2). In these experiments, we used extracts of LTRα3 (46) cells, as they express both wild-type TBP and a modified TBP which is tagged with the influenza virus HA epitope (FLU-TBP). Immunoprecipitations were performed with increasing amounts of nuclear extract, using anti-UBF antibody bound to protein A-agarose beads. The presence of TBP in the resulting immunoprecipitates was determined by probing Western blots of the immunoprecipitates with antibody 12CA5 to the influenza virus HA epitope.

FLU-TBP was detected in Western blots, probed with 12CA5, of immunoprecipitates generated with the anti-UBF antiserum from increasing amounts of nuclear extracts (Fig. 2A, lanes 7 to 10, top panel). The immunostained band increased in intensity in proportion with the amount of nuclear extract used in the immunoprecipitation reaction. This finding indicates that SL-1/TBP was coimmunoprecipitating with UBF. When the blot was stripped and reprobed with an anti-UBF antibody, a similar pattern was observed for UBF, which appears as a doublet of UBF1 and UBF2 (Fig. 2A, lanes 7 to 10, bottom panel). Neither TBP nor UBF interacted with either protein A-agarose beads alone (Fig. 2A, lanes 3 to 6) or preimmune antiserum (Fig. 2B).

To ensure that the band stained by antibody 12CA5 was indeed TBP and that the presence of the FLU epitope did not somehow affect the coimmunoprecipitation of TBP with UBF, a coimmunoprecipitation experiment similar to the one described above was carried out (Fig. 2C). The resulting blot was first probed with antibody 12CA5 then stripped, and reprobed with a mouse monoclonal antibody to human TBP. When the blot was probed with antibody 12CA5, the antibody recognized a single band of the appropriate mobility as well as several cross-reactive bands in the starting extract (Fig. 2C, lane 5). When the extract was immunoprecipitated with the anti-UBF antibody, only the band representing the epitope-tagged TBP coimmunoprecipitated with UBF (Fig. 2C, lanes 7 and 8). When the same blot was stripped and reprobed with the monoclonal anti-TBP antibody, that antibody recognized a doublet in the unfractionated extract (Fig. 2C, lane 1). This result is in agreement with previously published results demonstrating that the LTRα3 cell line expresses both epitope-tagged and endogenous TBP at a ratio of about 5:1 (46). This same doublet was coimmunoprecipitated by the anti-UBF antibody (Fig. 2C, lanes 3 and 4). The upper band of the doublet had the same mobility as the putative FLU-TBP recognized by antibody 12CA5. These results show that the band recognized by antibody 12CA5 is indeed TBP and that the presence of the FLU epitope does not affect the coimmunoprecipitation of TBP with UBF, as both epitope-tagged and endogenous TBP coimmunoprecipitated with UBF.

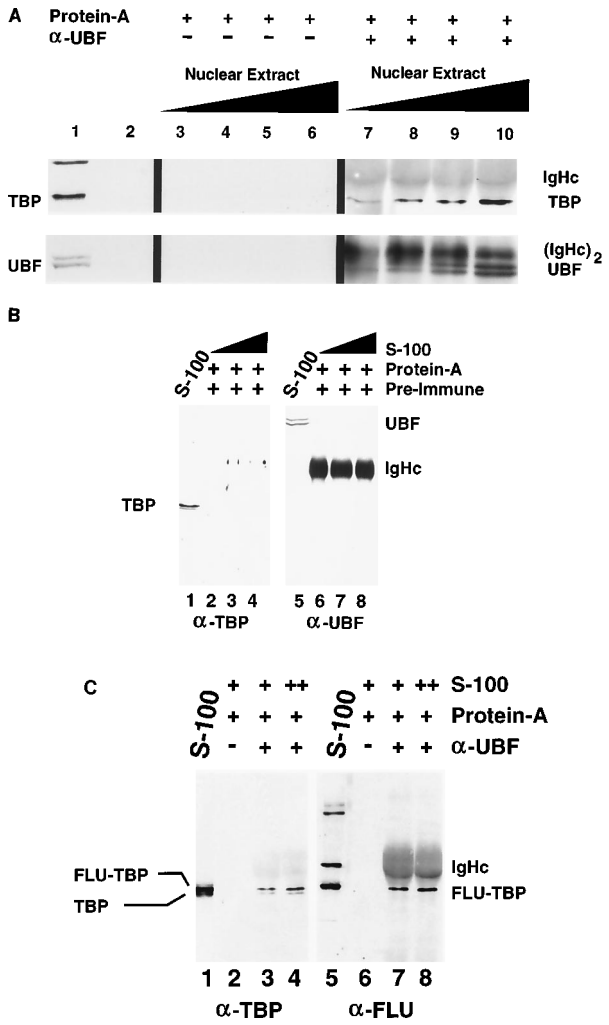

FIG. 2. Coimmunoprecipitation of TBP and UBF from nuclear extracts with an anti-UBF antibody. (A) Increasing amounts of LTRα3 nuclear extracts were incubated with 25 μl of packed protein A-agarose (lanes 3 to 6) or anti-UBF antibody (α-UBP) bound to protein A-agarose (lanes 7 to 10). Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed for HA-tagged TBP, using antibody 12CA5. The filter was then stripped and reprobed for UBF by using a polyclonal antiserum raised to recombinant UBF. Lane 1 contains 5 μl of LTRα3 extract as a positive control; lanes 3 to 6 and 7 to 10 contain immunoprecipitates from 10, 25, 50, and 100 μl of nuclear extract generated with protein A-Sepharose alone and anti-UBF antiserum bound to protein A-Sepharose, respectively. (B) Duplicate immunoblots of the precipitates generated following the incubation of preimmune Ig bound to protein A-Sepharose and increasing amounts (50, 100, and 200 μl, as indicated) of an S-100 extract of LTRα3 cells probed with either a monoclonal antibody to TBP (α-TBP; lanes 2 to 4) or the anti-UBF antiserum (lanes 6 to 8). Lanes 1 and 5 contain 10-μl aliquots of the initial extract. (C) Lanes 1 and 5 contain 5 μl of LTRα3 extract as a positive control. Lanes 2 and 6 contain protein A precipitates from 50 μl of extract. Lanes 3 and 7 and lanes 4 and 8 contain anti-UBF immunoprecipitates from 25 and 50 μl of nuclear extract, respectively. Lanes 1 to 4 were probed with a monoclonal antibody to TBP; lanes 5 to 8 were probed with a monoclonal antibody 12CA5 to the FLU epitope. (α-FLU).

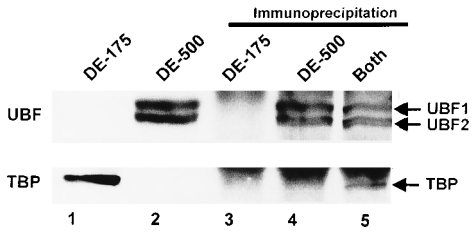

FIG. 3. Coimmunoprecipitation of TBP and UBF requires UBF. UBF-depleted nuclear extracts of LTRα3 cells (DE-175) and the complementary fraction containing crude UBF (DE-500) from N1S1 extracts were precleared with protein A-agarose; 100 μl of the precleared extracts was incubated with anti-UBF beads either alone or after being mixed for 30 min. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed for FLU-TBP by using antibody 12CA5. The filter was then stripped and reprobed for UBF by using a polyclonal antiserum raised to recombinant UBF. Lanes 1 and 2 contain 25 μl of UBF-depleted LTRα3 extract and partially purified UBF from N1S1 extracts, respectively, as positive controls. Lanes 3 to 5 contain anti-UBF immunoprecipitates of UBF-depleted LTRα3 extract, partially purified UBF from N1S1 extracts, and the two mixed together, respectively.

The previous experiment indicated that preformed complexes of UBF and SL-1/TBP could be immunoprecipitated from nuclear extracts. We next examined whether the UBF–SL-1/TBP complex could form de novo. Extracts of LTRα3 cells were fractionated by chromatography over DEAE-Sephadex, generating a fraction (DE-175) that was enriched for SL-1/TBP and depleted of UBF (39, 40). Extracts of Novikoff ascites cells were fractionated by chromatography over DEAE-Sephadex, generating a fraction (DE-500) enriched for UBF and devoid of SL-1/TBP (39, 40). The UBF used in these experiments did not contain detectable amounts of SL-1/TBP in either transcription experiments or Western blots with an anti-TBP antibody (data not shown). FLU-TBP was not detected in Western blots of the immunoprecipitates generated when the UBF-depleted extract was immunoprecipitated with anti-UBF, blotted, and probed with antibody 12CA5 (Fig. 3, lanes 3 and 4). However, when UBF-depleted extracts of LTRα3 cells and partially purified rat UBF were mixed prior to immunoprecipitation with anti-UBF, TBP was recovered in the immunoprecipitate (Fig. 3, lane 5). This result indicates that rat UBF and human SL-1/TBP can associate from partially purified fractions, consistent with the observation that rodent UBF can stimulate transcription dependent on human SL-1 (4). In addition, these results demonstrate that immunoprecipitation of TBP by the anti-UBF antibody is dependent on the presence of UBF.

We next examined the strength of the interaction between UBF and SL-1/TBP by performing the immunoprecipitation with anti-UBF beads in the presence of the ionic detergents SDS and deoxycholate. Under standard immunoprecipitation conditions, we used 0.2% NP-40 in order to inhibit nonspecific binding of UBF and SL-1/TBP to the immunoprecipitating beads. When increasing amounts of LTRα3 nuclear extract were immunoprecipitated with anti-UBF beads under these conditions, Western blot analysis of the immunoprecipitations with antibody 12CA5 demonstrated increasing recovery of TBP (Fig. 4). When 0.2% SDS and 0.2% deoxycholate were included in the immunoprecipitation mix, no TBP was recovered in the immunoprecipitate, but UBF was still immunoprecipitated by the anti-UBF beads (Fig. 4). This result demonstrates that the interaction between UBF and SL-1/TBP, while resistant to disruption by a nonionic detergent, is disrupted by moderate concentrations of ionic detergents. This finding suggests that the interaction between UBF and SL-1/TBP is of moderate strength and not due to some type of covalent interaction. This result is also consistent with the observation that UBF and SL-1 can be separated by chromatography over DEAE-Sephadex (40).

These experiments provide only indirect evidence that UBF may associate with SL-1. They demonstrate that UBF can associate with TBP. However, they do not discriminate between the possibilities that the TBP in the immunoprecipitates is free or is in complexes with, for example, SL-1, TFIID, or TFIIIB.

If SL-1 binds to UBF, it should be possible to demonstrate a UBF-dependent depletion of SL-1 activity from nuclear extracts by using the anti-UBF beads. As UBF-depleted extracts (DE-175) are capable of supporting transcription (39), merely depleting the extracts of any residual UBF would not significantly affect the ability of those extracts to carry out rDNA transcription. However, depletion of SL-1 would render the extracts inactive.

Anti-UBF beads were incubated with UBF-depleted extracts (DE-175; Materials and Methods) of N1S1 cells in the presence or absence of partially purified UBF from the same cell type. After removal of the immunobeads, the supernatants were assayed to determine their ability to support transcription by RNA polymerase I. Treatment of UBF-depleted extracts with anti-UBF beads had no effect on the transcriptional activity of the extract (Fig. 5, lanes 1 and 2). As one might predict, when UBF was added to these same UBF-depleted extracts during the incubation with protein A-agarose, the final supernatants were more active (Fig. 5, lane 3). This was expected, as the addition of UBF to the extract would stimulate rDNA transcription (40). However, treatment of the UBF-depleted extract with anti-UBF beads in the presence of partially purified UBF resulted in an extract that did not support rDNA transcription (Fig. 5, lane 4). This indicates that the inclusion of UBF in the immunoprecipitation reaction mixture resulted in the removal of a critical component from the extract, presumably SL-1.

An alternative interpretation of our results is that UBF interacts with RNA polymerase I (38) and that the extracts were depleted of the polymerase. Under the conditions used in the immunodepletion experiments, UBF was quantitatively removed from the N1S1 extracts, as demonstrated by the lack of immunodetectable UBF in the supernatants (Fig. 6) and its

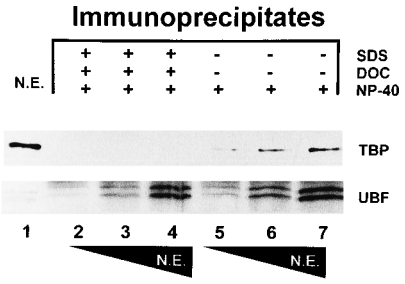

FIG. 4. Detergent blocks the coimmunoprecipitation of TBP and UBF. Nuclear extracts (N.E.) of LTRα3 cells were diluted 1:1 with C/0 buffer and precleared on protein A-agarose. The precleared extract was brought to 0.5% NP-40–SDS–deoxycholate (DOC) and diluted in C/10 buffer to bring the detergent concentration to 0.2% for each detergent. Precleared extract without detergent was also diluted in the same manner and brought to 0.2% NP-40. Then 75, 150, or 300 μl of the samples with only 0.2% NP-40 or all three detergents was immunoprecipitated with anti-UBF beads, and immunoblots were prepared as described in Materials and Methods. Lane 1 contains 5 μl of LTRα3 extract; lanes 2 to 4 contain immunoprecipitates from 15, 30, and 60 μl of starting extract, respectively, immunoprecipitated in the presence of all three detergents; lanes 5 to 7 contain immunoprecipitates from 15, 30, and 60 μl of starting extract, respectively, immunoprecipitated in the presence of NP-40 alone.

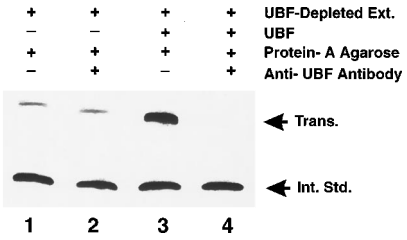

FIG. 5. Immunodepletion of SL-1 activity by anti-UBF requires the presence of UBF. The procedure for the SL-1 depletion experiments is described in detail in Materials and Methods. N1S1 nuclear extracts (Ext.), biochemically depleted of UBF, were incubated with protein A or anti-UBF bound to protein A beads in the presence (+) or absence (−) of UBF purified as described in Materials and Methods. Aliquots of 5 μl of the supernatants from samples incubated in the absence of UBF or 10 μl of the supernatants from samples incubated in the presence of UBF were tested for the ability to transcribe the rat rDNA promoter (0.02 μg of template DNA) in an in vitro transcription assay. Trans., transcript; Int. Std., internal standard added for recovery of nucleic acids.

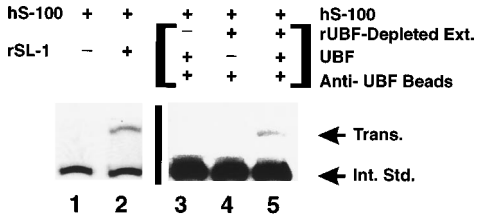

FIG. 7. Immunoprecipitation of SL-1 activity by anti-UBF requires the presence of UBF. The procedures for the immunoprecipitation and recovery of SL-1 are described in Materials and Methods. N1S1 nuclear extracts, depleted of UBF, were incubated with protein A or anti-UBF coupled to protein A-agarose beads in the absence or presence of UBF purified as described in Materials and Methods. Ten microliters of the material eluted from the immunoprecipitates was tested for the ability to reprogram 10 μl of an S-100 extract of human LTRα3 cells (hS-100) to transcribe from 1 μg of rat 45S rDNA promoter in an in vitro transcription assay. Lanes 1 and 2 were exposed for 16 h; lanes 3 to 5 were exposed for 72 h. rSL-1, rat SL-1; rUBF, rat UBF; Trans., transcript; Int. Std., internal standard added for recovery of nucleic acids.

presence in the immunoprecipitates which had been generated following incubation of the S-100 extracts with both the anti-UBF antibody and protein A-agarose. Parallel blots of the supernatants and immunoprecipitates were probed for RNA polymerase I. For these experiments, we used an antibody raised against a recombinant fragment of the 194-kDa subunit of rat RNA polymerase I (13). This antibody detected the 194-kDa subunit in the starting extract and in the supernatants following immunodepletion with the anti-UBF antibody. Moreover, the 194-kDa subunit was not detected in the immunoprecipitates (Fig. 6). Similar results were obtained when these blots were probed with an antibody raised to the 114-kDa subunit of RNA polymerase I (data not shown). These data, as well as the results of other experiments presented below, indicate that under the conditions used in these experiments, the anti-UBF antiserum did not deplete RNA polymerase I from the extracts.

The converse of the experiments just described would be the demonstration that SL-1 activity can be recovered from the immunoprecipitates formed with anti-UBF antiserum and the S-100. An experiment demonstrating this result is represented in Fig. 7. The assay for SL-1 was based on the fact that SL-1 activity is species specific. As shown in Fig. 7, lane 1, S-100 extracts of human LTRα3 cells did not support transcription from the rat rDNA promoter. However, when the LTRα3 extracts were supplemented with partially purified rat SL-1, the S-100 extract was able to transcribe the rat rDNA (Fig. 7, lane 2). In other words, the rat SL-1 reprogrammed the LTRα3 extract to transcribe the rat rDNA.

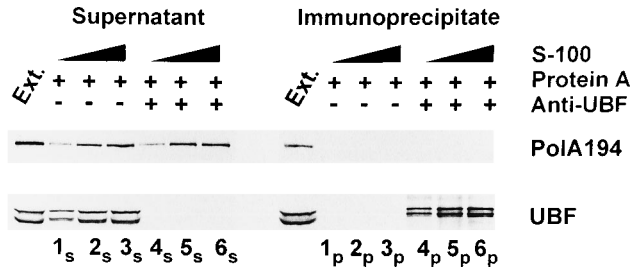

FIG. 6. RNA polymerase I does not immunoprecipitate with UBF. Increasing amounts (50, 100, and 200 μl) of S-100 extracts (wedges) of N1S1 cells were incubated with protein A-agarose in the absence (−) or presence (+) of an anti-UBF antiserum. One-fifth of the immunoprecipitates (lanes 1_p to 6_p) and one-tenth of the supernatants (lanes 1_s to 6_s) after immunoprecipitation were fractionated by SDS-PAGE, transferred to nitrocellulose, and probed with monospecific polyclonal antibodies to either the 194-kDa subunit of RNA polymerase I (PolA194) or UBF. The presence of immunoreactive material on the blots was determined by enhanced chemiluminescence as described in Materials and Methods. Ext., 10 μl of the initial extract was fractionated in parallel with the experimental samples to serve as a marker for UBF and PolA194.

When either partially purified UBF or UBF-depleted N1S1 extracts (DE-175) were immunoprecipitated with anti-UBF beads, the proteins recovered from the immunoprecipitates were not capable of reprogramming the LTRα3 S-100 extract (Fig. 7, lanes 3 and 4) to transcribe from the rat rDNA promoter. However, when the two fractions were first mixed and then immunoprecipitated with the anti-UBF antiserum, the proteins recovered from the immunoprecipitates were capable of redirecting the LTRα3 extract to recognize the rat rDNA promoter (Fig. 7, lane 5). This is a direct demonstration that the immunoprecipitates contained SL-1 activity. Moreover, as SL-1 activity was present in the immunoprecipitates only when UBF was present during the immunoprecipitation, this result is consistent with the model that UBF interacts with SL-1.

It has recently been demonstrated that UBF can bind directly to TBP (21). Thus, it was necessary to consider the possibility that the association between UBF and SL-1 reflects a nonspecific interaction between UBF and TBP. To address this question, we examined whether UBF could bind to TFIIIB, the TBP-containing component of the RNA polymerase III transcription apparatus. If UBF bound to TBP regardless of the complex with which it was associated, then one would expect to see UBF-dependent immunodepletion of TFIIIB activity by the anti-UBF beads.

S-100 extracts of N1S1 cells were treated with anti-UBF beads, and the supernatants were assayed for the ability to support transcription of either the Syrian hamster 5S RNA gene (14) by RNA polymerase III or the rat 45S rDNA promoter by RNA polymerase I. Transcription of the 5S gene results in the α-amanitin-sensitive synthesis of the predicted RNA of 118 nucleotides (Fig. 8, lanes 1 and 1′). Treatment with the protein A beads alone minimally inhibited both 5S RNA and 45S rDNA transcription (Fig. 8; compare lanes 1 and 2 and lanes 4 and 5). Treatment with anti-UBF bound to protein A beads had little effect on 5S RNA synthesis (Fig. 8, lanes 2 and 3). In contrast, treatment of the extracts with anti-UBF beads significantly reduced their ability to carry out transcription by RNA polymerase I (Fig. 8; compare lanes 5 and 6). These same results were obtained for S-100 extracts of LTRα3 cells (data not shown). These results demonstrate that if UBF binds to TFIIIB via TBP, this interaction is not nearly

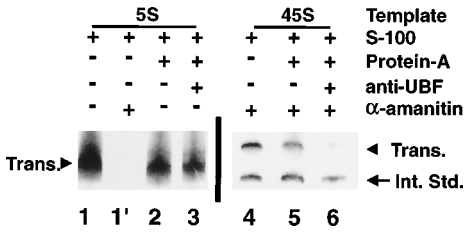

FIG. 8. Anti-UBF antibody immunodepletes N1S1 S-100 extracts of SL-1 but not TFIIIB activity. S-100 extracts of N1S1 cells were incubated with an anti-UBF antibody as described in Materials and Methods, and the resulting supernatants were tested for the ability to transcribe from either the 5S RNA gene or the 45S rDNA promoter. In either case, 20% of the supernatants were tested for the ability to transcribe 1 μg of template DNA in vitro. Lanes 1 to 3 contain 5S rDNA transcripts, and lanes 4 to 6 contain 45S rDNA transcripts (Trans.) generated from N1S1 S-100 extracts treated as indicated in the figure. Transcription and PAGE analysis of the transcription reactions were carried out as described in Materials and Methods. Int. Std., internal standard added for recovery of nucleic acids.

as strong or stable as the interaction between UBF and SL-1. Thus, it seems that UBF preferentially binds to SL-1.

DISCUSSION

The major finding of the current study is the demonstration that UBF and TBP associate in the absence of DNA. Specifically, we show that the TBP associated with UBF is part of the SL-1 complex, since SL-1 activity both is depleted in extracts immunodepleted by treatment with anti-UBF antibodies and can be recovered in the immunoprecipitates. Moreover, we demonstrate that both the depletion and the recovery of SL-1 activity were dependent on the presence of UBF. It has recently been reported that mouse UBF and a subunit of RNA polymerase I can associate in the absence of DNA (38). Analysis of the immunoprecipitates generated in these experiments, using antibodies to the 194-kDa subunit of rat polymerase I, failed to demonstrate the presence of RNA polymerase I. In addition, we have not been able to demonstrate such an association despite repeated attempts to do so using antibodies to two other subunits of RNA polymerase I (data not shown). Thus, it is unlikely that an association between UBF and RNA polymerase I can account for the results observed. Rather, the results of the current study are consistent with the hypothesis that SL-1 binds to UBF. Kwon and Green have reported that UBF can bind to TBP directly (21). Since we have demonstrated that UBF binds to SL-1, it could be argued that UBF binds to TBP regardless of the nature of the larger complex, i.e., SL-1, TFIID, or TFIIIB. However, extracts that were SL-1 depleted following treatment with an anti-UBF antiserum contained levels of TFIIIB similar to those in control, nontreated extracts, as demonstrated by their ability to transcribe the 5S RNA gene. Moreover, the addition of excess UBF to these immunodepletion experiments had no effect on 5S transcription (data not shown). These findings suggest that UBF binds to SL-1 preferentially over TFIIIB and that the binding of UBF to TBP is at least in part dependent on the context in which TBP is found. In the experiments of Kwon and Green (21), the same anti-UBF antibody used in this report was shown to inhibit transcription by RNA polymerase II. In this report, we demonstrate that this antibody can nearly quantitatively deplete SL-1 activity under the same conditions in which it did not affect the recovery of TFIIIB activity.

Moreover, it can be argued that the binding of UBF to TFIID may not be physiologically relevant. In the intact cell, the vast majority of UBF is localized to the nucleolus (37), where it interacts with SL-1 to direct transcription of the 45S rDNA genes. TFIID, however, is found in the nucleoplasm, where it directs transcription by RNA polymerase II. Thus, for the most part, intranuclear compartmentalization would keep these two factors separated.

One likely function for the association of SL-1 with UBF may be to facilitate the recruitment of SL-1 to the 45S rRNA promoter, since there are only a few hundred molecules of this transcription factor per cell (30). An analogous situation may exist with respect to transcription by RNA polymerase II in that several potent transcription activators, e.g., VP16, the adenovirus large E1A protein, and the product of the c-*myc* oncogene, can interact with TBP (15, 25, 26, 41). By analogy, the association of UBF and SL-1 may represent the mechanism by which they are recruited to the 45S rDNA promoter.

As discussed, the binding of UBF to SL-1 may be important in facilitating the formation of a stable complex between SL-1 and the 45S rDNA promoter. This seems likely, as SL-1 alone either fails to bind or only weakly binds to the CPE despite the fact that it has been shown to be critical for initiating transcription when the CPE is utilized (23, 40). Previous studies have suggested that the interaction between UBF and SL-1 may be important in mediating the effects of UBF. The work described in this report provides evidence that UBF and SL-1 can directly interact and support the hypothesis that this interaction may be important for optimal utilization of the 45S rDNA promoter.

We have recently identified a possible mechanism by which UBF-dependent transcription by RNA polymerase I may be negatively regulated through an interaction of UBF with the Rb protein (8). The physiological relevance of the Rb-UBF interaction is demonstrated in differentiating U937 cells. By associating with UBF, Rb protein downregulated rDNA transcription when U937 cells stopped proliferating and started to differentiate (8). In light of the role of Rb as a tumor suppressor protein in many cell types (12), this model provides an additional mechanism through which Rb exhibits its growth-suppressing effects. Since the association of UBF and SL-1 may be critical for rDNA transcription, it is interesting to speculate that Rb may compete with SL-1 for binding to UBF and thus modulate the ability of UBF to stimulate rDNA transcription.

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