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Biochemistry

Composition of transcription factor B-TFIID

[TATA-binding protein/TATA-binding protein-associated factors/*in vitro* transcription/(d)ATPase activity]

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ABSTRACT **Initiation of transcription by RNA polymerase II requires a TFIID factor, which can recognize the TATA element common to many promoters. Two distinct multisubunit TFIID factors can be resolved from extracts of mammalian cells, and both of them contain the well-characterized TATA-binding protein (TBP) and are capable of supporting RNA polymerase II transcription in an *in vitro* reaction system. The smaller complex, B-TFIID, was purified and its subunit composition was determined. B-TFIID consists of two subunits: the TBP and a TBP-associated factor (TAF) of 170 kDa. This TAF is specific for B-TFIID and appears not to be present in the D-TFIID complex. Furthermore, it was found that the highly purified B-TFIID fractions have (d)ATPase activity.**

Initiation of transcription by eukaryotic RNA polymerase II is a complex process requiring multiple proteins (for reviews, see refs. 1–3). Gene-specific transcription factors regulate the initiation frequency by controlling formation of an initiation complex that contains both basal factors (TFIIA, -B, -D, -E, -F, and -H) and RNA polymerase II. Assembly of this initiation complex occurs in an ordered fashion (4, 5). The first step is the binding of TFIID to a TATA element; subsequently, the other basal factors and polymerase II enter the complex in a sequential manner. Many of the basal factors have been purified to homogeneity by conventional chromatography, allowing elucidation of their subunit composition and cloning of their corresponding cDNAs.

TFIID has not been purified to homogeneity by conventional chromatographic methods. Recent experiments suggest that one of the difficulties in purification of TFIID might be its heterogeneity (6, 7). Identification and cloning of a gene encoding a yeast protein that could substitute for the human TFIID in an *in vitro* transcription reaction led to the isolation of a human cDNA encoding a 38-kDa protein (8–15). This protein (TATA-binding protein, TBP, also known as TFIIDτ) binds specifically to the TATA element and directs formation of the initiation complex. Antisera specific for human TBP were used to show that this protein does not exist as a free polypeptide in HeLa cell extracts (16). Instead, TBP is assembled into at least two high molecular mass complexes: one of 300 kDa (B-TFIID) and the other of >700 kDa (D-TFIID). Polypeptides associated with TBP in these complexes are referred to as TAFs [TBP-associated factors (17)]. Although the D-TFIID factor is the well-characterized TFIID activity in HeLa cell extracts, the B-TFIID factor contains the majority of the cellular TBP (16). These two TFIID complexes are equally efficient in supporting basal transcription in reconstituted reactions (16). However, the two activities are not equivalent, since transcription reactions reconstituted with the traditional D-TFIID factor are responsive to gene-specific transcription factors such as SP1, while reactions reconstituted with B-TFIID are not.

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The TBP is probably also a component of transcription factors for RNA polymerases I and III. For example, the SL1 factor, which is necessary for transcription by RNA polymerase I, is composed of TBP and three TAFs (18). Genetic and physical evidence suggests that TBP is also essential for RNA polymerase III activity (19–24). These TBP complexes are thought to be distinct from TFIID-type complexes in that the latter are specific for generation of an initiation complex containing RNA polymerase II.

The existence of the different forms of TFIID in cellular extracts has several implications for mechanisms of gene regulation. For example, B-TFIID could be a precursor in the assembly of more complex D-TFIID activities. Alternatively, B-TFIID and D-TFIID could be distinct mature complexes that respond to different regulators and serve different types of promoters. In this case, the sequence of the promoter and the nature of critical regulatory factors would dictate the type of TFIID activity used to form the initiation complex. The latter model is supported by mutational analysis, which provided evidence for functionally distinct processes recognizing TATA elements (25, 26). To elucidate the relationship between the two TFIID factors we have purified B-TFIID from HeLa cells by both conventional and immunoaffinity chromatography and have determined its subunit composition. Interestingly, our most highly purified B-TFIID fractions have an (d)ATPase activity.

MATERIALS AND METHODS

Purification of B-TFIID. Purification of the B-TFIID factor began with chromatography of 3.6 g of protein from HeLa whole cell extract (27) on a 500-ml phosphocellulose column (P11; Whatman) according to Samuels *et al.* (28). The 350 mM KCl fraction B (1.2 g of protein) was dialyzed against buffer A [20 mM Hepes–KOH pH 7.9/20% (vol/vol) glycerol/1 mM EDTA/1 mM dithiothreitol/1 mM phenylmethanesulfonyl fluoride] plus 50 mM KCl and applied to a 190-ml Q Sepharose FF column (Pharmacia). The column was washed extensively and developed by step elution, first with buffer A plus 200 mM KCl and then with buffer A plus 1 M KCl. The majority of the TBP (as judged by immunoblot analysis) eluted in the 200 mM KCl step. This fraction (320 mg of protein) was adjusted to buffer A plus 40 mM KCl and applied to a 45-ml phosphocellulose column. The column was developed with a linear gradient of 40–400 mM KCl in buffer A, and B-TFIID was eluted at 160 mM. Following this step, all buffers contained aprotinin at 1 μg/ml and pepstatin A (Sigma) at 1 μg/ml. B-TFIID-containing fractions (45 mg of protein) were pooled, adjusted to buffer A plus 50 mM KCl, and loaded on a Mono S FPLC column (Pharmacia). This column was developed with a linear gradient of 50–500 mM

Abbreviations: TFIID, transcription factor IID; TBP, TATA-binding protein; TAF, TBP-associated factor.

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KCl in buffer A, and B-TFIID was eluted at 150 mM KCl. The corresponding fractions (8.8 mg of protein) were pooled, adjusted to buffer A plus 400 mM KCl plus 0.01% Triton X-100, and chromatographed on a Superdex 200 PG column (Pharmacia). Insulin (Boehringer) was added to the eluted fractions at 100 μg/ml as a carrier. Two fractions corresponding to 300 kDa contained B-TFIID. The peak fraction contained 66% of the eluted B-TFIID activity and was adjusted to buffer A plus 50 mM KCl and loaded on a Mono Q FPLC column (Pharmacia). This column was developed with a linear gradient of 50–300 mM KCl in buffer A. B-TFIID was eluted in a broad peak from 100 to 170 mM KCl. In an alternative final step, the minor fraction from the gel filtration column was applied to a 1.5-ml hydroxylapatite (Bio-Rad) column after addition of 10 mM potassium phosphate, pH 7.6. The column was washed with 50 mM potassium phosphate, pH 7.6/10% (vol/vol) glycerol/5 mM dithiothreitol and developed with a linear gradient of 50–500 mM potassium phosphate in 10% glycerol/5 mM dithiothreitol.

Purification of Other Transcription Factors. Purification of RNA polymerase II and the basal transcription factor-containing fractions was exactly as described (28). The expression and purification of histidine-tagged human TBP from *Escherichia coli* strain BL21 were as described (29).

***In Vitro* Transcription Assay.** A standard TFIID-dependent transcription reaction mixture contained, in addition to the TFIID fraction, 0.3 μl of calf thymus RNA polymerase II, 0.5 μl of fraction [AB], 1.5 μl of fraction [CB] (see Fig. 1), 12 units of RNAsin (Promega), and 200 ng of pML(C_2AT)19Δ-51 (8, 30) in a 20-μl volume. Incubation conditions were as described (8) with the addition of bovine serum albumin (Sigma) at 0.1 mg/ml to stabilize proteins. RNA was extracted, processed, and quantitated as described (16).

Protein Analysis. Immunoblots were prepared and developed with anti-human TBP rabbit antiserum as described (16). For immunoprecipitation analysis, rabbit polyclonal anti-TBP sera were purified by affinity chromatography according to Harlow and Lane (ref. 31, pp. 313–315). This involved coupling bacterially expressed histidine-tagged human TBP to cyanogen bromide-activated Sepharose (Pharmacia) at 3 mg of protein per ml of matrix, as recommended by the manufacturer. The affinity-purified anti-TBP and control 12CA5 antibodies (32) were cross-linked to staphylococcal protein A-Sepharose beads (Pharmacia) by using dimethylpimelimidate (ref. 31, pp. 522–523) and blocked prior to immunoprecipitation in incubation buffer (buffer A plus 350 mM KCl, 0.1% Nonidet P-40, pepstatin A at 1 μg/ml, and leupeptin at 1 μg/ml) plus insulin (Boehringer) at 0.5 mg/ml. The D-TFIID sample was preabsorbed with protein A-Sepharose to remove bovine serum albumin from this fraction. All protein fractions were adjusted to the incubation buffer and incubated with the cross-linked antibodies for 3 hr at 4°C. The antigen–antibody complexes were washed four times with the incubation buffer, and bound proteins were eluted by boiling in SDS sample buffer. The polypeptides were analyzed on a 10% polyacrylamide/SDS gel and detected by silver staining according to Blum *et al.* (33). Protein concentrations were determined by using the Bio-Rad protein assay with bovine gammaglobulin (Sigma) as the standard.

ATPase Assay. ATPase activity was assayed by incubation of the indicated protein fractions (adjusted to buffer A plus 100 mM KCl) in a volume of 10–15 μl at 25°C for 4.5 hr. Reaction mixtures contained 40 mM Tris·HCl at pH 7.9, 50 mM KCl, 7 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, bovine serum albumin at 0.5 mg/ml, and 8–10 μM [α-^{32}P](d)NTP (25 Ci/mmol; 1 Ci = 37 GBq). DNA was included in the reaction mixtures where indicated. Reaction products were analyzed by thin-layer chromatography on polyethyleneimine plates and developed with 1.5 M LiCl or 0.5 M LiCl plus 1 M $NaHCO_2$. Reaction products were quantitated by using a PhosphorImager and ImageQuant 3.0 software (both from Molecular Dynamics, Sunnyvale, CA).

RESULTS

Purification of B-TFIID. Purification of the B-TFIID factor from HeLa whole cell extracts was performed by chromatography on six successive columns (Fig. 1). TBP-containing fractions were identified by immunoblot analysis using a rabbit serum raised against the N-terminal 139 amino acids of TBP (16). The TFIID-dependent transcription activity of the different fractions across the purification was assayed in a reaction mixture containing the adenovirus major-late core promoter (data not shown). This analysis showed that after the second column the TFIID activity strictly coeluted with TBP. In accordance with our previous findings, TFIID activity could not be detected in earlier fractions (16). This might suggest the presence of an inhibitor specific for B-TFIID in early column fractions; however, such an inhib-

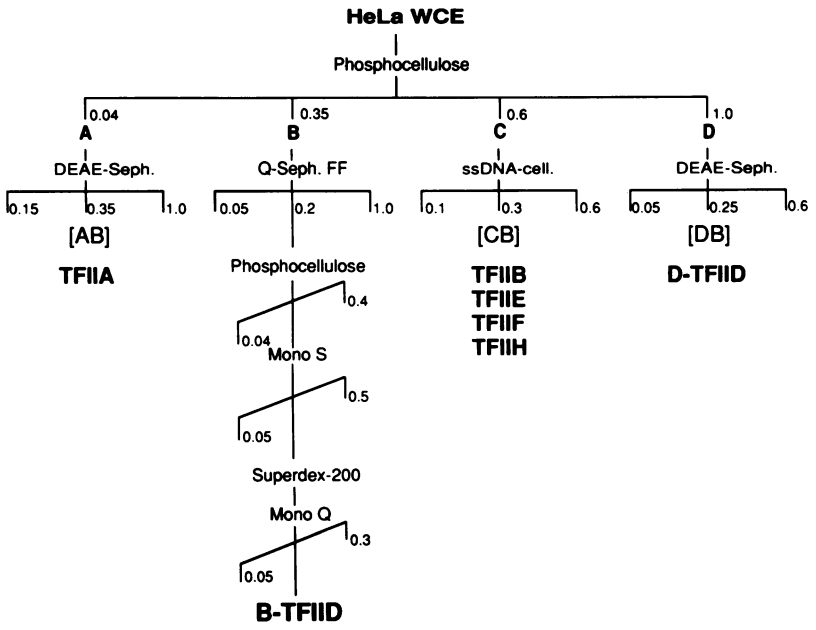

FIG. 1. Purification scheme for B-TFIID and the different general transcription factors from HeLa whole cell extracts. Horizontal lines represent step elutions, with the numbers indicating the molarity of the salt; oblique lines represent gradient elutions. The [AB] fraction contains TFIIA as indicated, whereas [CB] provides TFIIB, -E, -F, and possibly -H. The [DB] fraction provides the traditional TFIID activity, indicated here as D-TFIID. RNA polymerase II was isolated from calf thymus.

itor could not be detected in mixing experiments (data not shown).

Analysis of the fractions from the final Mono Q column are shown in Fig. 2. Fractions 27 and 28 were peak fractions when assayed both for transcription activity (Fig. 2*B*) and for the concentration of TBP (Fig. 2*C*). Three polypeptides (43, 80, and 170 kDa) coeluted with this B-TFIID activity as revealed by silver staining of an SDS/polyacrylamide gel (Fig. 2*A*). The 43-kDa polypeptide corresponded to TBP, which migrated slightly slower than its predicted size of 38 kDa (6, 16). The 80- and 170-kDa polypeptides were good candidates for components of the B-TFIID complex.

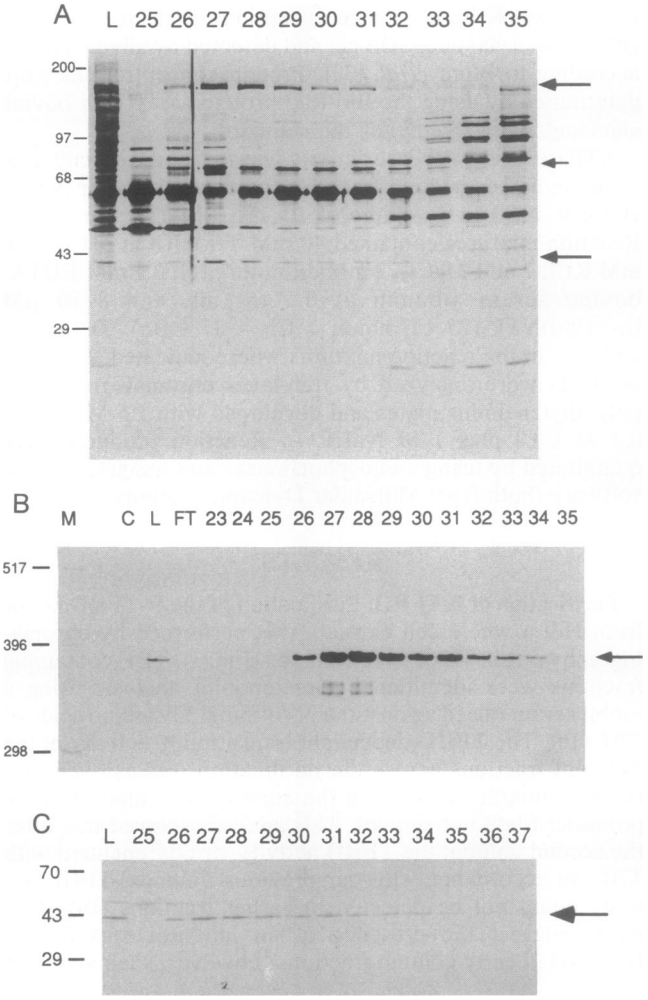

FIG. 2. Analysis of chromatographic fractions of the last column in the purification of the B-TFIID factor. (*A*) The different fractions of the Mono Q column were analyzed by silver staining of a 12% polyacrylamide/SDS gel. Fifteen microliters of the eluted fractions and 25 μl of the load (L) were applied to the lanes. The positions of protein markers are indicated to the left of the gel by their molecular mass in kDa. The arrows to the right indicate protein bands that comigrate with transcription activity. (*B*) Transcriptional analysis of the Mono Q fractions. Five microliters of the load (L) and of the eluted fractions and 6 μl of the flow-through fraction (FT) was adjusted to buffer A plus 100 mM KCl and analyzed in a TFIID-dependent transcription reaction using pML(C_2AT)19Δ-51 as the template (8, 30). This plasmid carries a 380-base pair guanosine-less cassette under control of the adenovirus core major-late (ML) promoter (−53/+10). The reactions analyzed in control lane C received only buffer A plus 100 mM KCl. The positions of DNA markers are indicated to the left by their length in nucleotides. The arrow indicates the correctly initiated RNA product. (*C*) Immunoblot analysis of the Mono Q fractions. Ten microliters each of the load (L) and of the eluted fractions were analyzed with rabbit antiserum specific for human TBP. The positions of prestained markers are indicated to the left by their molecular mass in kDa. The 43-kDa band indicated by the arrow represents TBP. The other protein bands of slower mobility result from nonspecific background staining (data not shown).

Immunoprecipitation Analysis of TFIID Complexes. To determine the subunit composition of the B-TFIID complex, pools of the peak fractions of two columns were analyzed by immunoprecipitation using affinity-purified TBP antibodies or control 12CA5 monoclonal antibodies directed against the hemagglutinin antigen of influenza virus (32). Antibodies were cross-linked to Sepharose beads, and similar amounts of B-TFIID activity from different steps in the purification protocol were subjected to the immunoprecipitation analysis. The 170-kDa polypeptide was specifically coimmunoprecipitated with the 43-kDa TBP (Fig. 3, lanes 5 and 7). The specificity of this coimmunoprecipitation is indicated by the fact that the 170-kDa protein was not a major polypeptide in the Superdex 200 PG load used in the immunoprecipitation (Fig. 3, lane 1). Comparison of lanes 5 and 7 indicated that the stoichiometry of the TBP polypeptide to the 170-kDa protein was similar at different steps in the purification. The immunoprecipitation analysis also indicated that the 80-kDa polypeptide was not stably associated with the B-TFIID complex. Since it was possible that the binding of the TBP antibody might interfere with binding of the 80-kDa protein and thus displace this protein from the complex, a B-TFIID fraction from the Superdex 200 column was further analyzed by chromatography on hydroxylapatite. Analysis of the peak fractions from this column by silver staining of SDS/

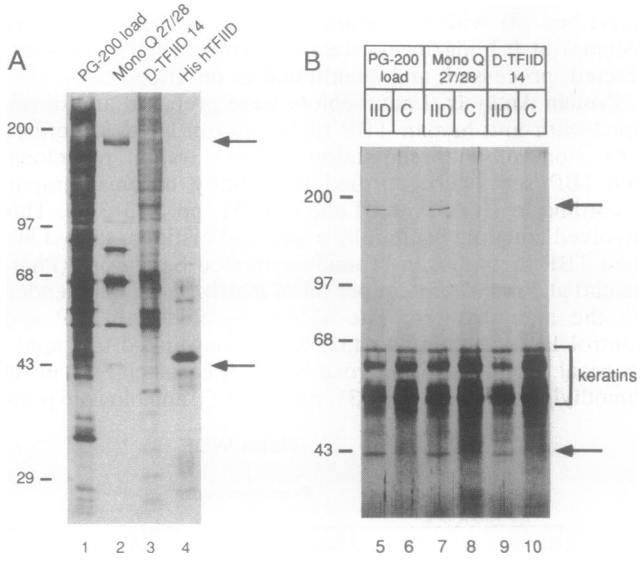

FIG. 3. Analysis of the immunopurified B-TFIID complex. Protein samples were separated by electrophoresis on a 10% polyacrylamide/SDS gel and were visualized by silver staining. (*A*) Silver staining of the fractions prior to immunopurification. Lane 1, 3.5 μg of the Superdex 200 PG load fraction; lane 2, 0.5 μg of the combined Mono Q fractions 27 and 28; lane 3, 1.5 μg of a D-TFIID fraction; lane 4, 200 ng of histidine-tagged recombinant human TBP, which is slightly larger than the endogenous TBP. (*B*) Silver-stain analysis of immunopurified TFIID fractions. Immunoprecipitations were performed with affinity-purified TFIID antibodies (lanes 5, 7, and 9) or control 12CA5 monoclonal antibodies (lanes 6, 8, and 10). The fractions used in the analysis are indicated above each pair of lanes: 10 μg of protein of the Superdex load, 1.4 μg of the Mono Q fractions 27 and 28, or approximately 500 μg of the D-TFIID-containing fraction. Based on transcription activity, about 5-fold more D-TFIID was used than B-TFIID. The arrows indicate the 43-kDa TBP and the 170-kDa component of B-TFIID. Bands migrating at about 50 kDa represent both keratins and IgG heavy chains. The positions of protein markers are indicated to the left, by their molecular mass in kDa.

polyacrylamide gels and reactivity with anti-TBP serum in Western blots clearly showed that the 80-kDa polypeptide did not elute in the fractions containing the 170-kDa protein and TBP (data not shown). This indicated that the 80-kDa polypeptide coincidentally coeluted with the B-TFIID complex on the Mono Q column and is not a subunit of B-TFIID. Taken together, these data show that the B-TFIID factor consists of two subunits: a 170-kDa polypeptide of unknown identity and a 43-kDa polypeptide, which is TBP.

If the B-TFIID complex is simply a precursor of the larger D-TFIID complex, then the 170-kDa polypeptide should be present in a stoichiometric amount relative to TBP in the D-TFIID preparation. This was tested by immunoprecipitation of a D-TFIID-containing protein fraction with anti-TBP antibodies (Fig. 3). Close inspection of lanes 9 and 10 indicates that at least five polypeptides (>200 kDa, 97 kDa, 95 kDa, 70 kDa, and 46 kDa) specifically coimmunoprecipitate with the 43-kDa TBP. The molecular masses of these polypeptides are very similar to the molecular masses of proteins present in the major D-TFIID complex characterized by Tjian and co-workers (6, 7) and Berk and co-workers (39). However, none of the coimmunoprecipitated proteins corresponded to the 170-kDa polypeptide present in the B-TFIID complex. This argues against a simple precursor/product relationship between B-TFIID and D-TFIID complexes.

The B-TFIID cofactor was purified about 250-fold in the last four chromatographic steps (Table 1). The degree of purification was determined relative to the Q Sepharose B-TFIID fraction, since transcription activity could be detected in B-TFIID-containing fractions only after this second column (see also ref. 16). Assuming complete recovery on the first two columns, the maximal purification of B-TFIID would be about 2600-fold. The recovery of B-TFIID activity during conventional chromatography was much higher than that of D-TFIID activity (Table 1; unpublished observations; refs. 5 and 35). This suggests that the B-TFIID complex is stable and homogeneous and that TBP and the 170-kDa TAF are probably tightly associated.

An ATPase Activity Copurifies with the B-TFIID Complex. In rat liver extracts a factor, δ, has been identified that is required for the basal transcription reaction and has an intrinsic (d)ATPase activity (34, 36). Interestingly, this ATPase activity is dependent on double-stranded DNA and is most efficiently stimulated by a TATA-box-containing sequence. Similarities in the molecular weight, chromatographic profile and basal transcription activity suggested that the B-TFIID factor might be the human equivalent of the rat δ factor. The most highly purified B-TFIID preparations were tested for dATPase activity in the presence of template DNA. Indeed, a (d)ATPase activity coeluted with B-TFIID (Fig. 4A). Whereas hydrolysis of ATP was efficient, the other ribonucleotides were essentially not used as substrates (Fig. 4B). Thus, the phosphatase activity is specific for adenosine. The K_m for dATP of this (d)ATPase was about 50 μM (data not shown), which is in accord with the K_m of most ATPase activities. The rate of turnover was estimated at about 309 fmol·min^{-1}·μl^{-1} for Mono Q fraction 27 (data not shown). These features are similar to those of the ATPase of δ (36). However, when the dATPase activity of fractions containing the B-TFIID factor was assayed for dependence on DNA, it was, in contrast to δ, found to be inhibited 2- to 3-fold by the addition of DNA (data not shown). This inhibitory effect was not dependent on the presence of a TATA box sequence (data not shown). We therefore conclude that B-TFIID is not the HeLa equivalent of the rat liver δ factor.

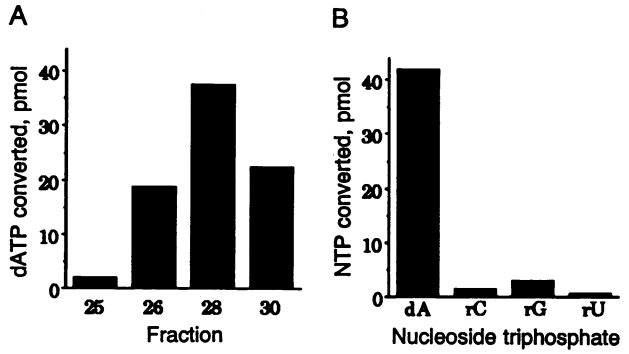

FIG. 4. An (d)ATPase activity cofractionates with the B-TFIID complex. (A) Analysis of dATPase activity of Mono Q fractions. The indicated fractions (3 μl each) were assayed for dATPase activity in the presence of 250 ng of pML(C_2AT)19Δ-51 plasmid. (B) Nucleotide specificity of the phosphatase activity. Mono Q fraction 27 (3 μl) was assayed for hydrolysis of the indicated nucleoside triphosphates in the presence of 150 ng of pML(C_2AT)19Δ-51 plasmid.

DISCUSSION

The TBP is found in a variety of complexes in extracts of mammalian cells. The predominant form of TBP complex is the B-TFIID factor, which is composed of a 170-kDa polypeptide and the well-characterized TBP. As noted previously, this complex is stable during purification and has a chromatography profile on a gel filtration column consistent with a 300-kDa factor. The discrepancy in molecular mass between the proposed 1:1 ratio of polypeptides for the complex and the gel filtration results may reflect an unusual shape for the complex.

B-TFIID is both smaller than and contains fewer subunit polypeptides than D-TFIID. The latter has a native molecular mass of approximately 700 kDa and contains a set of associated polypeptides, TAFs, of >200 kDa, 97 kDa, 95 kDa, 70 kDa, and 46 kDa. This complex was not extensively characterized in this study, and we assume that this subunit composition is equivalent to the previously described TFIID

Table 1. Purification of B-TFIID

Step	Protein, mg	Volume, ml	Activity, units	Specific activity, units/mg protein	Purification, fold	Yield, %
Whole-cell extract	3600	280	—	—		
Phosphocellulose	1200	280	—	—		
Q Sepharose FF*	300	125	137,000	460	1	100
Phosphocellulose	45	60	92,000	2,000	4.3	67
Mono S FPLC	8.8	3.4	90,000	10,200	22	66
Superdex 200 PG	1.0	12	25,000	25,000	54	18
Mono Q FPLC†	0.17	2.1	11,500	67,600	150	8.4
Mono Q 27/28	0.05	0.6	5,400	108,000	235	

Transcriptional activity was determined in reaction mixtures dependent on TFIID and using the core adenovirus major late promoter. Activity is given in arbitrary units.

*Purification was determined relative to the Q Sepharose fraction.

†The peak fraction of the Superdex 200 PG column contained 66% of the eluted activity, and 83% of this was used for subsequent purification.

complex, which contains six TAFs (>200 kDa, 125 kDa, 95 kDa, 78 kDa, 70 kDa, and 50 kDa; refs. 7 and 39). These studies suggest that the 170-kDa TAF is unique to the B-TFIID complex and argue against a simple model in which the intact B-TFIID is the precursor of D-TFIID. It remains possible that the B-TFIID complex is the precursor of the larger D-TFIID complex and that the 170-kDa subunit is released upon formation of D-TFIID. The abundance of the B-TFIID complex as compared with the D-TFIID complex argues against this possibility. We suggest that both the B-TFIID and D-TFIID complexes exist independently in the cell and are capable of supporting transcription by RNA polymerase II.

A precedent for functionally and physically distinct TBP–TAF complexes comes from the analysis of the transcription factor SL1, which is specific for RNA polymerase I. This factor is, in fact, a complex of TBP with three other proteins [110 kDa, 63 kDa, and 48 kDa (18)]. Analysis of RNA polymerase III transcription also suggests the involvement of TBP–TAF complexes (22), but such specific complexes have not yet been identified. The TBP is thought to be a component of the TFIIIB factor in view of the binding of this factor upstream of the site of initiation and its role in specifying RNA polymerase III binding. Although this factor has not been completely purified, studies involving both biochemical fractionation and UV crosslinking have not detected a polypeptide component of 170 kDa (37, 38). Thus, there is no evidence indicating that B-TFIID may be a transcription factor for polymerases other than RNA polymerase II. In fact, the observation that B-TFIID promotes initiation by RNA polymerase II *in vitro* suggests it has an equivalent activity *in vivo*.

B-TFIID is functionally distinct from the traditional D-TFIID (16). In contrast to transcription with D-TFIID, reaction mixtures containing B-TFIID do not respond to the transcriptional regulators SP1 or GAL4-AH and respond only weakly to the major-late transcription factor. It was also observed that the B-TFIID activity associates less stably with the adenovirus major-late promoter than either D-TFIID or recombinant TBP (16). This latter finding was supported by the inability to detect B-TFIID·DNA complexes when highly purified fractions were used under a variety of gel shift conditions and with different promoter fragments (data not shown). Obviously, these negative results might reflect the limited range of promoters tested. Alternatively, it is possible that a partial function of the 170-kDa TAF might be to prevent a stable association of B-TFIID with the standard subgroup of promoters that are highly active *in vitro*, thereby redirecting B-TFIID to a distinct class of core promoters or TATA elements. This latter model is consistent with the different DNA-binding properties of recombinant TBP and B-TFIID (16).

The observation that an (d)ATPase activity is associated with B-TFIID raises two interesting issues. Which of the two subunits is responsible for this (d)ATPase activity and is such an activity associated with all TBP·TAF complexes? Early studies using crude transcription extracts showed that hydrolysis of the β–γ bond of ATP or dATP is required for the initiation reaction (for review see ref. 2). Although this (d)ATP requirement appears to be a late step in the formation of the initiation complex, it cannot be attributed to any of the late-acting basal factors (such as TFIIE and TFIIF) that have been purified to homogeneity. Therefore, the observation of a (d)ATPase activity associated with B-TFIID raises the possibility that TBP·TAF complexes are responsible for the (d)ATP-requiring step in the initiation of transcription.

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