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Interactions between yeast TFIIIB components

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ABSTRACT

Yeast transcription factor TFIIIB is a multicomponent factor comprised of the TATA-binding protein TBP and of associated factors TFIIIB70 and B″. Epitope-tagged or histidine-tagged TFIIIB70 could be quantitatively removed from TFIIIB by affinity chromatography. TBP and B″ (apparent mass 160–200 kDa) could be easily separated by gel filtration or ion-exchange chromatography. While only weak interactions were detected between TBP and B″, direct binding of [^{35}S]-labeled TBP to membrane-bound TFIIIB70 could be demonstrated in absence of DNA. On tRNA genes, there was no basal level of transcription in the complete absence of TBP. The two characterized TFIIIB components (recombinant rTFIIIB70 and rTBP) and a fraction co-chromatographing with B″ activity were found to be required for TFIIIC-independent transcription of the TATA-containing U6 RNA gene *in vitro*. Therefore, beside the TFIIIC-dependent assembly process, each TFIIIB component must have an essential role in DNA binding or RNA polymerase recruitment.

INTRODUCTION

Specific transcription by RNA polymerase III (polIII) requires the participation of several general transcription factors. The sequential assembly of transcription initiation complexes on polIII genes has been particularly well studied in the case of yeast tRNA and 5S genes (for a review, see 1). The multisubunit protein TFIIIC is responsible for binding to the two intragenic promoter elements of tRNA genes (the A and B blocks), or to preformed TFIIIA·5S gene complexes. When bound, TFIIIC recruits factor TFIIIB, which by itself does not bind detectably DNA. Once assembled upstream of the transcriptional start site, TFIIIB is sufficient to position RNA polymerase III for transcription initiation (2, 3, 4). The TATA-binding protein (TBP) is part of TFIIIB and is involved in this multistep assembly process (5, 6). Thus, TFIIIB is the key general factor necessary for polIII initiation process, whereas TFIIIA and TFIIIC function as assembly factors (4). The central role of TFIIIB in gene activation makes it also a critical target for regulation (7, 8, 9, 10).

Although mammalian and yeast TFIIIB have been originally reported to consist of a single polypeptide of 60 kDa (11, 12), recent studies indicate that TFIIIB is a multiprotein complex comprising TBP (13, 14). Yeast TFIIIB factor is constituted of TBP and two components first identified by photocrosslinking experiments (15), the 70 kDa subunit (hereafter termed TFIIIB70), encoded by the *BRF1/PCF4/TDS4* gene (16, 17, 18), and a 90 kDa polypeptide not yet cloned. Recent data suggest that the 70 and 90 kDa polypeptides are class III (RNA polymerase III-specific) TBP-associated factors (TAFs) (19). Yeast TFIIIB activity could be immunopurified using antibodies directed to TBP. The immune complexes were not dissociated by high salt, and 4M urea was required to quantitatively elute the TAFs, among which were TFIIIB70 and a 90 kDa polypeptide (19). On the other hand, yeast TFIIIB could be resolved into two mutually dependent fractions, B′ and B″, on a strong cation-exchange resin (20). B′ is made of TFIIIB70 and TBP, whereas B″ contains the 90 kDa component (6). Recombinant TBP and TFIIIB70 proteins combined with the electrophoretically-purified 90 kDa protein were found to be sufficient to reconstitute all the known *in vitro* properties of TFIIIB (6). The B′ fraction is capable of assembling into the TFIIIC·tDNA complex, and the incorporation of B″ to the B′·TFIIIC·tDNA complex confers the ability to recruit the RNA polymerase for transcription initiation (20). Remarkably, after being assembled, the TFIIIB·DNA complex becomes high salt and heparin-resistant under conditions that dissociate TFIIIC (3). The TBP is retained in the heparin-resistant complex (5, 6, 16).

Concurrently with the studies on yeast TFIIIB, analysis of mammalian TFIIIB also disclosed a TBP-containing multicomponent factor (21, 22, 23, 24). TFIIIB activity could be chromatographically resolved into two complementary fractions, only one of which contained detectable amounts of TBP (21, 25, 26). The polypeptide composition of human TFIIIB is not yet clearly established but is probably complex. Using antibodies directed to TBP, Taggart *et al.* (23) identified one tightly associated polypeptide (TAF172) and another, more loosely associated fraction (TAF-L). Using a similar approach, Lobo *et al.* (21) described three polypeptides of 150, 82 and 54 kDa coimmunopurifying with TBP, and Chiang *et al.* (25) found an even more complex collection of polypeptides (190, 96, 87 and 60 kDa). The relationship between these factors is still uncertain and their role in class III transcription remains to be clarified. Class II TAFs are thought to mediate the action of transcriptional activators (13, 27). The situation is somewhat different in the case of class III TAFs. Hence, yeast TFIIIB70 is structurally and functionally related to the general basal factor TFIIB that is not a TAF.

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These considerations and the fact that, unlike mammalian TAFs, yeast mediators are not stably bound to TBP (13, 27) and that TBP is mostly in free form in yeast extracts (13), led us to reinvestigate the interaction of yeast TFIIIB components. Here, we show that TFIIIB can be easily dissociated into three active components, B″, TBP and TFIIIB70, all of them being required for transcription of TATA-less or TATA-containing genes. A direct interaction of TBP and TFIIIB70 could be demonstrated in absence of DNA.

MATERIALS AND METHODS

Site directed mutagenesis and construction of plasmids

Oligonucleotide-mediated mutagenesis was performed as described by the manufacturer, using a Muta-Gene kit (Bio-Rad). Uracil-enriched single-stranded pRSM3 (18) DNA was used to mutagenize the 5′ end of the *PCF4/BRF1/TDS4* (hereafter named *PCF4*) open reading frame encoding the 70 kDa subunit of TFIIIB (TFIIIB70). The sequences encoding the HA epitope YPYDVPVYA derived from the influenza hemaglutinin protein (28), or 6 histidine residues, were introduced after the initiation codon of *PCF4* using the oligonucleotides 5′-GTCATATACC-AATGgtcgactatccttacgacgttcctgattatgctCCAGTGTGTAAGAA-C-3′ and 5′-GTCATATACCAATGgtcgaccatcatcatcaccaccacC-CAGTGTGTAAGAAC-3′, respectively. The oligonucleotides contain a *SalI* restriction site, followed by the sequence encoding either the HA epitope or 6 histidine residues (lower case). The sequences in upper case are complementary to *PCF4* DNA. After oligonucleotide mediated mutagenesis of the pRSM3 single-stranded DNA, inserts of several transformants were sequenced to verify the site directed modification of the *PCF4* gene.

Construction of yeast strains

Plasmids harboring modified alleles of the *PCF4* gene were used to transform the SHy76 (17) haploid strain containing the *PCF4* gene on a plasmid with the chromosomal copy disrupted. The modified copies of *PCF4* were substituted for wild type *PCF4* by plasmid shuffling on 5′ FOA plates.

Transcription factors and RNA polymerase III

Yeast TFIIIC (factor τ), highly purified RNA polymerase III and TFIIIB* (TFIIIB depleted of TBP) activity were prepared as described (29, 30, 5). Yeast recombinant TBP (rTBP), expressed from plasmid pET3b(rTFIIDY) (a gift from J.M.Egly) in *E.coli* cells, was purified as described previously (5). Recombinant histidine-tagged TFIIIB70 was expressed in *E.coli* cells from plasmid pSH360 (a gift from Steve Hahn) and purified by chromatography on Ni^{2+}-NTA-Agarose (Quiagen) under native (G.Dieci, personal communication) or denaturing conditions (17).

TFIIIB fractionation

The TFIIIB factor was partially purified on a heparin-ultrogel (Pharmacia) column from cells expressing the wild type or a modified copy of *PCF4* as described (31, 32). Heparin-ultrogel fractions containing TFIIIB activity were pooled and used for affinity purification (see Figure 2). Chromatography on Ni^{2+}-NTA-Agarose (Quiagen) was performed as described by the manufacturer with minor modifications. Briefly, 8 ml of heparin-ultrogel fraction (1.45 mg/ml proteins) containing histidine-tagged TFIIIB were dialyzed against buffer A (20 mM Tris−HCl pH8, 10% glycerol, 10 mM β-mercaptoethanol, 1 mM PMSF, 100 mM ammonium sulfate), and then incubated with one ml of Ni^{2+}-NTA-Agarose beads equilibrated in the same buffer. After 1 hour at 4°C, the flow through fraction was removed and the resin was washed with 14 ml of buffer A, then 12 ml of buffer A containing 20 mM imidazole. Bound proteins were eluted stepwise with buffer A containing 250 mM imidazole in 400 μl fractions and analyzed for transcription factor activity and protein content by SDS-PAGE.

Immunopurification of epitope-tagged TFIIIB was performed by incubation of 5 volumes of heparin-ultrogel fraction (0.65 mg/ml) with one volume of monoclonal antibody 12CA5 (28) coupled to Protein A-Sepharose beads (Sigma), and equilibrated in the same buffer (20 mM Tris−HCl pH8, 1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol, 1 mM PMSF, 250 mM ammonium sulfate). After one night at 4°C, the flow through fraction (FT250, 0.65 mg/ml proteins) was collected, assayed for transcription factor activity and used for further purification of B″ activity on Superose 12, Mono Q or Q-Sepharose columns. The beads were washed and incubated with the same buffer containing 50 mM ammonium sulfate and 1 mg/ml of epitope peptide. Fast protein liquid chromatography (FPLC) on a Superose 12 column (Smart System, Pharmacia) was performed as described except that the buffer contained 250 mM ammonium sulfate instead of 370 mM KCl (33). FPLC over a Mono Q column (Smart System, Pharmacia) was performed on 0.8 ml of FT250 diluted to 50 mM ammonium sulfate with buffer B (20 mM Tris−HCl pH8, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 10% glycerol). The column was washed with buffer B containing 50 mM ammonium sulfate and 20% glycerol, then developped with a linear gradient of 50 to 250 mM ammonium sulfate in the same buffer followed by a step elution with 500 mM ammonium sulfate. Twenty fractions (100 μl) were collected. TBP activity and 90% of the proteins were recovered in the flow through fraction. For Q-Sepharose chromatography (5), FT250 fraction (0.8 ml, 0.5 mg protein) was dialyzed against buffer C (20 mM Tris−HCl pH8, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 10% glycerol, 50 mM ammonium sulfate), then applied to a Q-Sepharose column (0.6 × 2.5 cm) equilibrated in the same buffer. The column was washed with 4 ml of buffer C and proteins were eluted stepwise with buffer C containing 150 mM ammonium sulfate. Fractions (150 μl) were collected and assayed for B″ activity.

Transcription assays

Transcription assays were performed using two different templates: pYtG-wt DNA (29) harboring the $tRNA^{Glu}_3$ gene and pB6 DNA (34) containing the wild type U6 snRNA gene. Transcription reactions were carried out for 45 min at 25°C in 40 μl mixtures containing 20 mM Hepes−KOH pH7.9, 10% glycerol, 5 mM $MgCl_2$, 90 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 unit RNasin (Amersham), 0.6 mM ATP, GTP and C-TP, 0.03 mM [^{32}P]-UTP (2−10 Ci/mmol), 0.15 μg plasmid DNA, TFIIIC (30 ng), RNA polymerase III (50 ng) and TFIIIB. TFIIIB activity was provided by the partially purified heparin fraction (UGH, 1.4 μg proteins), or reconstituted using a mixture of rTFIIIB70 (90 ng), rTBP (15 ng) and B″ (Q-Sepharose fraction 7, 320 ng), or a mixture of TFIIIB* (Q-Sepharose fraction, 190 ng) and rTBP (15 ng), or a mixture of FT250 (1.3 μg) and rTFIIIB70 (90 ng). RNA transcripts were analyzed by polyacrylamide/urea gel electrophoresis and revealed by autoradiography.

Interaction of TFIIIB70 with [^{35}S]-TBP

The pET3b(rTFIIDY) plasmid harboring the TBP gene was linearized with BamHI and the gene was transcribed *in vitro* with T7 RNA polymerase. The RNA was translated in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]-methionine. [^{35}S]-labeled TBP migrated as a single labeled polypeptide chain by SDS-PAGE (data not shown). Recombinant TFIIIB70 purified under native or denaturing conditions was subjected to SDS-PAGE, and blotted onto nitrocellulose. The filters were incubated for 1 hr in buffer D (20 mM Hepes−KOH pH7.9, 0.1 mM EDTA, 5% glycerol, 1 mM DTT, 5mM $MgCl_2$, 100 mM KCl) containing 5% low fat milk, washed with buffer D, then incubated overnight at 4°C in buffer D in the presence of [^{35}S]-labeled TBP protein (100000 cpm/ml). The filters were then washed for 10 min at 4°C, twice with buffer D and once with buffer D without glycerol. The [^{35}S] background was reduced by washing the filters with 15% trichloro-acetic acid then 50% ethanol. Full size and partially proteolyzed TFIIIB70 polypeptides were located by anti-TFIIIB70 antibodies (kindly provided by Steve Hahn). Immune complexes were visualized by antibodies tagged with alkaline phosphatase (Promega Biotec) and labeled polypeptides were revealed by autoradiography.

RESULTS

Physical interaction between TFIIIB70 and TBP

The 70 kDa subunit of TFIIIB (TFIIIB70) is thought to interact with various components of the class III transcriptional machinery. In the original partition of TFIIIB into B′ and B″ (20), TBP was found to be in B′ together with TFIIIB70 (6). While the co-elution of these two components might have been coincidental, it hinted that TBP interacted more strongly with TFIIIB70 than with B″. This putative interaction was investigated by a Farwestern blotting experiment. rTFIIIB70 expressed in *E.coli* cells and purified on Ni^{2+}-NTA-Agarose beads in native or denatured form migrated as three major polypeptides of 70, 60 and 36−38 kDa in SDS-PAGE. All three polypeptides reacted with an anti-TFIIIB70 serum (Figure 1). These polypeptides were transferred to a membrane and probed with [^{35}S]-labeled TBP. As shown in Figure 1, all three polypeptides bound TBP with equal efficiency as judged from the relative band intensity in the Western blot and in the autoradiogram. Smaller polypeptides not recognized by the antibodies did not bind TBP. TBP binding was optimal at 0.1−0.2 M KCl and was much reduced at 50 mM or 0.4 M salt (data not shown). Under the optimal conditions, [^{35}S]-labeled TBP did not bind to any polypeptides present in B″ fraction from the Mono Q column, or to bovine serum albumine (data not shown). As discussed below, it is not clear whether the full size rTFIIIB70 and its proteolyzed byproducts were purified through the histidine tag or by an endogenous metal binding site. N-terminal analysis of the TBP-binding TFIIIB70 fragments would provide a coarse mapping of the TBP-binding region. The reciprocal Farwestern experiment was performed by probing rTBP blotted on a membrane with [^{35}S]-labeled TFIIIB70. No binding signal, however, was detected under these

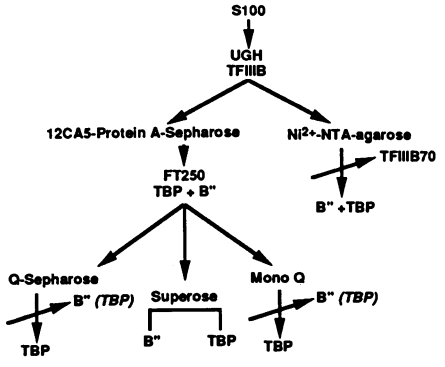

Figure 2. Fractionation of TFIIIB factor and its components, TFIIIB70, TBP and B″ from yeast S100 extracts. Crossed vertical and diagonal arrows represent flow through fractions and gradient elutions, respectively. The presence of substoichiometric amounts of TBP is indicated within parentheses.

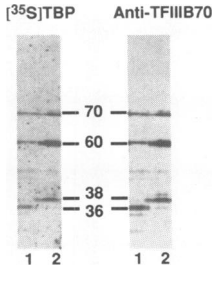

Figure 1. Physical interactions of TFIIIB70 and TBP. Histidine-tagged TFIIIB70 was purified from *E.coli* cells under denaturing (lane 1; 2 μg) or native (lane 2; 3 μg) conditions, subjected to SDS-PAGE, transferred onto a membrane, and probed with [^{35}S]-TBP (as described in Materials and Methods). Labeled polypeptides were revealed by autoradiography. The same membrane was then incubated with an anti-TFIIIB70 serum, and immune complexes were visualized using antibodies tagged with alkaline phosphatase. The molecular weight of the major polypeptides is indicated.

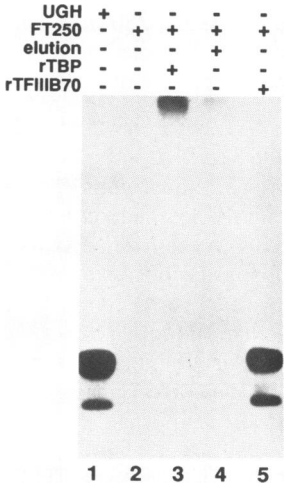

Figure 3. Immunopurification of epitope-tagged TFIIIB. Heparin-ultrogel fraction (UGH) containing epitope-tagged TFIIIB was incubated with 12CA5-Protein A-Sepharose beads. The flow through fraction (FT250) was collected, the beads were washed, then incubated with the epitope peptide (elution). The fractions were assayed alone or in combination with recombinant TFIIIB70 (rTFIIIB70) or TBP (rTBP) with the $tRNA^{Glu}_3$ gene as template, as indicated. Transcripts were analyzed by polyacrylamide/urea gel electrophoresis and revealed by autoradiography.

conditions (data not shown), a negative result may be due to the inadequate renaturation of TBP on the membrane.

Dissociation of tagged TFIIIB70 from TFIIIB

To investigate the class III factors stably associated with TFIIIB70 in yeast extracts, we used an immunopurification procedure that was not expected to disrupt preformed protein complexes (Figure 2). TFIIIB70 was epitope-tagged at its N-terminus and expressed in yeast cells from its own promoter. The cells grew normally in the absence of the wild type gene, indicating that the N-terminus extension did not impair TFIIIB70 function. Epitope-tagged TFIIIB, partially purified on heparin-ultrogel (UGH), was able to form the ternary complex TFIIIB·TFIIIC·tDNA, as evidenced by gel shift assays, and to react with a monoclonal antibody directed to the epitope (data not shown). This indicated that the epitope had been retained and was accessible to antibodies.

The tagged, partially purified TFIIIB fraction was applied to a column of immobilized monoclonal antibody in the presence of 250 mM ammonium sulfate to decrease non specific interactions (Figure 3). The flow through fraction was analyzed in transcription assays for the presence of TFIIIB70, TBP and B″ activity (B″ is required to restore TFIIIB activity in a reconstituted transcription system containing recombinant TFIIIB70 and TBP). As seen in Figure 3, the flow through fraction (FT250) had lost an essential factor since it was unable to replace the input TFIIIB UGH fraction in the reconstituted transcription system (compare lanes 1 and 2), even in the presence of recombinant TBP (rTBP, lane 3). Full transcriptional activity was recovered by supplementing the FT250 fraction with recombinant TFIIIB70 alone (rTFIIIB70) (lane 5). The FT250 fraction supplemented with rTFIIIB70 was also able to form the ternary complex TFIIIB·TFIIIC·tDNA in gel shift assays (data not shown). Therefore, TFIIIB70 must have been retained on the column, leaving TBP activity and B″ in the flow through fraction. Attempts to elute TFIIIB70 activity from the antibody column using high concentrations of the epitope peptide were unsuccessful (lane 4). As the 250 mM salt concentration in the loading buffer may have disrupted protein−protein interactions, the experiment was repeated at 100 mM salt. Under these conditions, most of the TFIIIB activity was recovered in the flow through fraction (data not shown). Therefore, we suspected that the retention of TFIIIB70 on the antibody column was independent of the presence of the epitope. Indeed, untagged TFIIIB70 was retained efficiently at high salt on the antibody column, leaving again B″ and TBP activity in the flow through fraction. We also observed that tagged or untagged TFIIIB70 activity was retained on Protein A-Sepharose without antibodies and could not be eluted by increasing salt concentrations.

The selective adsorption of TFIIIB70 on the antibody column suggested a very weak interaction, if any under the conditions used, between this factor and the other components of TFIIIB, B″ and TBP. One could imagine that strong hydrophobic interactions between TFIIIB70 and the column might have disrupted a preexisting protein complex. To explore this possibility, TFIIIB70 was tagged differently, by adding 6 histidine residues to the N-terminus of the protein. Recombinant histidine-tagged TFIIIB70 purified on Ni^{2+}-NTA-Agarose columns was fully active in transcription assays (17). Histidine-tagged TFIIIB70 expressed in yeast cells harboring a chromosomal disrupted version of the *PCF4/BRF1/TDS4* gene supported normal growth. TFIIIB activity was partially purified from these cells on heparin-ultrogel, as above, then applied batchwise to Ni^{2+}-NTA-Agarose beads (Figure 2), in the presence of 100 mM ammonium sulfate. Remarkably, as in the case of epitope-tagged TFIIIB70, the flow through fraction contained B″ and TBP activities that could be revealed by adding rTFIIIB70 (Figure 4A, compare lanes 2 and 3). TFIIIB70 activity was fully retained on the nickel beads and could be eluted stepwise by 250 mM imidazole (Figure 4A, lanes 4−11). Among several polypeptides present in the eluted fraction, there was a major band of 60 kDa. This 60 kDa polypeptide was not recognized by anti-TFIIIB70 antibodies that recognized exclusively a 70 kDa polypeptide (data not shown). As shown in Figure 4B, the imidazole eluted protein fraction contained only TFIIIB70 activity since both rTBP and B″ fraction were required for transcription of the $tRNA^{Glu}_3$ gene. The low level of transcription observed with B″ alone (lane 3) corresponds to trace amounts of TBP present in this B″ fraction (see Figure 7, lane 14). This experiment confirmed the previous conclusion that TFIIIB70 is not strongly associated with B″ and TBP. To explore the role of the histidine tag on TFIIIB70 binding, an untagged TFIIIB fraction was treated similarly with Ni^{2+} beads. Surprisingly, untagged TFIIIB70 activity was also selectively absorbed on the beads, leaving B″ and TBP in the

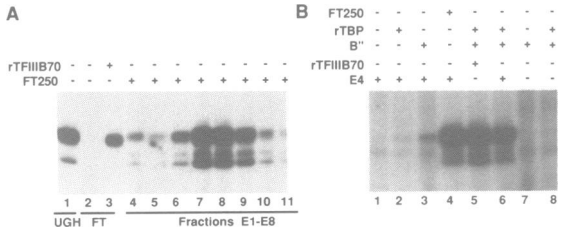

Figure 4. Chromatography of histidine-tagged TFIIIB on Ni^{2+}-NTA-Agarose. Heparin-ultrogel fraction (UGH) containing histidine-tagged TFIIIB was incubated batchwise with Ni^{2+}-NTA-Agarose beads. The flow through fraction (FT) was collected, the beads were washed, and proteins were eluted with 250mM imidazole (fractions E1−E8). **(A).** Dissociation of TFIIIB components. Fractions were assayed for transcription factor activities with the $tRNA^{Glu}_3$ gene as template, by adding rTFIIIB70 or the FT250 fraction (containing TBP and B″, see Figure 3), as indicated. **(B).** Elution fractions contain only TFIIIB70. Fraction E4 (2μl) was assayed alone or in combination with rTBP, rTFIIIB70 and FT250 (containing B″ and TBP), as indicated. B″ activity was provided by adding the Q-Sepharose fraction 7 (320 ng ; see Figure 7).

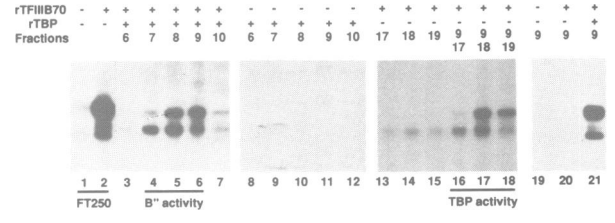

Figure 5. Resolution of TBP and B″ by gel filtration on Superose 12. The FT250 fraction (30 μg) depleted of TFIIIB70 (lanes 1 and 2) was subjected to gel filtration on a Superose 12 column. The proteins eluted as a function of their size were assayed with the $tRNA^{Glu}_3$ gene as template. Fractions (2 μl) were assayed for B″ activity by supplementing with rTBP and rTFIIIB70 (lanes 3−12 and 19−21). B″ activity was recovered in fractions 7−9. TBP activity, assayed in the presence of rTFIIIB70 and fraction 9 (2 μl, lanes 16−18), was recovered in fractions 17−19. Note that B′ fraction 9 has no TBP activity (lanes 19−21).

flow through fraction, and could be eluted with 250 mM imidazole. The protein probably bound via a metal binding site, possibly the two cysteine pairs of the N-terminal region (in the case of histidine-tagged TFIIIB70, it is not clear which site, the endogenous or the tag, reacted preferentially with the metal beads). This interesting property of TFIIIB70 was not further explored.

Resolution of B″ and TBP

To examine the possible existence of a B″·TBP complex, the FT250 fraction (depleted of TFIIIB70, see above) was subjected to gel filtration over a Superose 12 column (Figure 2) in the presence of 250 mM ammonium sulfate. The proteins eluted as a function of their size were assayed for B″ activity by complementing the transcription mixture with recombinant TBP and TFIIIB70 (Figure 5). The peak of B″ activity was eluted in fractions 8 and 9 (Figure 5, lanes 5, 6) which, compared to the elution profile of size markers, corresponded to a protein of 160−200 kDa. This indicated the existence of TFIIIB90 as a dimer, or its association with some other component(s). TBP activity was assayed by complementing the transcription reaction with fraction 9 containing B″ activity (see above) and rTFIIIB70. As shown in Figure 5 (lanes 16−18), TBP activity was eluted separately, in fractions 17−19, as expected for a 27 kDa protein. No TBP activity coeluted with B″ (i.e. no transcription activity was observed in B″ peak with rTFIIIB70 alone, see Figure 5, lane 20).

To reveal a weak interaction between B″ and TBP, the FT250 fraction was dialyzed to lower the salt concentration, then chromatographed on an analytical scale on a Mono Q column (Figure 6A). Free TBP being a highly basic protein is not retained on this type of column. In Western blots, TBP was found in the flow through fraction only (Figure 6C, lane FT). B″ activity, tested in the presence of rTFIIIB70 and rTBP, was entirely retained on the column and was eluted by a salt gradient in essentially two fractions, at 130 mM salt (Figure 6A; Figure 6B, lanes 10, 11). Nevertheless, a weak transcription activity was detectable in the B″ peak upon the addition of rTFIIIB70 (no rTBP added) (Figure 6B, lane 17). This residual TBP activity at the level of B″ probably reflected some functional interaction between these two components.

B″ and TBP activities could also be separated on a preparative scale on Q-Sepharose column (Figure 7). TBP activity, tested in the presence of TFIIIB* (TFIIIB depleted of TBP, see 5), was recovered in the flow through fraction (Figure 7, lane 4). Further addition of rTBP did not increase the transcription activity (Figure 7, lane 5). B″ activity was eluted at 110−120 mM salt (Figure 7, lanes 8−12) Again, the B″ fraction exhibited a weak transcription activity independent of the addition of rTBP (Figure 7, lane 14).

TBP, TFIIIB70 and B′′ are required for U6 RNA synthesis

In the above experiments, transcription activity was tested using a $tRNA^{Glu}_3$ gene as template. We were interested in determining whether the three components of TFIIIB (TBP, TFIIIB70 and B″) were also required for transcription of the yeast U6 RNA gene (SNR6). This gene harbors a TATA box at −30 and can be transcribed by RNA polymerase III in the presence of a

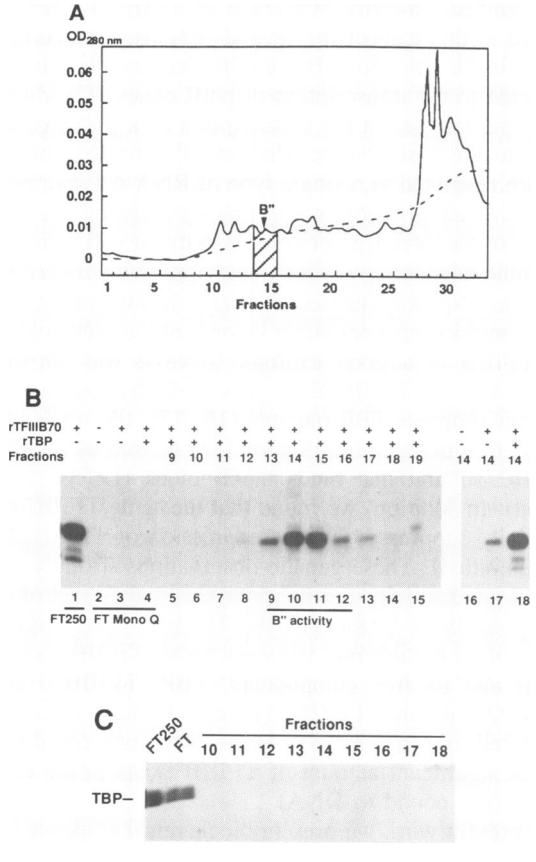

Figure 6. Resolution of TBP and B″ by chromatography on Mono Q column. **(A).** The FT250 fraction (0.5 mg) was chromatographed on a Mono Q column as described in Materials and Methods. Bound proteins were eluted by a salt gradient (dotted line) and detected by measuring the OD280nm (full line). The hatched box indicates the fractions containing B″ activity. **(B).** Transcription assay. Column fractions (2 μl) were assayed for B′ activity with the $tRNA^{Glu}_3$ gene as template by supplementing the mixtures with rTBP and rTFIIIB70, as indicated. Lane 1, FT250 ; lanes 2−4, flow through fraction (FT) ; lanes 5−18, eluted fractions. B″ activity was eluted in fractions 13−16. Fraction 14 contained a trace of TBP activity (lanes 16−18). **(C).** Immunodetection of TBP. Proteins contained in FT250 (3.25 μg), in the Mono Q flow through fraction (FT, 3 μg) and in fractions 10−18 (30 μl) were separated by SDS-PAGE (11% acrylamide), transferred onto nitrocellulose and probed with an anti-TBP antiserum (a gift from J.M.Egly). The position of TBP-immune complexes is indicated (TBP).

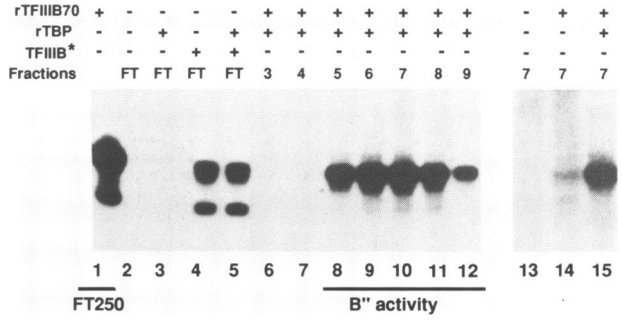

Figure 7. Resolution of TBP and B″ by Q-Sepharose chromatography. The FT250 fraction (0.5 mg) was chromatographed on a Q-Sepharose column as described in Materials and Methods. Column fractions were assayed for TBP and B″ activities with the $tRNA^{Glu}_3$ gene by supplementing the transcription mixtures with rTBP, rTFIIIB70, and 190 ng of a TFIIIB* fraction (TFIIIB depleted of TBP), as indicated. Lane 1, FT250 ; lanes 2−5, flow through fraction (FT ; 0.6 μg) ; lanes 6−15, column fractions (2 μl). B″ activity was recovered in fractions 5−9. Fraction 7 retained traces of TBP (lanes 13−15).

partially purified TFIIIB fraction (35). Figure 8 shows that the SNR6 gene could be specifically transcribed, like the $tRNA^{Glu}_3$ gene, in the presence of the FT250 fraction (that contains B″ and TBP) supplemented by rTFIIIB70 (Figure 8, lane 1). There was no transcription in the absence of rTFIIIB70 (Figure 8, lane 2). Transcription factor activity, using the SNR6 gene as template, was eluted at the level of the B″ peak (Q-Sepharose fractions 5 to 9, see Figure 7) and required the addition of both rTFIIIB70 and rTBP (Figure 8, compare lanes 3–8 to lanes 9–14). Therefore, the same three components of TFIIIB are required for tRNA and U6 RNA synthesis.

DISCUSSION

We have investigated the interactions between yeast TFIIIB components. We developped methods that easily dissociated and resolved TBP, TFIIIB70 and B″. A stable interaction could be demonstrated, in absence of DNA, between TFIIIB70 and TBP, and a weak interaction was observed between TBP and B″. All three components were required for transcription of the U6 snRNA gene in a TFIIIC-independent reaction.

While we found no evidence for a stable, multisubunit TFIIIB factor, a stable interaction was observed in absence of DNA between TFIIIB70 and TBP in Farwestern blotting experiments. TFIIIB is structurally related to TFIIB (16, 17, 18). A TBP-binding motif in TFIIB was mapped by deletions, mutagenesis and limited proteolysis to the conserved repeats (36, 37, 38). The N-terminal zinc-finger motif is not important for interaction with TBP. The smallest truncated TFIIIB70 derivative that bound TBP in our experiments (36 kDa) had the size expected to include the two repeated motifs, assuming that the fragments were retained on the Ni^{2+} column by the N-terminal metal binding domain. Substoichiometric amounts of TBP activity were present in the B″ fraction eluted from Mono Q or Q-Sepharose columns. This observation raised the interesting possibility that a low level of basal transcription may occur in the complete absence of TBP especially since no traces of TBP could be visualized in Western blots. This issue has been raised previously (5, 6). However, this hypothesis is ruled out by the fact that the B″ fraction, size fractionated in high salt, was devoid of basal activity in the absence of rTBP (see Figure 5, lane 20).

In vitro transcription of the U6 RNA gene in a reconstituted system led to the intriguing observation that this TATA-containing gene could be transcribed in the absence of TFIIIC despite the presence of a B-block element (35). A partially purified TFIIIB fraction containing TBP provided all the factor activity needed (33). It was interesting to see if TFIIIB70 and B″ components of TFIIIB were required for transcription in the absence of TFIIIC. Indeed, we demonstrated that rTFIIIB70 and purified B″ were needed, in addition to rTBP, for *in vitro* TFIIIC-independent transcription. Each component must have an essential role in DNA binding or RNA polymerase III recruitment. The interaction of TFIIIB70 with the C34 subunit of RNA polymerase III was recently demonstrated in yeast cells (39).

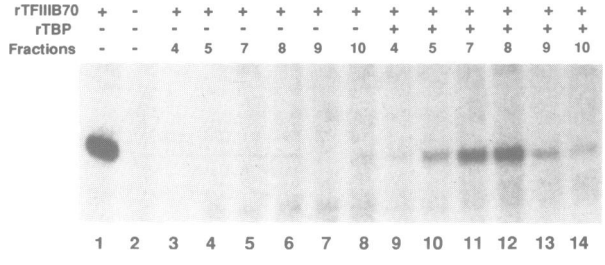

Figure 8. The same components of TFIIIB are necessary for tRNA and U6 RNA synthesis. The Q-Sepharose fractions containing B″ activity (see Figure.7) were assayed for transcription of the U6 snRNA gene by supplementing the mixtures with rTBP and rTFIIIB70, as indicated. Lanes 1 and 2, FT250 (1.3 μg) ; lanes 3–14, Q-Sepharose fractions 4–10 (2 μl). The migration of U6 RNA is indicated. U6 RNA synthesis required fractions with B″ activity, rTBP and rTFIIIB70.

The nomenclature of the general transcription factors that is widely accepted has conveyed two concepts that deserve to be formulated. As transcription factors are named by the form of RNA polymerase that they activate, this has tacitly implied that these components are strictly specific for a particular class of RNA polymerase . The second concept is that each factor corresponds to a stable molecular entity that can be physically isolated as such. These two concepts have obvious implications as to possible mechanisms of gene regulation and they deserve to be examined carefully. The case of TFIIIB is particularly instructive in this respect for several reasons. One component of TFIIIB is the ubiquitous factor TBP that was first thought to be restricted to the transcription of polII genes. The discovery that TBP participates in the transcription of a polIII gene (33, 40, 41) was the first evidence that not all general transcription factors are restricted to a single type of RNA polymerase. The existence of shared factors suggesting mechanisms for a cross-regulation of the three transcription systems, it will be important to determine whether any other transcription factors are shared amongst the RNA polymerases. Similarly, it is important to know whether a multicomponent factor is, or is not, a stable molecular entity. TFIIIB is a good example since it was shown that overexpression of TFIIIB70 in yeast cells by increased gene dosage can suppress TBP mutants (16, 17), the weak binding of TFIIIC to a mutated tRNA gene (18) as well as a mutant of the TFIIIC subunit that binds the B block (Lefebvre *et al.*, submitted). In addition, we found that the same TFIIIC mutant was partially suppressed by overexpression of TBP (Lefebvre *et al.*, submitted). These genetic observations strongly suggest that polIII gene activity can be regulated by the level of one of the components of factor TFIIIB. This, in turn, implies the existence of an equilibrium between native TFIIIB (not bound to DNA) and its free components, TBP, TFIIIB70 and B″ (ieTFIIIB90), and that TFIIIB70 level is limiting in the cell as in whole-cell extracts (3, 18). At the limit, one could wonder whether a significant amount of TFIIIB exists in the cell in a free form (not bound to DNA).

In the present work we present biochemical evidence that, at least in yeast, TFIIIB is not a stable molecular entity, in contrast to the multisubunit RNA polymerases or the TFIIIC factor. TFIIIB70 or TBP could be easily removed from partially purified TFIIIB. The resolution of TFIIIB in two fractions, B′ (TFIIIB70 and TBP) and B″, on Mono S columns (20), provided the first example of TFIIIB lability. These results contrast with the observation of Poon and Weil (19) who isolated TFIIIB activity and others TAFs by immunopurification using antibodies directed to TBP. TFIIIB70 was clearly identified among the TBP-associated polypeptides eluted at high urea concentrations. Several explanations may explain this discrepancy. First, there was no direct evidence that TFIIIB70 was only bound via TBP on the antibody column. A weak binding to TBP may favor a subsequent strong interaction with the Protein A-Sepharose. Alternatively,

the immunopurification being performed on a crude TFIIIB fraction, the TFIIIB components retained by the antibodies may be bound to DNA. Indeed, it has been shown that TFIIIB·DNA complexes remain associated even after stringent treatments with heparin or high salt concentrations (3). On the other hand, one could argue that all the chromatographic systems used in the present work disrupt a stable, preexisting TBP·TFIIIB70·B″ complex. It is conceivable that binding of TFIIIB70 to the nickel column through an important metal binding domain caused the dissociation of TBP and B″. However, different interpretations must then be invoked to explain the dissociation of TFIIIB by the Mono S, Q-Sepharose or the antibody−Protein A-Sepharose chromatography steps: as judged from the elution properties of TFIIIB70 activity, strong hydrophobic interactions are involved in its association with the Protein A-Sepharose, ionic interactions with the Q-Sepharose and Mono S columns, and metal coordination with the Ni^{2+} column. Alternatively, it could be that the TFIIIB fraction used by Poon and Weil (19) contained an additional component, not essential for basal transcription factor activity, that locks the TFIIIB components into a stable complex. A novel polIII transcription factor, named TFIIIE, has been recently purified from yeast nuclear extracts (42). Distinct from known TFIIIB or TFIIIC components, this factor could be a candidate as a TFIIIB activation factor.

By analogy with the polII TAFs, the TFIIIB components are considered as polIII TAFs (13, 14). With the pre-assembly of TFIIIB, the polIII system would benefit from the pre-assembly of all the transcriptional components: RNA polymerase III, with its 16 subunits, TFIIIB, and the multisubunit assembly factor and chromatin antirepressor (1, 43) TFIIIC (6 subunits). The present work, however, suggests a multistep pathway of TFIIIB assembly on the DNA, as also suggested by genetic suppression data (16, 18, Lefebvre *et al.*, submitted). Recent studies on mammalian TFIIIB appear to lead to the same conclusion as TFIIIB activity was found to be separable from TBP (K.Seifart, personal communication). It would be interesting if polII TAFs were also present in free and bound forms to generate subfamilies of TFIID molecules. While this work was being submitted, Joazeiro *et al.* (44) showed that the same TFIIIB components were required for transcription of TATA-less and TATA-containing polIII genes.

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