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Cofractionation of the TATA-binding protein with the RNA polymerase III transcription factor TFIIIB

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ABSTRACT

We have investigated the requirement for TBP (TATA-binding protein) in transcription mediated by RNA polymerase III (pol III) in fractionated HeLa cell extracts. Two activities, TFIIIB and TFIIIC, found in phosphocellulose fractions PC B and PC C respectively, have been defined as necessary and sufficient, with pol III, for in vitro transcription of tRNA genes. Depletion of TBP from PC B, using antibodies raised against human TBP, is shown to inhibit the pol III transcriptional activity of the fraction. Furthermore, TBP is present in fractions with human TFIIIB activity, and a proportion of TBP co-fractionates with TFIIIB over four chromatographic purification steps. TFIIIB fractions are capable of supplying TBP in the form necessary for pol III transcription, and cannot be substituted by fractions containing other TBP complexes or TBP alone. The use of a 5S RNA gene and two tRNA templates supports the general relevance of our findings for pol III gene transcription. Purified TFIIIB activity can also support pol II-mediated transcription, and is found in a complex of ~230kD, suggesting that TFIIIB may be the same as the previously characterized B-TFIID complex (1,2). We suggest that transcription by the three RNA polymerases is mediated by distinct TBP – TAF complexes: SL1 and D-TFIID for pol I and pol II respectively, and TFIIIB for pol III.

INTRODUCTION

In order to accurately initiate transcription, the three multisubunit eukaryotic RNA polymerases require accessory factors (3). Initially it was considered that each polymerase would utilize a distinct set of accessory factors in transcription of its class of genes. However, recent experimental advances suggest that the different polymerases use similar strategies, and occasionally the same factors, in expression of their target genes. The first example of a protein involved in transcription by all three polymerases is TBP, the TATA-binding protein initially identified as a subunit of TFIID.

Early biochemical studies defined TFIID as the pivotal factor in assembly of pol II preinitiation complexes on mRNA promoters. Binding of TFIID to the TATA element in such promoters is the first step in the assembly pathway (4,5; reviewed in 6). Complementary DNAs encoding the TBP component of TFIID have been cloned from *Saccharomyces cerevisiae* (7–11), and subsequently from human (12–14), Drosophila (15,16), *Arabidopsis thaliana* (17), the fission yeast *Schizosaccharomyces pombe* (18,19), and mouse (20). Comparison of the predicted amino acid sequences reveals a phylogenetically conserved 180 residue C-terminal 'core' domain (see ref. 21 for review). Outwith the core, the TBPs show divergence in both the length and sequence of the N-terminal region. The conserved core contains a central basic region flanked by 34 residue direct repeats, and a homology to prokaryotic sigma factors which first suggested that the region was important in DNA binding (7,11). Indeed, mutagenesis of TBP proteins showed that this C-terminal domain binds the TATA box, and interacts with other general pol II factors to restore basal transcription to a TFIID-depleted extract (7,12,15,22–24), suggesting an evolutionary conservation of function. Although response to acidic transcriptional activators can be mediated via the C-terminal regions of human and yeast TBP (25), the N-terminus may be required for response to other activators (12,14).

TBP is also necessary for TATA-dependent pol III-mediated transcription of human U6 snRNA genes in vitro (26,27) and can bind the U6 TATA element in vitro (27; K.A.S. unpublished data). Given the central role of the TATA element in determining pol III specificity of U6 expression (28–30) this suggests that TBP requires associated factors to participate in pol III recruitment to U6 promoters, and polymerase choice in general. TBP has been subsequently revealed as a general polymerase cofactor necessary for in vitro transcription of TATA-less pol III templates (31), pol I templates (32), and certain TATA-less pol II templates (33). The involvement of TBP in transcription by all three nuclear polymerases has been confirmed by genetic and biochemical studies in yeast (34,35). Loss of TBP expression from temperature sensitive mutant strains correlated with rapid decreases in transcription from a range of templates when assayed both in vivo and in cell extracts.

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In the absence of TATA-binding, TBP recruitment to TATA-less promoters presumably results from association with other proteins. Indeed, TBP is detected exclusively in multiprotein complexes within human and Drosophila cell extracts (32,33,36–38, see ref. 39 for review). The classical pol II TFIID complexes, originally isolated from a high salt phosphocellulose fraction (PC D) of HeLa or Drosophila cell extracts have been shown to contain TBP and several TBP-associated factors (TAFs) that are required for response to certain transcriptional activators (37,38). A second human TBP complex capable of supporting pol II transcription has recently been purified from a low salt phosphocellulose fraction (PC B) and termed B-TFIID, distinguishing it from the high salt TFIID fraction (renamed D-TFIID; see refs. 1 and 2). Although both B-TFIID and D-TFIID support basal pol II transcription, only the latter supports activated transcription, again implicating subsets of TAFs as important in mediating the response to specific activators. That TAFs also function in directing polymerase choice was suggested by the identification of TBP in the promoter selectivity factor SL1, a multisubunit factor essential for pol I transcription (32). The three TAFs found in SL1 are distinct from those in D-TFIID complexes (32). These findings prompted speculation that the three polymerases utilized distinct TBP–TAF complexes (40,41): SL1 and D-TFIID for pol I and pol II respectively, and a third, unidentified complex for pol III.

Two activities found in HeLa extract phosphocellulose fractions have been defined as necessary and sufficient, together with pol III, for in vitro transcription of tRNA genes (42). These factors, TFIIIB and TFIIIC, are found in the PC B and PC C fractions respectively, from which they have been partially purified (43,44). Pol III is found in both the PC B and PC C fractions (42). Binding of TFIIIC to the conserved intragenic A and B boxes initiates tRNA transcription complex assembly (reviewed in 45). TFIIIC has been resolved into two activities (44,46). TFIIIC2 is a multisubunit factor with a molecular weight of ~500kD which binds with high affinity to the B box. TFIIIC1 is a smaller multisubunit activity and binds the A box, although this occurs only after TFIIIC2 is bound at the B box (47). TFIIIB associates with TFIIIC only after TFIIIC is bound, and stabilizes the interaction between the factors and the promoter (48).

Transcription of 5S RNA promoters requires, in addition to TFIIIB and TFIIIC, the 5S gene-specific factor TFIIIA (42). Binding of TFIIIA to the intragenic control region (ICR) is the first step in complex formation, and precedes TFIIIC association with the promoter (49). Subsequent recruitment of TFIIIB to the TFIIIA/C-ICR complex is the rate-limiting step in initiation (50,51). Although *Xenopus laevis* TFIIIA was the first eukaryotic transcription factor to be cloned (52), TFIIIB and TFIIIC from multicellular eukaryotes have proven difficult to purify and clone.

Elegant in vitro experiments by Geiduschek and colleagues have shown that TFIIIB plays the role of initiation factor in transcription of yeast 5S RNA and tRNA genes (53). TFIIIC activity is retained in one entity with distinct A and B box-binding domains (54; also termed τ, 55). Following TFIIIC binding, TFIIIB is positioned upstream of the start site in a TFIIIC-dependent manner (53,56). Stable association of TFIIIB with the promoter facilitates the recruitment of pol III to the start site, and subsequent initiation. Once tightly bound, TFIIIB can direct multiple rounds of initiation, even in the absence of TFIIIC (in tRNA genes) or TFIIIA and TFIIIC (in 5S genes) (53). The use of photocrosslinking has established that two polypeptides of 90kD and 70kD are TFIIIB components closely associated with DNA in this upstream complex (56,57). Stable assembly of TFIIIB is therefore the critical step in pol III initiation, and TFIIIC and TFIIIA act to position TFIIIB in yeast 5S and tRNA transcription. The central role of TFIIIB in transcription of other classes of pol III templates is suggested by its requirement for transcription of both vertebrate and yeast U6 genes in vitro (58,59).

Given the evidence above, and the observation that 75% of human cellular TBP fractionates into PC B (1) we wished to address whether this fraction contained a TBP–TAF complex capable of supporting pol III transcription. Here we report that immunodepletion of TBP from the HeLa PC B fraction renders the fraction inactive in pol III transcription, and that this is due to loss of TFIIIB activity. Furthermore, part of the TBP present in PC B is shown to cofractionate over multiple chromatographic steps with TFIIIB activity. After extensive purification TBP and TFIIIB activity are both found in a complex of ~230kD in size.

MATERIALS AND METHODS

Fractionation of HeLa cell extracts

Nuclear extracts (~10 mg/ml protein) were prepared from HeLa cells as described (60) and fractionated over phosphocellulose (P11; Whatman, U.K.) as described (42). The flowthrough fraction (PC A, 100mM) was collected and then KCl step elutions performed at 350mM (PC B), 600mM (PC C), and 1000mM (PC D) in buffer D (20mM HEPES pH 7.9, 10% (v/v) glycerol, 0.2mM EDTA, 3mM DTT, 0.2mM PMSF). Peak protein fractions were pooled and dialyzed back to 100mM KCl in buffer D prior to use in transcription or immunodepletion.

Before DEAE-Sephadex chromatography, the PC B fraction was dialyzed into 10% (v/v) glycerol, 5mM $MgCl_2$, 20mM HEPES pH 7.9, 3mM DTT, containing 50mM ammonium sulphate. The dialyzed fraction (2.2 mg/ml protein) was loaded onto the column at 3mg protein/ml of bed resin. The flowthrough was collected (DS 0.05M), before eluting with the same buffer containing 135mM (DS 0.135M) and 1M (DS 1.0M) ammonium sulphate. The fractions were dialyzed to 100mM KCl in buffer D prior to use. The DS 0.135M fraction (0.75 mg/ml) was further fractionated over an FPLC 1ml Mono Q column (Pharmacia). After loading in buffer D (100mM KCl), the column was eluted with a linear gradient from 100 to 600mM KCl. The 0.5ml fractions were dialyzed to 100mM KCl in buffer D before being assayed.

Mono Q fractions active for TFIIIB activity were pooled (2.6 mg/ml) and adjusted to the elution buffer (buffer D containing 20% (v/v) glycerol, 400mM KCl) prior to gel filtration chromatography. 200μl of sample was applied and eluted from a Superose 12 column (Pharmacia) at a flow rate of 80 μl/min. Collected fractions (400μl) were dialyzed to 100mM KCl in buffer D before being assayed. The calibration standards for the gel filtration column were apoferritin (443kD), β-amylase (220kD), albumin (66kD), and carbonic anhydrase (29kD).

The D-TFIID fraction used in Figures 4 and 5 was kindly supplied by Jean-Marc Egly at the Institut de Chimie Biologique, Strasbourg.

Overexpression and purification of recombinant human TBP

A full length human TBP cDNA (residues 1–335) bearing a 6 histidine 'tag' at the N-terminus (kindly provided by Alexander Hoffmann and Robert Roeder, The Rockefeller University, New York) was placed under the control of a T7 RNA polymerase

promoter and transformed into *E.coli* BL21(DE3) lysE (61). 500ml cultures were grown in SuperBroth supplemented with 0.4% glucose, 100 μg/ml ampicillin, 25 μg/ml chloramphenicol at 30°C up to OD_{600} ~0.7. Cultures were then induced by addition of IPTG to 1mM and grown for 2 more hours. Cells were harvested by centrifugation at 3000×*g* for 10 min at 4°C, washed with 100ml of 0.1M NaCl, 1mM EDTA, 10mM Tris-HCl pH 8, and spun again. The cell pellet was resuspended in 30ml of ice-cold lysis buffer (0.5M NaCl, 20% glycerol, 20mM Hepes, pH 7.9, 1mM EDTA, 20mM 2-mercaptoethanol, 1mM PMSF, 0.1% NP-40, 20 μg/ml pepstatin A, 20 μg/ml leupeptin, 20 μg/ml aprotinin) and sonicated (10×30 seconds) on ice with a Branson Sonifier B-15 at maximal setting. Lysates were cleared by centrifugation at 100,000×*g* for 1 h at 4°C, and the supernatants loaded onto a 10ml-DEAE-Sephadex A-25 column preequilibrated in lysis buffer at 4°C. The flowthrough fraction was mixed with 1ml of Ni^{2+}-NTA resin (Diagen, Germany) preequilibated in lysis buffer, and rotated for 2 hours at 4°C. The mix was loaded into a BioRad Econocolumn, and the resin washed with 10ml of buffer D and 3ml of buffer D containing 20mM imidazol. Recombinant hTBP was eluted with buffer D containing 100 mM imidazol, frozen in liquid nitrogen and stored at −80°C.

Preparation and purification of polyclonal anti-hTBP serum

Histidine-tagged recombinant hTBP, prepared as described above, was further purified by preparative SDS-PAGE. Gels were lightly stained with 0.2% Coomassie brilliant blue in water and the hTBP band was excised and crushed in phosphate-saline buffer prior to injection of rabbits (New Zealand White). Antisera were pooled and the IgG fraction obtained by ammonium sulfate fractionation and subsequent chromatography over DEAE-cellulose (DE-52; Whatman, U.K.).

Immunodepletion of phosphocellulose fractions

IgG fractions from preimmune and hTBP sera were coupled to protein A-Sepharose beads (Pharmacia) at a ratio of 25mg IgG/ml of beads as described (62). Immunodepletion of phosphocellulose fractions was performed by rotating 300μl of each fraction with 100μl of coupled beads for 4 h at 4°C. Beads were pelleted by centrifugation at 5K for 3min and supernatants were immediately frozen in liquid nitrogen. Depletion was checked by immunoblot analysis (see below); more than 90% of hTBP was consistently depleted from fractions with this method.

Immunoblotting analysis

Proteins were separated on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting in a Trans-Blot Cell (BioRad) in transfer buffer (25mM Tris, 40mM glycine, 0.05% SDS, 20% methanol) at 80 V for 2 hours at 4°C (all other steps were at room temperature). Protein transfer was checked by Ponceau-S staining for a few min followed by destaining in water. TBP was detected with ECL reagents (Amersham) as indicated by the manufacturer. This involved the following steps. The membranes were blocked in 5% nonfat dry milk, 0.1% Tween-20, 1×PBS, 0.02% NaN_3 with gentle rocking for 1h. After washing with PBS containing 0.05% Tween-20 (2×5min) they were incubated with anti-hTBP antibodies (IgG purified fraction diluted in PBS/0.05% Tween-20) with gentle shaking for 1h. After washing with PBS/0.05% Tween-20 (3×5min) they were incubated with donkey anti-rabbit antibodies (Amersham) for another hour. Membranes were finally washed (5×5min) in PBS/0.05% Tween-20, blotted dry and exposed to X-ray sensitive film.

RNA polymerase II *in vitro* transcription assays

Chromatographic fractions were assayed for hTBP activity using a complementation assay which included 3μl each of HeLa phosphocellulose fractions PC A and PC C. G-less transcription reactions (63) were in a final volume of 25μl and contained 7.5mM $MgCl_2$, 1.2mM 3'-O-me-GTP (Pharmacia), 0.4mM ATP, 0.4 mM CTP, 2 units RNAse T1, 1.25mM DTT, 2% polyethylene glycol 8000 (Sigma), 20μCi α^{32}P-UTP(800 Ci/mmol), 33mM Hepes, 50mM KCl, 10% glycerol and 200ng of template (the Adenovirus 2 Major Late promoter construct AdML404[180] (26). Transcription reactions were allowed to proceed for 60 min at 30°C and samples were processed as described (64). Transcripts were analyzed on 8% denaturing polyacrylamide gels, dried and exposed to X-ray sensitive film (XAR 5, Kodak) for 24 hours at −80°C with intensifying screens.

RNA polymerase III *in vitro* transcription assays

Pol III transcription reactions for tRNA templates were performed exactly as in (26). The tRNA templates used were the nematode $tRNA^{Pro}$ gene (Mcet1 in ref. 65), and the *Xenopus laevis* $tRNA^{Phe}$ gene pJ5 (pPhe in ref. 66; kindly provided by Daniel Scherly, Dept. de Génétique et Microbiologie, Geneva). Reaction conditions for 5S RNA templates were identical to those for tRNA transcription. The 5S template was the *Xenopus laevis* 5S (Xls 560−764 in ref. 67). Alpha-amanitin was always present at a final concentration of 2 μg/ml, which is sufficient to prevent pol II transcription. Precipitated products were separated by electrophoresis on 8% denaturing polyacrylamide gels and detected by autoradiography at −80°C.

RESULTS

Immunodepletion of TBP from the PC B fraction inactivates pol III transcription

To study pol III transcription at the biochemical level we first prepared nuclear extracts from exponentially growing cultures of HeLa cells (60). Extracts active for pol III transcription were fractionated over phosphocellulose into PC A, -B, -C, and -D according to (42) (see Materials and Methods), and the fractions assayed for transcription of two tRNA templates and a 5S RNA gene. As expected (42), PC B and PC C are necessary in combination for efficient tRNA transcription (Figure 1A, lanes 1−3; Figure 1B, lane 1); cross contamination is minimal as either fraction alone generates only negligible Mcet1 $tRNA^{Pro}$ transcription (Figure 1A). Addition of PC A or PC D had no stimulatory effect on the transcription reconstituted with PC B and PC C (data not shown). To investigate the involvement of TBP in the observed transcription, we performed immunodepletions of PC B and PC C using rabbit serum raised against recombinant human TBP, or the corresponding preimmune serum. After incubating the fractions with the antibodies coupled to protein A-Sepharose beads, the beads were removed and the fractions assayed in transcription. Complementation assays using the various immunodepleted or control fractions were performed to determine whether removal of TBP from either fraction affected pol III activity. Control depletions carried out with the preimmune serum-coupled beads have essentially no effect on the ability of PC B and PC C to

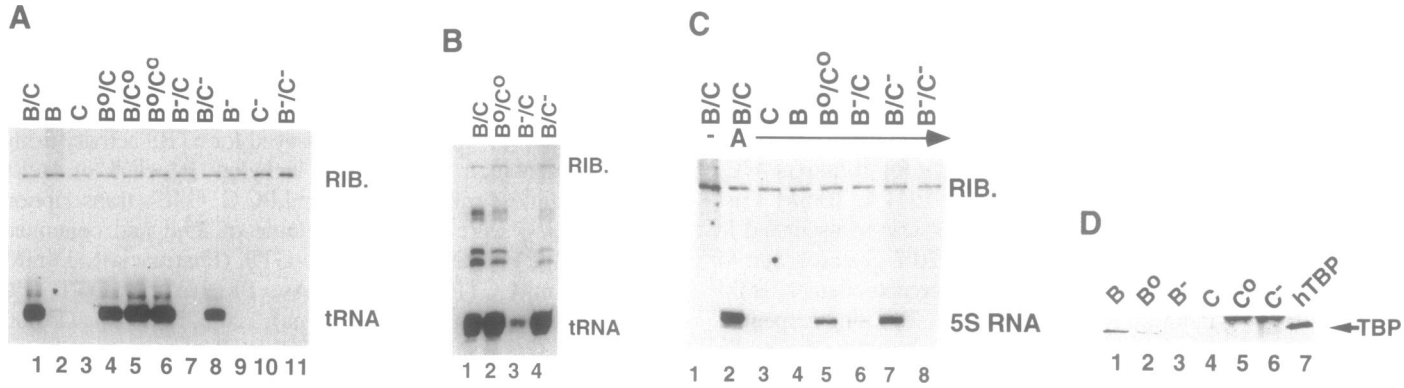

Figure 1. TBP depletion of the PC B fraction inactivates expression of pol III genes. **(A)** Transcription reactions were performed as described in Materials and Methods using phosphocellulose fractions of a HeLa cell nuclear extract. 5μl of each PC fraction was used as indicated above the lanes. 100ng of the nematode $tRNA^{Pro}$ gene Mcet1 (65) was present in each reaction. Alpha-amanitin was present at 2 μg/ml. Fractions denoted (B) or (C) are control phosphocellulose fractions. Fractions denoted (B°) or (C°) were immunodepleted with pre-immune serum coupled to protein A-Sepharose beads (see Materials and Methods). Fractions denoted (B^-) or (C^-) were immunodepleted with anti-TBP serum coupled to protein A-Sepharose beads. tRNA transcripts are indicated. RIB is a riboprobe added to the reactions after transcription to control for sample recovery. **(B)** Transcription reactions were as above, using 100ng of the *Xenopus laevis* $tRNA^{Phe}$ gene pJ5 (66) as template. 5μl of each PC fraction was used as indicated above the lanes. tRNA transcripts are indicated. The other signals migrating above are precursor tRNAs extending ~20 and ~60 nucleotides further downstream than the processed tRNA (Daniel Scherly; personal communication). **(C)** Reaction conditions for 5S RNA gene transcription were as above, using 250ng of the *Xenopus laevis* 5S RNA gene (Xls 560−764; 67) as template. 3μl of each PC fraction was used as indicated above the lanes. 5S RNA transcripts are indicated. **(D)** Immunoblot analysis of TBP in the PC fractions required for pol III transcription. PC B and PC C fractions (15μl), before and after immunodepletion with antibody-coupled protein A-Sepharose beads, were separated on a 12.5% SDS-PAGE gel. Immunoblot analysis was performed as described in Materials and Methods. The position of TBP is indicated. The strong background signal in the C° and C^- fractions is attributable to antibody released into the fraction during depletion.

support transcription of either tRNA template (Figure 1A, compare lanes 4−6 with lane 1; Figure 1B, lanes 1−2). In contrast, immunodepletions with the TBP serum inhibit tRNA expression; comparing lanes 11 and 6 (Figure 1A) shows that TBP depletion of both fractions results in complete loss of Mcet1 tRNA transcription. Lanes 7 and 8 reveal that this effect is due almost exclusively to inactivation of PC B function in PC B^-, as PC C^- is virtually equivalent to PC C in its ability to complement PC B (compare lanes 8 and 1). However, removal of TBP in PC C^- has some effect, as the tRNA signal drops when switching from PC C to PC C^- in complementation to PC B^- (compare lanes 7 and 11). Similar effects are observed when assaying $tRNA^{Phe}$ expression (Figure 1B, lanes 3−4), with TBP immunodepletion of PC B resulting in a much greater decrease in transcription than PC C depletion. The use of an RNA recovery control transcript (RIB.) in such experiments verifies that the absence of test signals is a genuine effect on transcription, and not simply due to a loss of RNA products during sample preparation.

To investigate whether the loss of tRNA transcription upon TBP depletion of PC B was affecting an activity required by other pol III genes, we assayed transcription of a Xenopus 5S RNA gene in the reconstituted system. Figure 1C shows that, as expected, efficient 5S transcription requires PC A in addition to PC B and PC C (lanes 1 and 2), confirming the need for TFIIIA (42). The reduction in transcription upon omission of PC B (lane 3) or PC C (lane 4) demonstrates the requirement for all three fractions. In the presence of PC A (which is devoid of detectable TBP; data not shown), TBP depletion of both PC B and PC C results in a loss of 5S transcription greater than that observed with control depletion (compare lanes 8 and 5). The lower signal strength in lane 5 c.f. lane 2 may be a consequence of dilution of the fractions incurred during immunodepletion (see Materials and Methods); that a similar decrease in tRNA transcription was not observed (Figures 1A and 1B) may reflect the greater transcriptional activity of the tRNA templates in our system. As with the tRNA templates, however, it is depletion of TBP from PC B, rather than PC C, which is responsible for the decreased 5S transcription (Figure 1C, lanes 6−8). TBP depletion of PC B yields a loss of 5S signal equivalent to omission of PC B in the same background (compare lanes 6 and 3).

To investigate whether the observed effects on pol III transcription correlated with a specific removal of TBP, we performed an immunoblot analysis of the relevant fractions. In agreement with Timmers and Sharp (1), we reproducibly detect TBP in both the PC B and PC D fractions of HeLa extracts (data not shown). Figure 1D shows that the TBP serum recognizes an antigen in PC B (lane 1) of apparent mobility ~45kD, migrating slightly faster than recombinant human TBP recognized in lane 7; this slight discrepancy may reflect the presence of the six histidine 'tag' on the recombinant TBP, which is predicted to add ~2kD to its mass. That this antigen in PC B is TBP was further supported by the inability of the preimmune serum to detect a protein of comparable mobility (data not shown). Depletion of PC B with preimmune serum results in a slight drop in TBP signal (compare lanes 1 and 2), presumably reflecting the dilution effects described above. Comparing lanes 1 to 3 confirms that the TBP antibodies were effective in removing TBP from PC B, as the signal in PC B^- is undetectable (lane 3). No TBP signal is detected in any of the three PC C fractions (lanes 4−6). It is possible that low levels of TBP (undetectable by our immunoblot analysis) were present in PC C and subject to depletion. Three lines of evidence support this. First, we occasionally could detect TBP at low levels (relative to PC B or PC D) in PC C fractions, using the same immunoblotting assay. Second, the PC C fractions utilized can support a low level of TBP function in pol II transcription assays (26; data not shown). Finally, we observed an effect on tRNA expression (albeit minor compared to that arising from PC B depletion) when using PC C^- instead of PC C (Figure 1A). We conclude that TBP immunodepletion of PC B inactivates pol III transcription, and that this effect correlates with physical removal of TBP. Depletion of PC C has only a comparitively minor effect that may be due to slight cross contamination with TFIIIB activity.

Cofractionation of TBP with TFIIIB activity

As stated in the Introduction, the pol III factor TFIIIB is present in PC B. To test if the loss of PC B activity upon TBP depletion reflected removal of TFIIIB activity, we fractionated PC B further to obtain more highly purified TFIIIB fractions. Following DEAE-Sephadex (DS) chromatography (43; see Materials and Methods) we assayed the flowthrough and step eluted fractions for ability to complement PC C in support of tRNA transcription (Figure 2A). Addition of PC B or DS load fractions to PC C generates more efficient transcription than does PC C alone (lanes 1–3). Comparing lanes 4–6 reveals that essentially all TFIIIB activity resides in the DS 0.135M ammonium sulphate fraction, as described (43). The minor activity detected in the DS 1.0M fraction (compare lanes 2 and 6) will be addressed below. As polymerase III in PC B elutes in the DS 1.0M fraction (43), we conclude that our PC C contains sufficient polymerase to support tRNA expression, and that the DS 0.135M fraction is enriched over PC B for TFIIIB activity (lanes 1 and 5).

We then assayed the same DS fractions for their ability to complement PC C$^-$ for tRNA expression; the aim being to more rigorously test whether the TFIIIB containing fraction identified in Figure 2A was also capable of supplying the TBP necessary for pol III transcription (31; Figure 1). Figure 2B shows that indeed, of the DS fractions, DS 0.135M complements most efficiently for tRNA transcription (lane 4). The DS 0.05M flowthrough fraction again does not support pol III transcription (lane 3). In this experiment, as in Figure 2A, the DS 1.0M fraction generates some tRNA transcription above background (compare lanes 5 and 1). The observation that a minor proportion of TFIIIB cofractionates with RNA polymerase III over several column matrices (68) may account for the complementation by the DS 1.0M fraction observed with PC C$^-$ (in Figure 2B), or PC C (in Figure 2A).

The ability of DS 0.135M to replace PC B in both these assays suggested that this fraction contained both TFIIIB activity and the TBP-containing activity necessary for pol III initiation. Support for this comes from immunoblot analysis of the DS fractionation (Figure 2C). The DS load fraction contains TBP (indicated by an arrow) migrating just above another reactive protein (lane 2). Some of this TBP partitions into the DS 0.05M flowthrough, along with the smaller cross-reactive protein (lane 3). However, much of the TBP from PC B is detected in the DS 0.135M fraction (lane 4), with none detectable in the DS 1.0M step (lane 5).

Although TBP is present in both the DS flowthrough and 0.135M ammonium sulphate fractions, only the latter is active in pol III transcription of tRNA and 5S genes, suggesting that TBP requires associated factors (TAFs) specific to that fraction for its function in TFIIIB activity. The different chromatographic behaviour, and inactivity in our TFIIIB assay, of TBP in the DS 0.05M fraction may reflect the nature of associated TAFs in this fraction. Given the coincidence of TFIIIB activity with TBP presence in DS 0.135M, we sought to determine whether these two activities remained coincident in one complex, or were separable upon further chromatography. The DS 0.135M was fractionated on a Pharmacia Mono Q (MQ) column using a linear salt gradient from 100 to 600mM KCl. The fractions were first assayed by immunoblotting for TBP (Figure 3A). TBP in the PC B and DS 0.135M (MQ load) fractions is shown in lanes 1 and 2. All of the TBP binds the matrix, as none is detectable in the flowthrough fraction (lane 3). Quantitative elution of TBP

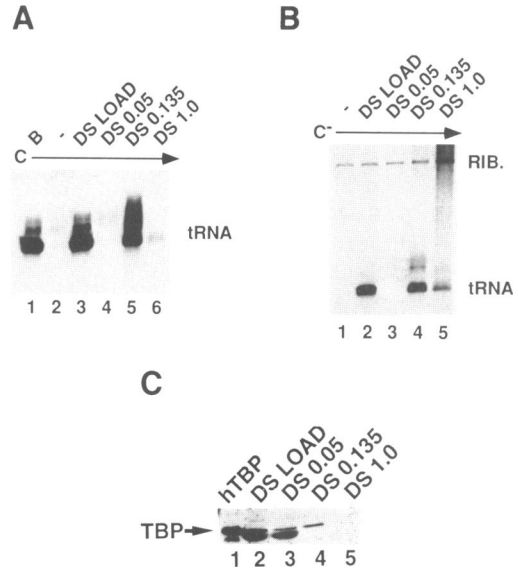

Figure 2. TFIIIB addition can reverse the effect of TBP-depletion. **(A)** Purification of TFIIIB from PC B. Mcet1 transcription reactions were performed as in Fig. 1. PC B fraction was fractionated over DEAE Sephadex (DS) and the fractions assayed for TFIIIB activity by complementation of PC C. Each reaction contained 5μl of PC C. This was supplemented with 5μl of PC B (lane 1), buffer D (lane 2), the DEAE Sephadex load, flowthrough and step eluted fractions (lanes 3–6). tRNA transcripts are indicated. **(B)** The TFIIIB fraction contains the TBP necessary for pol III transcription. Mcet1 transcription reactions were as above. The DS fractions were assayed for their ability to complement PC C$^-$. Each reaction contained 3μl of PC C$^-$. This was supplemented with 3μl of the indicated fractions (lanes 2–5), or buffer D (lane 1). tRNA transcripts are indicated. **(C)** Immunoblot analysis of the DEAE-Sephadex fractionation of PC B performed as in Figure 1D. The position of TBP is indicated.

occurs between 300 and 400mM on the gradient, with fraction 10 (lane 12; 330mM) representing the peak. Fractions flanking and including the TBP peak were then analyzed for TFIIIB activity in the tRNA transcription complementation assay with PC C$^-$ (Figure 3B). Individually, both the MQ load fraction (DS 0.135M) and PC C$^-$ are inactive for tRNA expression (lanes 1–2); however, in combination they support efficient tRNA synthesis (lane 3). The MQ flowthrough and MQ 8 fractions, shown above to contain no detectable TBP, are inactive for complementation (lanes 4–5). Lanes 6–8 show that the MQ fractions 9, 10, and 11 retain TFIIIB activity, peaking in MQ 10. MQ 10 was also found to retain TFIIIB activity from DS 0.135M when assaying transcription from the Xenopus tRNA^{Phe} template in the same experimental system (data not shown). Comparing Figures 3A and 3B we conclude that TBP cofractionates with TFIIIB activity over three columns. Closer inspection reveals that although MQ 11 contains less detectable TBP than MQ 9 (Figure 3A), the former has greater transcriptional activity (Figure 3B). This may reflect one of two possibilities; that TBP was being partially separated from other factors required for TFIIIB activity during the gradient elution, or that TFIIIB activity did cofractionate with TBP and the reduced activity in fraction MQ 9 was rather due to the presence of an inhibitor in the fraction. To test the former, we performed experiments mixing fractions on either side of the activity peak in various ways or by repeating the Mono Q chromatography with shallower gradient profiles using either a pool of MQ 9–11, DS 0.135M, or PC B fractions as input. No evidence was obtained to suggest that TFIIIB activity was divisible (data not

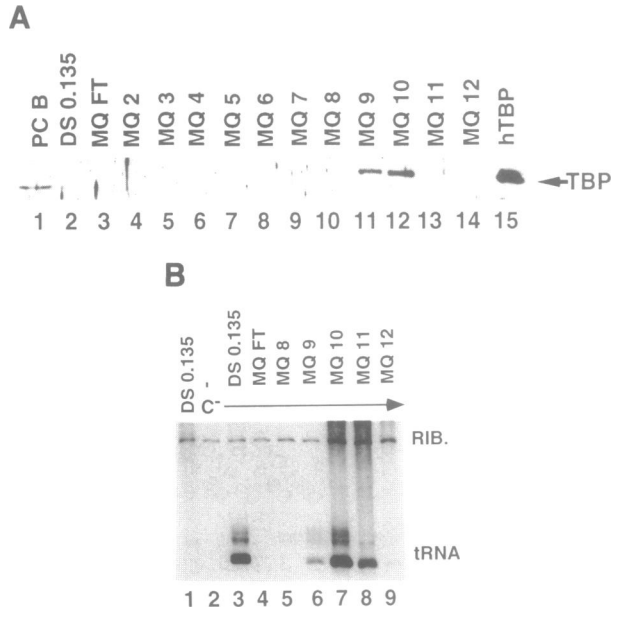

Figure 3. TBP cofractionates with TFIIIB activity over Mono Q. **(A)** Immunoblot analysis (as above) of the Mono Q fractionation of DS 0.135M. The position of TBP is indicated. **(B)** Peak TBP fractions retain TFIIIB activity in pol III transcription. Mcet1 transcription reactions were performed as for Figure 2B, using 4μl of PC C$^-$ and 4μl of each complementing fraction as indicated above the lanes. Lane 1 contained 4μl of the DS 0.135M fraction alone. tRNA transcripts are indicated.

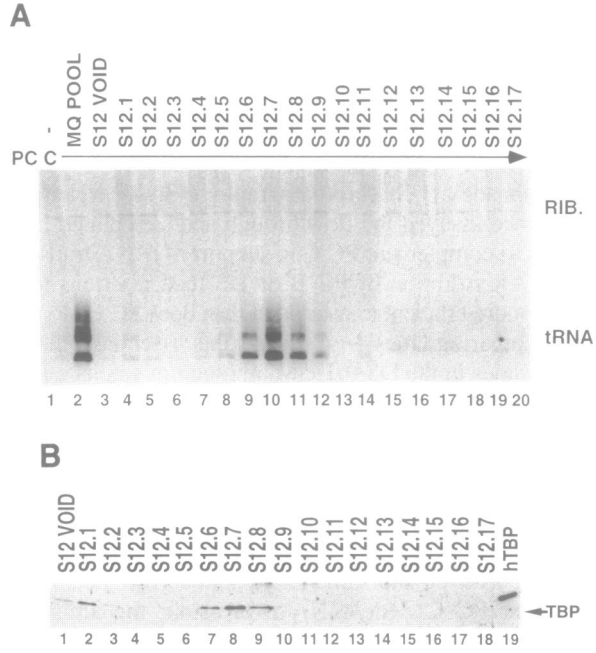

Figure 4. TBP and TFIIIB activities copurify in a complex of ~230kD. **(A)** Mcet1 tRNA transcription reactions were performed as in Materials and Methods with 3μl PC C in each reaction. This was complemented with 6μl of buffer D (lane 1), the Mono Q pool loaded onto the Superose 12 column (lane 2), and the eluted S12 fractions (as indicated above the lanes). Alpha-amanitin was present at 2 μg/ml. **(B)** Immunoblot analysis of the Superose 12 fractionation performed as in Figure 1D. 100μl of each S12 fraction was used. The position of TBP is indicated.

shown). However, mixing experiments showed that the addition of MQ 8 to MQ 10 inhibited the TFIIIB activity of MQ 10 (data not shown), arguing for the presence of an inhibitor and the latter possibility stated above, that TFIIIB does cofractionate with TBP.

To separate TFIIIB activity away from inhibitors, and address directly the question of whether TFIIIB is a multiprotein TBP complex, gel filtration chromatography was used. Active TFIIIB fractions, purified by sequential phosphocellulose, DEAE-Sephadex, and Mono Q chromatography, were pooled and size fractionated on a Superose 12 (S12) (Pharmacia) gel filtration column. The eluted fractions were tested for TBP content by immunoblotting and TFIIIB activity in pol III transcription. Figure 4A shows that the TFIIIB activity of the MQ pool, as assayed by the ability to complement PC C in support of $tRNA^{Pro}$ transcription, elutes in fractions S12.6–9, peaking in S12.7 (lanes 1–2, and 9–12, fraction nos. are at the top of the figure). The same fractions were also active in support of 5S RNA transcription in reactions containing PC A and PC C (data not shown). Immunoblot analysis revealed that while part of the TBP was present as higher molecular weight complexes (Figure 4B, lanes 1–3) that are inactive in pol III transcription (Figure 4A, lanes 3–5) much of the TBP cofractionates with TFIIIB activity in fractions S12.6–8, peaking in fraction S12.7 (Figure 4B, lanes 7–9). Separation of molecular weight standards under the same conditions shows that fraction S12.7 corresponds to a molecular mass of ~230kD(+/−50kD), suggesting that TBP and TFIIIB activity copurify in a complex.

TBP present within TFIIIB fractions can support pol II-mediated transcription

To test whether the TBP resident in the TFIIIB fractions was capable of supporting basal level pol II activity, we assayed them in a TBP-dependent reconstituted pol II transcription system. This comprised the PC A and PC C fractions of a HeLa nuclear extract. PC A contributes the factors TFIIA and TFIIJ (69), and PC C contains the pol II enzyme and the essential pol II basal factors, TFIIB, TFIIE, TFIIF, and TFIIH (see ref. 70). In combination, PC A and PC C are inactive for transcription of the pol II Adenovirus major late (AdML) promoter (Figure 5, lane 1), coupled to a G-less cassette reporter (63). Efficient AdML transcription is observed when recombinant human TBP is added to the reaction (lane 12), demonstrating the TBP-dependence of the system. In light of our observations that PC C contains residual TBP (see above), this TBP is presumably insufficient for, or inactive in, support of transcription in this assay. When the PC B and PC D fractions were similarly assayed for TBP function, only PC D was active (lanes 2–3). Further purification of PC D to yield a D-TFIID fraction potentiates a greater TBP response as measured by AdML transcription (lanes 3–4). The inability of PC B to support a similar TBP response despite the clear demonstration of its presence (Figure 1B) has been previously reported and attributed to the presence of an inhibitor of pol II TBP function in that fraction (1). Those authors demonstrated that further fractionation of PC B was necessary in order to detect the pol II TBP activity they defined as B-TFIID (1,2). In agreement with their findings, we also observe that further fractionation of PC B, in this case into DS 0.135M, unmasks the pol II TBP activity of PC B (compare lanes 2 and 5). Lanes 6–11 clearly demonstrate that this pol II TBP activity elutes in the Mono Q fractions 9, 10, and 11, corresponding to those fractions active for TFIIIB function (see Figure 3B) and enriched for TBP (Figure 3A). Fraction 10 exhibits the best

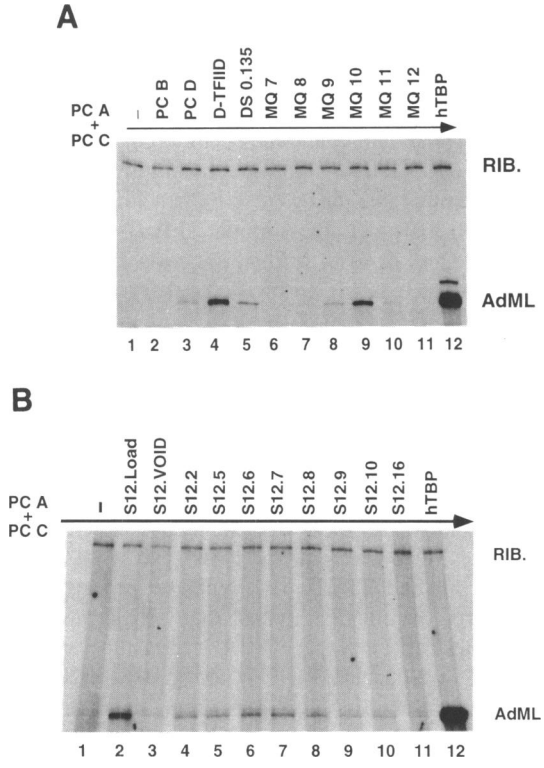

Figure 5. TBP within TFIIIB fractions is active for pol II transcription. Testing TBP-containing fractions for support of Adenovirus Major Late transcription in a TBP-dependent reconstituted system. All reactions contained 3μl PC A and 3μl PC C. **(A)** The PC A and PC C fractions were complemented with 3μl of buffer D (lane 1), PC B (lane 2), PC D (lane 3), 3μl of partially purified D-TFIID fraction (lane 4; kindly provided by J.-M. Egly, Institut de Chimie Biologique, Strasbourg), 3μl DS 0.135M (lane 5), 3μl of Mono Q fractions 7−12 (lanes 6−11), or 1μl recombinant human TBP (~20ng protein) (lane 12). Correctly initiated AdML transcripts are indicated. **(B)**. Exactly as in 5A, except that the PC A and PC C fractions were complemented with the Superose 12 fractions indicated above the figure (lanes 3−11).

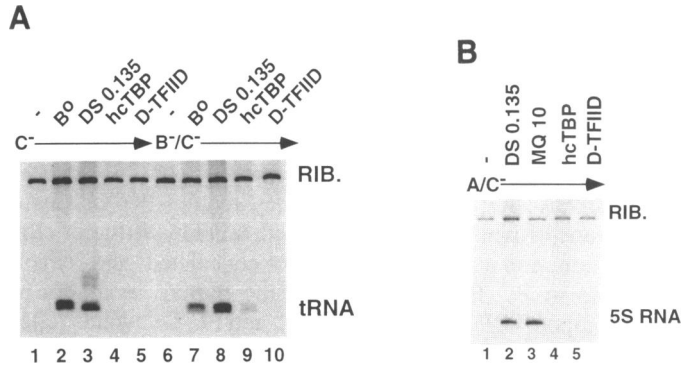

Figure 6. TFIIIB cannot be replaced by TBP or D-TFIID in pol III transcription. **(A)** Mcet1 tRNA transcription reactions were performed as for Figure 2B, using, in lanes 1−5, 3μl of C⁻, and in lanes 6−10, 3μl of both B⁻ and C⁻. These reactions were complemented with 3μl of buffer (lanes 1 and 6), B° (lanes 2 and 7), DS 0.135M (lanes 3 and 8), 2μl of recombinant human core TBP (lanes 4 and 9), or 4μl partially purified D-TFIID fraction (lanes 5 and 10; as used in Figure 4). tRNA transcripts are indicated. **(B)** 5S RNA transcription reaction conditions were as in Figure 1C; each reaction contained 3μl each of PC A and PC C⁻. These reactions were complemented with 3μl of buffer D (lane 1), DS 0.135M (lane 2), MQ 10 (lanes 3), 2μl of recombinant human core TBP (lane 4), or 4μl partially purified D-TFIID fraction (lane 5). 5S RNA transcripts are indicated.

correlation between TBP content and pol II function. Although MQ 9 contains an amount of TBP only slightly less than MQ 10, only a minor proportion is active in the pol II assay, perhaps suggesting the presence of an inhibitor in that fraction (as for TFIIIB activity in the same fraction, see above). The transcription supported by recombinant TBP, the DS 0.135M and MQ fractions was mediated by pol II as it was sensitive to low (1 μg/ml) concentrations of α-amanitin (data not shown). When the Superose 12 fractions were tested in this assay, basal pol II activity, like TFIIIB activity (Figure 4A), was seen to peak in Fraction 7 (Figure 5B, only a subset of the fractions are shown).

Thus we conclude that the TBP present within TFIIIB fractions can also support pol II transcription. The potential relationship between TFIIIB and the previously characterized pol II TBP−TAF complex, B-TFIID (1,2), will be discussed later.

TFIIIB cannot be replaced by TBP or D-TFIID in pol III transcription

To test the model proposed by Pugh and Tjian (40) and Sharp (41) that specific TBP−TAF complexes are implicated in polymerase choice, we attempted to replace our partially purified TFIIIB fractions with other forms of TBP in support of pol III-mediated transcription. The tRNA complementation assays shown in Figure 6A (lanes 1−5) allow us to stringently test different TBP forms for their pol III activity, as PC C⁻ has no detectable TBP (see Figure 1). As in Figure 2B, efficient complementation of PC C⁻ for Mcet1 tRNA expression is supported by control serum-depleted PC B° and DS 0.135M fractions (Figure 6A, lanes 1−3). Neither recombinant human core TBP (lane 4) or partially purified D-TFIID fraction (lane 5) support any tRNA complementation above background although both are active for pol II AdML transcription to a level higher than the DS 0.135M fraction (Figure 5, data not shown).

Similar results are obtained when measuring 5S RNA expression with the same PC C⁻ fraction, in combination with PC A (Figure 6B). The ability of PC B to provide the TBP component necessary for pol III transcription in this system has already been demonstrated (Figure 1C, lane 7). Lanes 2 and 3 of Figure 6B show that the DS 0.135M and MQ 10 fractions also retain this ability, whereas recombinant TBP core and D-TFIID fractions are inactive (lanes 4 and 5).

These results argue strongly for the existence of specific TAFs present in TFIIIB fractions, required to form a pol III-functional complex with TBP. That these postulated TAFs are to be found in PC B is further supported by interpretation of the remaining experiments in Figure 6A. Here the same TBP-containing fractions were assayed for complementation of PC C⁻ and PC B⁻ in combination. Again, efficient tRNA transcription requires PC B° or DS 0.135M fractions (lanes 6−8). The D-TFIID fraction is again inactive in this assay (lane 10). However, addition of the TBP core, now in the additional presence of PC B⁻, allows an intermediate recovery of tRNA transcription (lane 9). In comparing lanes 4 and 9 we suggest that the higher level of activity conferred by TBP addition in lane 9 reflects an association of the exogenous TBP with residual TAFs present in PC B⁻. This association generates the functional TBP−TAF complex specific to PC B capable of supporting pol III transcription. The inability of TBP alone to recover a full level of tRNA transcription could not be overcome by adding more, or less, recombinant TBP core protein, or recombinant full length

TBP (data not shown). A likely cause of this effect is that the depletion of TBP from PC B has removed not just TBP alone, but TBP in association with TAFs. Although this level of depletion was sufficient to inactivate pol III transcription (see Figure 1), it may have left quantities of the proposed TFIIIB TAFs sufficient to allow complex reformation upon TBP addition. Alternatively, full TFIIIB activity may require two classes of TAFs, one of which is codepleted with TBP by virtue of close association, and a second which is not codepleted, and remains available in PC B^-. In this case, the partial restoration of activity upon TBP addition to PC B^- and PC C^- would reflect the absence of the codepleted TAF. Support for the stability of at least some TBP−TAF associations in the conditions of the experiment comes from the inability of the TBP present (and active for pol II transcription, see Figure 5) in D-TFIID to exchange and form the pol III active complex in the presence of PC B^- (lane 10).

DISCUSSION

TBP association with TFIIIB activity

In this paper we have presented evidence that the general pol III activity TFIIIB is a TBP-containing complex. Three lines of experimental evidence support this conclusion. First, immunodepletion of TBP from the HeLa phosphocellulose PC B fraction, known to contain TFIIIB, leads to inhibition of the pol III transcriptional activity of the fraction. Secondly, TBP cofractionates with HeLa cell TFIIIB activity over four chromatographic purification steps. And finally, TFIIIB fractions are capable of supplying TBP in the form necessary for pol III transcription, and cannot be substituted by other TBP complexes or TBP alone.

The general relevance of our findings to pol III gene transcription is supported by the use of two tRNA templates from different organisms, Xenopus and nematode, and a 5S RNA gene from Xenopus. We and others have also found TFIIIB to be necessary for pol III transcription of vertebrate U6 snRNA genes in vitro (58; K.A.S. and J.D.L unpublished data). Thus TFIIIB appears to be, like TBP itself (26,27,31,34,35), necessary for expression of both intragenic (5S/tRNA classes) and extragenic (U6/7SK class) pol III promoters.

Other evidence in support of an association between TFIIIB activity and TBP comes from studies in yeast. Transcription assays with a truncated *S.cerevisiae* U6 snRNA gene fragment in vitro (59) showed that TBP and TFIIIB cofractionated from yeast extracts during several steps of purification. Others have shown yeast TFIIIB to contain at least two other polypeptides, of ~70kD (denoted B′) and ~90kD (B″), both of which can be photocrosslinked to upstream regions of yeast tRNA and 5S genes, and are necessary for the transcription of these genes (56,57). Genetic evidence for TBP association with yeast TFIIIB comes from the isolation of an essential gene (called TDS4) encoding the B′ polypeptide (71). This gene was identified as an extragenic suppressor of a temperature sensitive mutant of yeast TBP, suggesting a specific functional interaction between TBP and the TDS4 gene product. The same gene has also been isolated as a suppressor of a tRNA gene A block promoter mutation, and designated PCF4 (72).

Our data show that mammalian TFIIIB function in pol III transcription is also dependent on cofractionating TBP, as the use of immunodepletion with anti-TBP antibodies allowed us to control the contribution of TBP from the other PC fractions required to assay pol III transcription. HeLa TFIIIB activity has been reported to reside in a ~60kD protein, as assayed by complementation with the PC C fraction (43). However, our demonstrations that immunodepleted TFIIIB cannot be replaced by recombinant TBP alone, and the coelution of TBP and TFIIIB in a complex of ~230kD upon gel filtration provide a more convincing argument that, as in yeast, human TFIIIB has multiple subunits. The report that Drosophila TFIIIB is detected as a complex of ~260kD (73) further suggests that multiprotein TFIIIB complexes exist in a range of eukaryotes.

Polymerase-specific TBP−TAF complexes?

With the identification of TFIIIB as a TBP-TAF complex, the tentative designation of polymerase specific TBP complexes becomes possible. That TFIIIB is distinct from D-TFIID and SL1, the TBP complexes active for pol II and pol I initiation respectively, is clear from its different fractionation profile. However, the pol II-competent B-TFIID complex (1,2) is purified from the same PC B fraction as TFIIIB. To what extent does our partially purified TFIIIB resemble the reported B-TFIID complex? Three arguments suggest that TFIIIB and B-TFIID share properties, and are therefore possibly the same.

The first is that our DS 0.135M, MQ 10 and Superose 12 fractions were active in TBP-dependent basal level pol II transcription assays (Figure 5) as well as in pol III assays (Figures 2−4). Secondly, this pol II activity was not apparent in the starting HeLa PC B fraction. Further purification of PC B was necessary to detect the pol II TBP function, as reported previously for B-TFIID (1). Finally, both TFIIIB activity and TBP coelute on gel filtration chromatography in a complex of apparent molecular weight ~230kD, a mass comparable with that reported for B-TFIID (~300kD; ref. 1). The same Superose 12 TFIIIB fractions also retained pol II TBP function when assayed with the TBP-dependent AdML promoter (Figure 5), suggesting that the 230kD complex has coincident pol II and pol III activities. Some TBP fractionated as larger (500kD or more) complexes on the Superose column (Figure 4B). These fractions were inactive in pol III transcription and barely active in basal pol II assays. Since the Superose fractionation had to be carried out in the absence of non-ionic detergents to preserve pol III activity, it is likely that the large complexes represent non-specific aggregates.

If the two activities are coincident, the question arises as to how the same complex can function in both pol II- and pol III-mediated transcription? We suggest that the pol II function of TFIIIB/B-TFIID may be incidental, perhaps due to TBP alone, since recombinant TBP is also active in the pol II TBP activity assay (Figure 4). The proposed association of TFIIIB specific TAFs with TBP might not block the availability of TBP domains necessary for participation in basal level pol II transcription. Consistent with this model, that only the TBP component of TFIIIB/B-TFIID is required in the observed pol II transcription, is the previously described inability of B-TFIID to respond to activators like Sp1, a property it shares with recombinant TBP (1). In this interpretation, the TAFs proposed to be present in the TFIIIB/B-TFIID fraction could be viewed as 'active' in pol III transcription and 'neutral' in pol II transcription.

A more complex scenario could invoke the removal or inactivation of the TFIIIB TAFs as necessary for TBP function in pol II transcription. In such a model, postulated factors would interact specifically with TBP, or the proposed TAFs, to promote remodelling or dissociation of the TFIIIB/B-TFIID complex, thus

facilitating TBP participation in pol II transcription. Recent evidence supports the existence of factors with comparable activities (69,74,75). For example, the recently cloned Dr1 protein, identified as a repressor of basal level pol II transcription, can bind TBP or D-TFIID in vitro, and precludes the association of promoter-bound TBP with the other basal pol II factors TFIIA and TFIIB (75). TFIIA itself has been proposed to function in promoting transcription through an antirepression mechanism involving removal or inactivation of a negative component (or TAF) from native TFIID (69). As we assayed for pol II TBP function in the presence of TFIIA (supplied in PC A), the observed TBP dependent transcription may have arisen via this mechanism.

Thus, the presence or absence of distinct TAFs in TBP complexes could play a role in polymerase selection. The striking homology of the TDS4/PCF4 gene product to the mammalian pol II TFIIB factor has already led to the proposal that TFIIB homologs may play a role in 'bridging' promoter-bound TBP complexes to RNA polymerases (71,72).

Recruitment of TBP–TAF complexes to gene promoters

Having considered how TBP–TAF complexes may function in polymerase choice, it is also important to consider how they are targetted to their respective gene classes.

In the case of TATA-containing pol II promoters this appears to be straightforward, as the D-TFIID complex displays sequence specific binding to TATA elements (6). However, in certain TATA-less pol II promoters, TBP recruitment requires factors bound upstream, such as Sp1, in addition to other activities resident within the D-TFIID fraction (33,36,76). Similarly, in pol I transcription or in pol III transcription of tRNA or 5S RNA genes, neither SL1 nor TFIIIB have intrinsic sequence-specific DNA binding activity, and are dependent on other factors for their assembly onto promoters. In pol I transcription, SL1 recruitment occurs by protein–protein interaction with the upstream binding factor UBF, which binds sites in the rDNA promoter (77,78). In pol III transcription, protein-protein interactions are essential in assembly of TFIIIB into initiation complexes on intragenic promoters. In both yeast and mammalian systems, TFIIIB recruitment occurs only after binding of TFIIIC (on tRNA promoters) and TFIIIA/TFIIIC (on 5S promoters) (48–51,53).

Accumulating evidence also suggests that TFIIIB is involved in the transcription of upstream pol III promoters like those of the vertebrate U6 genes (26,27,58). If TFIIIB is needed for global pol III transcription, how is it recruited to such extragenic promoters? As TFIIIC and TFIIIA, the assembly factors used by the intragenic promoters, are dispensable for vertebrate U6 expression (58,79; K.A.S. and J.D.L., unpublished data), other U6 transcription factors must act to recruit TFIIIB. Two factors, TBP and the PSE (Proximal Sequence Element) binding factor PBP bind the human U6 proximal promoter and participate in its transcription in vitro (26,27,58,80). Given the proximity of their binding sites, TBP and PBP may, either separately or together, facilitate recruitment of TFIIIB in a manner analogous to TFIIIC in tRNA assembly. In this scenario two TBP molecules, or complexes, could be required for U6 snRNA transcription.

Stable binding of yeast TFIIIB is known to induce DNA bending around the initiation site (81). Recent studies have shown that TBP binds in the minor groove of the TATA element through its C-terminal direct repeats (82,83). Furthermore, TBP also bends the AdML TATA element upon binding (84). Whether the ability of both TFIIIB and TBP to induce DNA bending is essential for assembly of functional pol II and pol III initiation complexes, and whether a similar phenomenon is mediated by SL1 in pol I transcription, remain to be tested. However, it is tempting to speculate that the RNA polymerases utilise a common initiation factor, TBP, and a common mechanism involving DNA bending, in their different initiation pathways.

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